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ORIGINAL ARTICLE

Amitriptyline aggravates the fibrosis process in a rat model of infravesical obstruction

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Summary

Infravesical obstruction (IVO) secondary to benign prostatic hypertrophy can affect up to 50% of men over 50 years old and may cause serious and irreversible alterations throughout the urinary tract, especially in the bladder. Therapeutic approaches are currently limited. Amitriptyline has recently been described as an analgesic, anti-inflammatory and myorelaxant in some experimental models. The objective of this study was to investigate the effects of amitriptyline hydrochloride on the process of fibrosis in a bladder outlet obstruction model in rats. Male Wistar rats were subjected to IVO and studied at intervals of 1 and 14 days postprocedure. The rats were randomly divided into five groups: sham, IVO1-T, IVO1-NT, IVO14-T and IVO14-NT. Bladder tissue was processed for histopathology, immunohistochemistry and RT-PCR. The IVO14 groups presented bladder fibrosis, smooth muscle cell hypertrophy and bladder wall thickening. The IVO14-T group demonstrated a higher intensity of fibrosis, higher macrophage infiltration rate and higher gene expression of Transforming growth factor (TGF) *Tgf-β1*. Thus this data shows that in this experimental mode amitriptyline had an amplifying effect on the process of fibrosis as a whole.

Keywords
amitriptyline, bladder outlet obstruction, fibrosis, osteopontin, transforming growth factor-beta1

Infravesical obstruction (IVO) secondary to benign prostatic hypertrophy is a common condition in adult men, affecting 50% of men over 50 years of age, causing alterations in the morphology and physiology of the urinary bladder. The chronicity of this disorder can lead to irreversible alterations not only in bladder tissue but also in the function of the entire urinary system, which may induce chronic renal failure (Fry 2004, Mirone *et al.* 2004; Nitti 2005; Oka *et al.* 2009).

Structurally, smooth muscle cell hypertrophy and increased intercellular collagen are observed owing to mechanical stress imposed by the IVO. Alterations in the bladder depend on both the duration and severity of the IVO, but the crucial factor regarding the loss of functional integrity of the bladder is intense collagen deposition that characterizes the process of bladder fibrosis (Cortivo *et al.* 1981; Buttyan *et al.* 1997; Flynn *et al.* 2002).

Inflammation usually precedes fibrosis, because the recruitment of inflammatory cells to the site of injury is a part of the process of wound healing. This recruitment is mediated by potent chemoattractant cytokines (Bouchelouche *et al.* 2006; Kisseleva & Brenner 2008). In this context, transforming growth factor (TGF) TGF-β1 is a strategic molecule in the process of tissue fibrosis. It stimulates cell chemotaxis and collagen synthesis and inhibits collagen degradation, and these effects promote fibrogenesis (Chen *et al.* 1994; Baskin *et al.* 1996; Monga *et al.* 2001). It is noteworthy that TGF-β1 activates fibroblast differentiation into myofibroblasts *in vitro* and *in vivo* (Gu *et al.* 2007). Myofibroblasts, in turn, increase osteopontin (Opn) expression, which can also enhance the transition of fibroblasts to myofibroblasts, thus creating a vicious cycle (Pereira *et al.* 2006).

Opn is considered a proinflammatory and profibrotic cytokine, secreted by epithelial cells of several tissues, including

the urothelium. Some authors have suggested that its presence maintains epithelium integrity and promotes adhesion, conferring impermeability to the urothelium (Sodek *et al.* 2006). During the inflammatory processes and wound healing, Opn is detected at high levels and promotes the initial influx of macrophages. Opn is described as a regulator of macrophage functions (Ophascharoensuk *et al.* 1999; Giachelli & Steitz 2000). On the other hand, Opn also seems to have a protective function on fibroblasts and interstitial renal tubule cell survival, as well as acting as an intrinsic inhibitor of cartilage inflammation (Attur *et al.* 2001; Sodek *et al.* 2006).

