Influence of oxidative stress on inducing micturition dysfunction following chronic infravesical obstruction and the protective role of an antioxidant diet – association of in vivo and in vitro studies in rats

Sérgio Bisogni, Fabio Thadeu Ferreira, Arnaldo Amstalden Neto, Leandro Oliveira Chiarelli, Valdemar Ortiz

Geriatric Urology Service, Federal University of São Paulo (UNIFESP), SP, Brazil.

ABSTRACT

Objective: This study was developed to determine whether the generation of free radicals, induced by ischemia followed by reperfusion in a model of chronic intravesical obstruction in rats, would lead to damage in the detrusor. It also investigates the possible protective action of the flavonoid galangin on the tissue lesion induced by lipid peroxidation.

Materials and Methods: Twenty-one male rats were divided into three groups of seven animals each. Group A was subjected to a sham procedure; group B to partial obstruction of the bladder neck; and group C to partial obstruction of the bladder neck, but also received a diet rich in the flavonoid galangin. All the animals were subjected to urodynamic evaluation and then sacrificed. The bladders were sent for enzymatic tests.

Results: The urodynamic showed that group B developed significantly greater numbers of involuntary contractions of the detrusor, greater post-micturition residue and lower compliance. The group A presented TEAC levels greater than to the group B. Comparative analysis of group A, B and C demonstrated significantly greater malondialdehyde levels in group B in relation to groups A and C. The group B presented smaller contraction amplitudes than did groups A and C, in electrically stimulated contractions.

Conclusions: That oxidative stress is implicated in the damage to the detrusor muscle following a period of chronic intravesical obstruction. We show, for the first time, that administration of an antioxidant prior to and following the start of chronic obstruction makes it possible to avoid the cellular lesions that cause detrusor dysfunction.

INTRODUCTION

Overactive Bladder Syndrome (OBS) is a complex of symptoms that include micturitional urgency with or without urge incontinence, frequency and nocturia (1), without any demonstrable anatomical cause. The International Continence Society regards OBS as a syndrome for which the physiopathology involved is not precisely known (1).

OBS is a recently defined entity. Thus, its prevalence and natural history have not been well studied. In our setting, Telöken et al. (2) conducted a population survey involving 848
people aged between 15 and 55 years, and 18.9% reported having symptoms compatible with OBS.

The symptoms of OBS have many causes, and a variety of factors may contribute towards the pathogenesis of the disease (3).

Recently, experimental studies have demonstrated a positive association between the triggering of OBS and the generation of free radicals (4,5). The results from an experimental study involving smooth muscle fragments subjected to periods of repeated electrical stimulation demonstrated high levels of lipid peroxidation products, and decreased smooth muscle contractility of the bladder, proportional to the level of oxidative stress (4). Masick et al. (5) demonstrated the existence of deterioration in muscle contraction, and also the emergence of oxygen-reactive species, in the bladders of animals subjected to chronic urethral obstruction. These authors suggested that the formation of free radicals was an important factor in decreasing the detrusor contractility observed in patients with benign prostatic hyperplasia.

Over recent years, there has been great interest in developing new therapeutic methods for treating HBP. The main objective is to develop drugs that selectively inhibit the involuntary contractions of the detrusor, thereby diminishing the symptoms without affecting micturition contraction.

Antioxidants protect the organism against free radicals and are important agents for maintaining an adequate state of health. Flavonoids are an important source of antioxidants. Among the more than 4000 different types of flavonoids, galangin is a flavonol that has important biological activities at low concentrations and has a low toxicity index, which makes it a flavonoid with properties of interest for clinical studies regarding its biological benefits.

By utilizing an in vivo model, the present authors have investigated the action of oxidative stress on the bladder musculature and the possible protective action from the use of antioxidants. Thus, the principal objective of this study was to determine whether the oxidative stress induced by ischemia followed by reperfusion, in a model of chronic infravesical obstruction in rats, would lead to damage of the detrusor musculature.

MATERIAL AND METHODS

Arrangement of the groups

Twenty-one immature male rats weighing between 250 and 300 grams and aged 6–8 weeks were utilized in the experiment. These animals were divided into three groups of seven animals each (the sample size was determined statistically). They were kept under adequate conditions of hygiene in a climate-controlled vivarium and received controlled feed.

Group A was submitted to a sham procedure consisting of opening and closing the abdominal wall without positioning an obstructive ring on the bladder neck. Four weeks after the procedure, the animals were submitted to urodynamic evaluation and then sacrificed to remove the bladder.