The majority of the therapeutic approaches for IVO described in the literature involve pharmacotherapy and surgical intervention (Lepor 2005; Nitti 2005; Oka *et al.* 2009). Although this drug is not indicated in patients undergoing bladder outlet obstruction, recent studies have reported the anti-inflammatory and muscle relaxant effects of amitriptyline hydrochloride (Achar *et al.* 2003, 2009), which has been already used clinically to alleviate disorders of the urinary system, such as enuresis, interstitial cystitis and detrusor instability, and to induce the extrusion of urinary calculi. Thus, this aim of this study was to investigate the action of amitriptyline hydrochloride on the process of fibrosis caused by IVO in the rat.

Methods

Animals

The experimental procedures were performed according to the rules of the Ethics Committee of the Federal University of São Paulo. Adult male Wistar rats were subjected to IVO and studied at intervals of 1 and 14 days after the procedure. The rats were divided into five groups: sham (n = 3); IVO1-T (n = 5),comprising animals submitted to IVO and treated with amitriptyline, sacrificed 1 day after surgery; IVO1-NT (n = 5), comprising animals submitted to IVO without any treatment and sacrificed 1 day after surgery; IVO14-T (n = 5), comprising animals submitted to IVO treated with amitriptyline, sacrificed 14 days after surgery; and IVO14-NT (n = 5), comprising animals submitted to IVO without any treatment and sacrificed 14 days after surgery.

Surgical procedures

The induction of partial outlet obstruction was performed by adapting the standard protocol described by Mattiasson and Uvelius (1982). Access to the bladder neck was achieved with minimal prostate dissection, and instead of lower urinary tract catheterization, a 20G angiocatheter was placed juxtaposed externally and a 3-0 silk ligature tied gently but snugly around the infravesical region. The rats were anaesthetized with ketamine (150 mg/kg) and xylazine (5 mg/kg) intraperitoneally. After the induction of partial obstruction, the angiocatheter was removed and the procedure was completed by reconnecting incision and suturing in layers. The sham group was subjected to the same surgical manipulation, but without the obstruction procedure. All the rats received a single prophylactic dose of ceftriaxone sodium 50 mg/kg.

Tissue preparation for analyses

At the end of the experiment, the rats were anaesthetized and the urinary bladder was removed, placed in a recipient with saline and sectioned in the craniocaudal direction. After sectioning, a fragment of the material was immediately frozen in liquid nitrogen for use in RT-PCR, and the other part was fixed in 10% formalin.

Pharmacological treatment

The pharmacological treatment consisted of the administration by gavage of amitriptyline hydrochloride (20 mg/kg/day). This dose was chosen as an average of the doses used for urinary disorders in the literature (Glazener & Evans 2000; Van Ophoven & Hertle 2005; Kelada & Jones 2007). Treatment was initiated 48 h before the procedure and continued once daily for the entire period of the experiment.

Histopathology

Fragments of urinary bladder were paraffin-embedded and cut into 3-μm sections, and the samples were then stained using haematoxylin and eosin (HE), Masson's trichrome (MT), picrosirius red (PR) and Verhoeff (VF). The criteria used in the interpretation of bladder tissue fragments were thickness (mm) and muscular hypertrophy (present, 1; or absent, 0). A semiquantitative scoring was performed to determine the presence and intensity of inflammatory infiltrate and the deposition of collagen and elastic fibres, as following: absent, 0; low, 1; moderate, 2; and intense, 3. Evaluation of these parameters was performed by two independent pathologists.