Group B was submitted to a surgical procedure to create partial obstruction of the bladder neck (as described below). Four weeks after the procedure, the animals were submitted to urodynamic evaluation and then sacrificed to remove the bladder.

Group C was submitted to a surgical procedure to create partial obstruction of the bladder neck (as described below). Four weeks before the procedure, an antioxidant-rich diet rich was introduced (containing the flavonoid galangin). After the procedure, the diet was maintained for four weeks afterwards, when the animals were submitted to urodynamic evaluation and then sacrificed to remove the bladder.

Antioxidant diet

Group C received a normal purine diet, like the other groups, but with the addition of a high proportion of the flavonoid galangin (30 µM/day). The diet was devised by the chemistry laboratory of the institution at which the study was conducted.

The addition of galangin did not alter the color, texture, smell or flavor of the diet.

The animals’ daily intake of this diet was controlled so that all of them received 40 g of feed/day.
Description of the procedure for creating the obstruction

Under anesthesia using xylazine (0.87 mg/kg intraperitoneally) and ketamine (43.3 mg/kg intraperitoneally), the abdominal cavity was opened up in layers and the periurethral space was delicately dissected. A silver ring of 2 mm in diameter was positioned around the bladder neck. This model made possible the institution of a gradual obstruction process parallel to the animal’s growth, similar to a model for urological diseases that cause infravesical obstruction (6).

Urodynamic evaluation

Under anesthesia using urethane (1 mg/Kg intraperitoneally) (the drug of choice for urodynamic evaluations in animals, that enable micturitional assessment), two children’s angiographic catheters were inserted percutaneously into the bladder (24-gauge, 1-2 cm, Baxter Healthcare AS). One of the catheters was connected to an infusion pump with a capacity of 0.23 mL/min and the other to a pressure transducer. The volume urinated was collected using a graduated pipette connected to the pressure transducer (Baxter) in order to measure the volume urinated. Ten micturition cycles were evaluated and the following parameters were observed: presence and frequency of involuntary contractions of the detrusor during bladder filling (contractions for which the maximum amplitude reached one-third of the maximum detrusor pressure during micturition), maximum detrusor pressure, compliance, residual volume, micturition volume and bladder capacity.

Following the urodynamic evaluation, the animals were sacrificed and the bladder was removed at the level of the bladder neck and processed for enzymatic and electrophysiological tests. After removing the bladder, it was sectioned longitudinally between the ureteral openings: the left half was sent for electrophysiological tests and the right half for enzymatic tests.

Tissue preparation for enzymatic tests

The right half of the bladder, composed of smooth muscle and mucosa, was exposed to liquid nitrogen, weighed, dispersed in 1.15% KCL and homogenized to a concentration of 50 mg/mL for 10s, using a polytron. The mitochondria and microsomes were then isolated through different centrifugation runs, as described previously (7).

Malondialdehyde (MDA)

Samples of bladder tissue (processed previously, as described above) were placed in glass tubes, and 1.15% 0.05M KCL and 1 mM FeSO4 were introduced into 500 mL of tissue extract. The samples were incubated at 37° C for 60 min. At the end of the incubation, the reaction was halted by means of adding 150 mL of 40% trichloroacetic acid. The material was put into tubes and centrifuged at 1000 x G for 1 min. After centrifuging, 100 mL of the supernatant was incubated with 750 mL of perchloric acid (PCA) and thiobarbituric acid (TBA) (2.3% PCA, 0.53% TBA, pH 7.4) at 100° C for 60 min and then in ice for 10 min. To each tube, 2 mL of 1-butanol was added, and the tubes were inverted for 2 min. The tubes were centrifuged at 1000 x G for 5 min and the top layer was measured fluorometrically at 532 nm. Total quantification of proteins was done by means of the Micro BCA protein analyzer, and the results were expressed as nmoL MDA/mg protein (7).

The MDA levels were described in relation to the total protein levels.

Total antioxidant capacity – trolox (TEAC)

To measure the total antioxidant capacity, a previously standardized technique was utilized (8,9). In short, the total antioxidant capacity was analyzed on the basis of the ability of a compound to eliminate the ABTS radical in 6 min. ABTS was produced the reaction between 7 mM ABTS and 2.45 mM of potassium persulfate in water. This solution was stored in a darkened environment for 12-16 h before use. The extinction coefficient of ABTS at 734 nm is 1.5 x 104/moL/cm.

The solution containing ABTS was diluted with phosphate (PBS) at pH 7.4, until final absorption of 0.70 ± 0.02 at 734 nm was achieved, at 37° C. Ten microliters of an antioxidant were added to 990 µL of ABTS and the absorption at 734 nm was evaluated. This result was compared with a reaction without antioxidant, in which 10 µL of the solvent was added to 990 µL of ABTS. The reduction in absorption after adding the antioxidant
was determined. The TEAC of the bladder tissue from the animals was calculated by adding the supernatant from the tissue to the ABTS solution. The tissue reacted instantly with the ABTS and the reaction was complete within a few seconds. The decrease in absorption at 734 nm was monitored over this period.

The TEAC levels were described in relation to the total protein levels.

Preparation of tissue for physiological evaluation

Fragments of 2 x 2 cm, consisting of the mucous layer, submucous fat and conjunctive tissue, were removed from the dorsum of the bladder cap with the aid of a binocular microscope. Similar-sized fragments of longitudinal smooth muscle, of 2 mm in diameter and 5 mm in length, were extracted for performing experiments. Only one fragment was taken from each animal, so as to utilize the greatest number of animals possible.

Electrophysiological evaluation

Organ bath: Each fragment was positioned vertically in an organ bath containing 5 mL of a standard nutrient solution (Krebs solution: 118 mM NaCl, 5.6 mM KCl, 25 mM NaHCO3, 1.3 mM NaH2PO4, 2.5 mM CaCl2, 1.2 mM MgSO4, 6.1 mM glucose; pH 7.4, 95% O2, 5% CO2), between two clips that were connected to a voltage transducer. An electrical field was generated (Hameg® Instruments) between two platinum electrodes. The input and output flow of bath solution was controlled by a system positioned at the bottom of the organ bath. The temperature was kept at 37°C by using a water heating system.

Protocol: The muscle fragments were individually inserted into the organ bath and incubated in the Krebs solution. Just after positioning the fragment in the organ bath, the “L0” point representing the length associated with maximum isometric strength was determined at a temperature of 37°C. After determining L0, the fragments were left to rest for a 30-minute period and then were subjected to different stimuli: electrical (EFS), Ach (10 µmol/L) and ATP (10 µmol/L). An interval of 10 minutes was maintained between applying each stimulus, in order to avoid muscle fatigue.

Statistical evaluation

The results were expressed as mean ± standard deviation, when appropriate. The differences in maximum strength and time constant between pre and post-treatment readings were evaluated using SPSS 8.0 for Windows, by means of student’s t-test and ANOVA with Dunnet’s post-hoc test. The significance level chosen was 95% (p < 0.05). It was unnecessary to normalize the readings for maximum strength and time constant, since the fragments were similar in size to reduce the variability in maximum basal amplitude.

RESULTS

Urodynamic evaluation

Table-1 summarizes the urodynamic findings of the different groups. The results demonstrated that the obstructed group (group B) developed significantly greater number of involuntary contractions of the detrusor during the bladder filling phase (p < 0.05), and also diminished maximum micturition flow (p < 0.05), increased post-micturition residue (p < 0.05) and decreased compliance (p < 0.05).

There was no significant difference between groups A and C (p > 0.05).

Total antioxidant capacity

Tissue TEAC measurements were obtained from 40 samples obtained from the supernatant of each animal. The results are represented in Figure-1.

The animals in group A (sham procedure) presented statistically greater TEAC levels (111.57 ± 9.50 μM/mg*mL-1) than did group B (infravesical obstruction without antioxidant-rich diet) (50.02 ± 9.80 μM/mg*mL-1). Group C (obstructed plus antioxidant-rich diet) presented TEAC of 80.54 ± 9.99 μM/mg*mL-1, which was statistically lower than group A and greater than group B.

The highest TEAC values (in group A) suggest that the bladders in these animals were exposed to the least oxidizing damage. In contrast, the lowest TEAC values (in group B) suggest that these animals had a low antioxidant capacity, i.e. the greatest oxidizing damage.
Malondialdehyde

The MDA levels were expressed as µM MDA/mg*mg-1 protein (Figure-2).

Since the MDA levels correlated negatively with the total antioxidant capacity, it can be assumed that the low MDA levels represented high antioxidant potential in the samples studied.

Comparative analysis of groups A (sham), B (obstructed) and C (obstructed + galangin) demonstrated that there were significantly greater MDA levels in group B than in groups A and C (respectively 0.19 ± 0.069; 0.72 ± 0.03 and 0.17 ± 0.04).