Immunohistochemical study (IHC)

For IHC, 3-μm-thick sections from vesical blocks had antigen retrieval enhanced by autoclaving slides in sodium citrate buffer (pH 6.0) for 15 min. Endogenous peroxidase activity was quenched by 10 min of incubation in a 3% hydrogen peroxide methanol buffer. The slides were then incubated with anti-CD68 (H-255, Santa Cruz Biotechnology) and anti-Opn antibodies (AKm2A1, Santa Cruz Biotechnology, Santa Cruz, CA, USA), both at 1:100 dilution, and antibodies for anti-TGF-β1 (V. Santa Cruz Biotechnology) at 1:200 dilution in bovine serum albumin (BSA), in a refrigerator at 4 °C for 16–18 h. This was followed by three washes in PBS solution, pH 7.4, for 5 min, following which the slides were incubated with secondary antibody diluted in PBS, pH 7.4, for 30 min at room temperature. Antigen–antibody complexes were detected by the avidin–biotin–peroxidase method (Kit LSAB DAKO, Carpinteria, CA, USA)

using 0.06% diaminobenzidine (DAB) (Sigma) as a chromogenic substrate. The slides were finally counterstained with Harris haematoxylin for 30 s and then examined under a light microscope. The intensity of positive staining was analysed according to the following parameters: no staining, 0; weak staining, 1; moderate staining, 2; and intense staining, 3.

RNA extraction and quantitative real-time PCR

Total bladder RNA was extracted from frozen samples using TRizol® LS Reagent (GIBCO, Invitrogen, CA, USA) according to the manufacturer's recommendations. RNA quality was verified via formaldehyde–agarose gel electrophoresis and by measuring the A_{260}/A_{280} absorption ratio, with a Spectrophotometer NanoDrop ND-1000. cDNA synthesis was performed using a total of 2 μg of RNA, and cDNA was amplified with a Promega kit (Promega, Madison, WI, USA). The cDNA was then amplified using specific primers: GADPH: 5′-TTC TCT TGT GAC AAA GTG GAC ATT G-3′ (sense) and 5′-TGA ACT TGC CGT GGG TAG AGT-3′ (anti-sense); TGF-β1: 5′-GAG AGC CCT GGA TAC CAA CTA CTG-3′ (sense) and 5′-GAT CCA CTT CCA ACC CAG GTC-3′ (anti-sense); Opn: 5′-AGA AAC GGA TGA CTT TAA GCA AGA-3′ (sense) and 5′-CTC TCT GCA TGG TCT CCA TCG-3′ (anti-sense). The samples were then submitted, in triplicate, to 40 thermal cycles on the ABI PRISM® 7300 sequence detector. The ADS program ADS version 1. 9 (Applied Biosystems, Foster City, CA, USA) was used to analyse the dissociation curves. The reactions were performed using the SyBer® Green PCR Master Mix kit (Applied Biosystems).

Statistical analysis

The results are expressed as the mean ± SD. The Mann–Whitney test was used for group comparisons and to test the hypothesis in relation to each of the variables. A 5% level of significance was established for all analysed parameters.

Results

Obstruction caused vesical inflammatory cells infiltration

All obstructed groups presented significant inflammatory cell infiltration when compared to sham group (Figure 1b–e). IVO14 groups showed marked inflammatory process, comprised predominantly of macrophages as can be seen in tissues stained with anti-CD68 (Figure 1g).

Obstructed animals presented thicker bladder wall with hypertrophy and fibrosis

The thickness of the bladder wall increased progressively in the IVO rats compared to the sham group. Hypertrophy of the muscular wall was also evident ($P \leq 0.05$) in all rats of the IVO14 groups (Figure 1c,e). Bladder fibrosis followed a similar pattern, but the IVO14 groups ($P \leq 0.05$) showed more severe fibrosis compared to the sham and IVO1 groups (Figure 2a–c). In a comparison between the IVO14 groups, the amitriptyline-treated rats had a higher intensity of fibrosis. The data and statistical tests are presented in Figure 2d.