In vitro evaluation – response of the detrusor musculature to electrical stimulation

Table 1 - Findings from the urodynamic evaluation performed four weeks after the sham procedure (group A), partial obstruction of the bladder neck using a silver ring (group B) and chronic obstruction together with the use of the flavonoid galangin (group C). The data are expressed as means.

<table>
<thead>
<tr>
<th></th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presence of ICD</td>
<td>absent</td>
<td>present</td>
<td>absent</td>
<td>not applicable</td>
</tr>
<tr>
<td>Frequency of ICD/min</td>
<td>0</td>
<td>4</td>
<td>1</td>
<td>0.01 B vs. A + C</td>
</tr>
<tr>
<td>MaxDP</td>
<td>28.7</td>
<td>51.2</td>
<td>31.4</td>
<td>0.03 B vs. A + C</td>
</tr>
<tr>
<td>Compl</td>
<td>1.32</td>
<td>0.08</td>
<td>1.3</td>
<td>0.03 B vs. A + C</td>
</tr>
<tr>
<td>Rvol</td>
<td>0.3</td>
<td>2.1</td>
<td>0.4</td>
<td>0.04 B vs. A + C</td>
</tr>
<tr>
<td>Mvol</td>
<td>2.7</td>
<td>0.4</td>
<td>2.3</td>
<td>0.01 B vs. A + C</td>
</tr>
<tr>
<td>MaxCC</td>
<td>3.1</td>
<td>2.5</td>
<td>2.9</td>
<td>0.3</td>
</tr>
</tbody>
</table>

ICD: involuntary contraction of detrusor; MaxDP: maximum detrusor pressure (cmH2O); Compl: bladder compliance (mL/cmH2O); Rvol: residual volume (mL); Mvol: micturitional volume (mL); MaxCC: maximum cystometric capacity (mL).
The data related to the detrusor response to different electrical frequencies are shown in Figure-3. At the frequencies of 16 and 34 Hz, it was observed that the animals in the obstructed group (B) developed contractions of significantly lower amplitude ($p < 0.05$) than did the sham group (A) and the obstructed group that received galangin (C).

In a preliminary study, it had been observed that the muscle contractions induced by electrical stimulation were totally suppressed by tetrodotoxin (10-6M), which is a nervous response inhibitor. Thus, the results obtained from the present study indicate that chronic obstruction leads to damage to the nervous response that is induced by the release of neurotransmitters at the presynaptic terminations.

In vitro evaluation—response of the detrusor musculature to acetylcholine

The data related to the detrusor response to acetylcholine are presented in Figure-4. The amplitudes of the muscle contraction induced by acetylcholine (Ach) (10 μmol/L) are shown in the figure. The group data are labeled as follows: Group A: sham; Group B: obstructed; Group C: obstructed + galangin.

**Figure 3 - Response of the detrusor musculature to different electrical stimulation (1, 2, 4, 8, 16 and 32 Hz).**

**Figure 4 - Response of the detrusor musculature to acetylcholine (Ach) (10 μmol/L).**
acetylcholine in the different groups were statistically similar (p > 0.05). Thus, it is suggested that the damage to the detrusor musculature induced by ischemia/reperfusion does not affect the muscarinic receptors.

In vitro evaluation – response of the detrusor musculature to ATP (adenosine triphosphate)

The data related to the detrusor response to ATP are shown in Figure-5. The amplitudes of the muscle contraction induced by ATP in the different groups were statistically similar (p > 0.05). Thus, it is suggested that the damage to the detrusor musculature induced by ischemia/reperfusion does not affect the purinergic receptors.

DISCUSSION

In this study, data from in vivo and in vitro evaluations were combined to assess detrusor function following a period of partial infravesical obstruction, and also the protective role of the flavonoid galangin on the detrusor response to chronic obstruction. Specifically, in vivo urodynamic evaluation data and in vitro isometric parameters and enzymatic tissue marker data were utilized to prove the hypothesis that the generation of free radicals has a role in detrusor damage after a period of chronic infravesical obstruction.

The possible role of oxidative stress in the physiopathology of bladder dysfunction has still not been elucidated, despite various studies conducted in this respect, in different centers around the world. Zhao et al. were the first investigators to suggest that a bladder submitted to acute distention would initially present a period of ischemia, followed by a period of reperfusion, which would lead to the generation of free radicals and tissue lesions (10). More recently, a study developed by Levin et al. demonstrated a possible relationship between a period of ischemia followed by reperfusion and the etiology of the bladder dysfunction subsequent to a period of chronic infravesical obstruction (7). These authors formulated the hypothesis that a state of oxidative stress would be present in a situation of chronic obstruction and that this would lead to the formation of metabolic end products that would cause damage to the phospholipids of the lipid membrane of the muscle cells. However, they did not find enough data to prove their hypothesis.

In the present investigation, two of the most widely used and accepted markers were utilized to demonstrate the existence of oxidative stress in the tissue (MDA and TEAC). MDA, which is the final product from the end product of lipid peroxidation induced by free radicals, has been implicated as the most important cause of destruction and damage in cell membranes. This occurs because the polyunsaturated fatty acids of cell membranes are degraded by this process, with consequent rupture of membrane integrity. Membrane peroxidation may lead to changes in membrane fluidity and permeability, and may also increase the protein degradation and cell lysis rates (11).

The total antioxidant capacity (TEAC), which is another marker used for indirectly determining the levels of oxidative stress in tissue, has been widely used for homogenized tissue from rats (12). This analysis assesses the antioxidant activity against the ABTS radical.

In the present study, the MDA concentration in the group subjected to the chronic obstruction procedure (B) was significantly greater than in the animals subjected to the sham procedure (A) and/or chronic obstruction with concomitant

Figure 5 - Response of the detrusor musculature to ATP (10 μmol/L).

Group A: sham; Group B: obstructed; Group C: obstructed + galangin.
use of galangin (C). It was also observed that the total antioxidant capacity in group B was statistically lower than in the other two groups, which indicates the presence of significant oxidative damage in the samples studied. When taken together, these findings corroborate the hypothesis that high levels of lipid peroxidation are associated with chronic infravesical obstruction. We can suggest that the damage to the detrusor musculature induced by infravesical obstruction (proven via the low amplitudes of contractions induced by electric stimulation and the occurrence of bladder dysfunction during the urodynamic evaluation) is at least partially related to the formation of free radicals and cell membrane damage.

There is growing evidence that flavonoids have beneficial effects and are of special interest for preventing and treating specific diseases that are related to free radical generation (13). In the present study, the hypothesis tested was that one antioxidant (the flavonoid galangin) would have the capacity to avoid tissue damage induced by oxidative stress in the detrusor cells of guinea pigs. Galangin was chosen from among other flavonoids because it has been demonstrated to be one of the flavonoids with greatest relaxant power of smooth musculature (14). Furthermore, galangin is the most lipophilic of the flavonoids, which makes it appropriate for intravesical use due to the facility with which it penetrates the barrier formed by bladder mucosa (15).

The mechanism through which galangin protects the detrusor from tissue lesions following a period of chronic obstruction is a matter for speculation. Capasso & Tavares suggested in a study on rats in which they verified the action of this flavonoid on the amplitudes of bladder muscle contractions, that it would act on type L calcium channels (16). Levin et al. demonstrated that, when muscle tissue was exposed to free radical generation, there was an accumulation of calcium in the intracellular medium, which would be responsible for cell damage. Thus, they suggested that galangin would act through type L calcium channels, thereby avoiding increased transportation of calcium ions into cells (17). There would, of course, be a need for studies to elucidate the exact mechanism for the action of galangin, in order to characterize the diseases for which galangin could become part of the therapeutic arsenal.

In summary, the findings described in the present study allow the conclusion that oxidative stress has a role in the damage of the detrusor musculature resulting from a period of chronic infravesical obstruction. We also have, for the first time, shown that administration of an antioxidant prior to and following the beginning of chronic obstruction makes it possible to avoid the cellular lesions that cause detrusor dysfunction.

From a broader perspective, it is suggested that benign prostate hyperplasia (BPH) is associated with induced free radical formation. Free radicals would therefore be at least partially responsible for the different degrees and types of micturition dysfunction observed during the evolution of these patients. Together with this hypothesis, it is also suggested that the use of antioxidants would have a protective role against micturition dysfunction during the follow-up of patients with BPH.

CONCLUSIONS

These results allow the conclusion that oxidative stress is implicated in the damage of the detrusor musculature following a period of chronic infravesical obstruction. We also have, for the first time, shown that administration of an antioxidant prior to and following the beginning of chronic obstruction can avoid the cellular lesions that cause detrusor dysfunction.

CONFLICT OF INTEREST

None declared.

REFERENCES


Correspondence address:
Dr. Fabio Thadeu Ferreira
Rua Duque de Caxias, 780
10º andar /102-104.
Campinas, SP, 13015-311, Brazil
E-mail: fabio.urologia@gmail.com