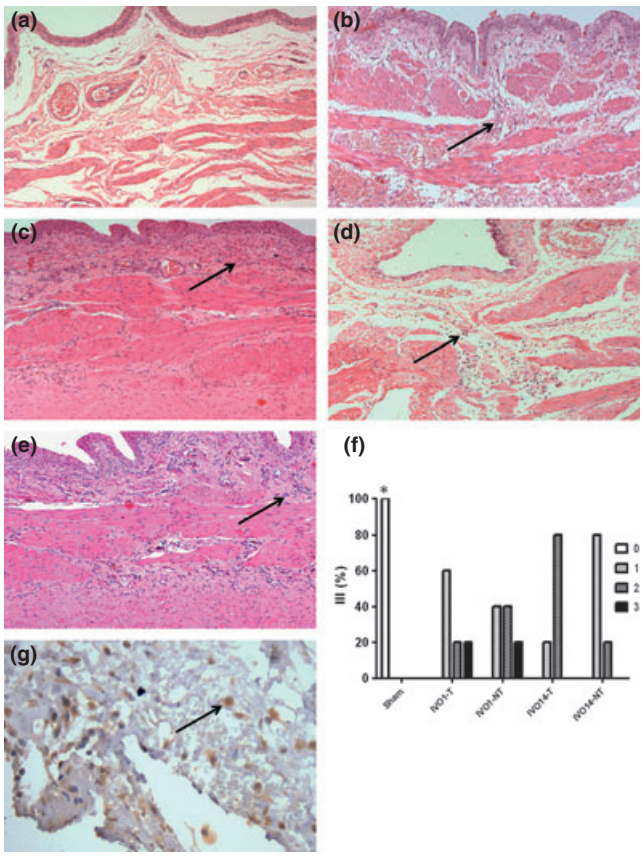

Figure 1 Inflammatory infiltration and thickness of bladder wall. Representative histological sections of (a) sham-operated, (b) IVO1-NT, (c) IVO14-NT, (d) IVO1-T and (e) IVO14-T groups are shown. The inflammatory infiltration was present in all obstructed rats (b, c, d, e and g arrows). (g) Representative micrography of macrophage infiltration in tissue stained with anti-CD68, IVO14-NT. Significant increase in thickness of bladder wall as well as detrusor hypertrophy after 14 days of obstruction (c and e). (f) Index of inflammatory infiltration (III) (0: absent; 1: low; 2: moderate; 3: intense); the highest inflammation index was seen in IVO14-T. *($P < 0{,}05$) *vs.* IVO1-T, IVO1-NT, IVO14-T and IVO14-NT; (HE; a, b, c, d, e ×100) (IHQ CD68; g ×400).

Obstruction caused higher gene expression of profibrotic cytokines

Analysis of *Tgf-β1* gene expression verified that all obstructed groups presented statistically significant higher values compared to the sham group, (Figure 3a). In comparisons between the treated and untreated groups, amplification of *Tgf-β1* was significantly higher in the IVO14-T group compared to the IVO14-NT group ($P \leq 0.05$). Regarding

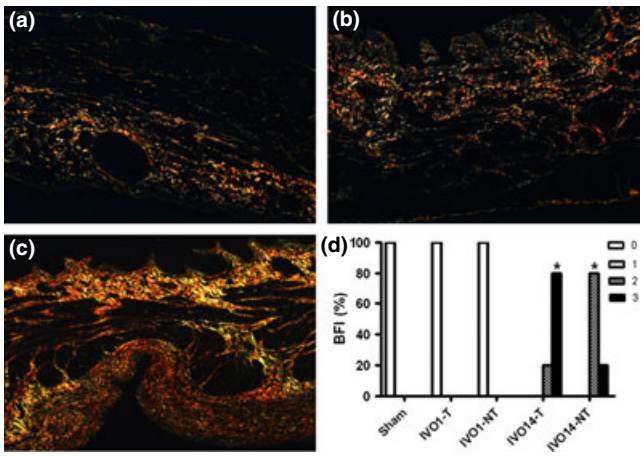

Figure 2 Bladder fibrosis. (a) Sham and (b) IVO1 showing normal collagen deposition; (c) IVO14 presenting intense collagen deposition; in graphic depiction of fibrosis index (d), we can observe that the degree of fibrosis is highest in IVO-14T (a, b and c) picrosirius red with polarization; (d) Bladder fibrosis index (BFI) (0: absent; 1: low; 2: moderate; 3: intense), *($P < 0.05$) *vs.* sham, IVO1-T and IVO1-NT.

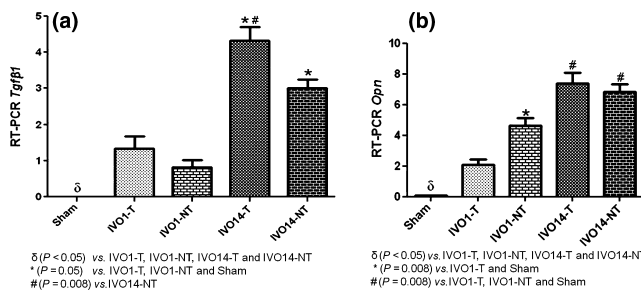

Figure 3 mRNA expression of *Tgf-β1* and *Opn*. (a) Gene expression of *Tgf-β1* is higher than all obstructed groups(δ), mRNA expression is significantly higher in the IVO14-T group compared to IVO14-NT(#), and IVO14 groups present higher expression in relation to IVO1 groups(*). (b) *Opn* gene expression to obstructed groups showed statistically significant increase compared to the Sham group(δ). IVO14 groups present higher expression in relation to IVO1 groups(#). For the same time interval, mRNA expression to *Opn* was significantly higher to IVO1-NT(*).

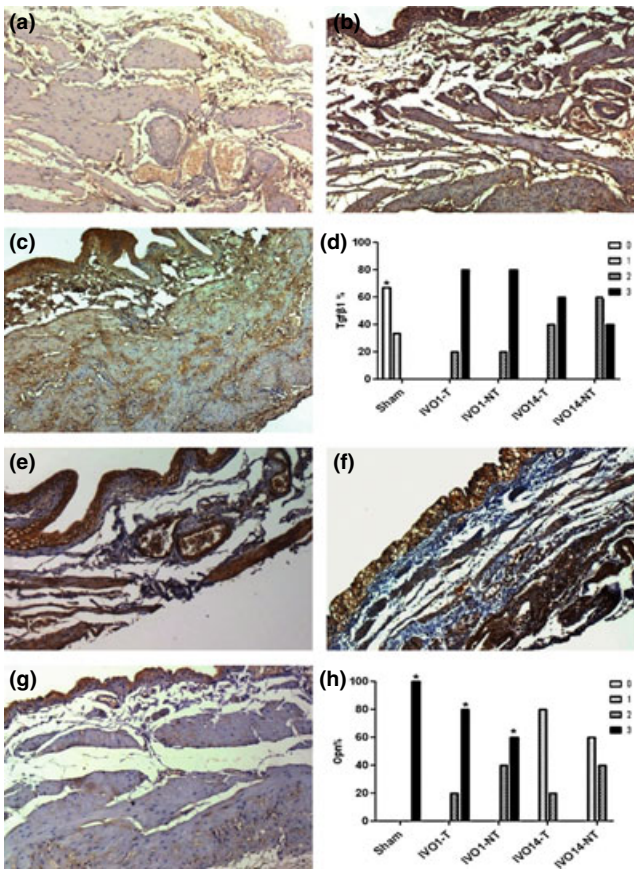

Figure 4 Immunohistochemistry of Tgf-β1 and Opn. Representative pictures of sham, IVO1 and IVO14 groups are shown. Tgf-β1 stained in bladder tissue to IVO1 (b) and IVO14(c) and low expressed to sham group (a); thus, Tgf-β1 expression was significantly higher in all obstructed groups. (d) Index of Tgf-β1 protein expression (0: absent; 1: low; 2: moderate; 3: intense); *($P < 0.05$) *vs.* IVO1-T, IVO1-NT, IVO14-T and IVO14-NT. Opn protein expression was significantly higher to Sham (e) and IVO1(f) groups in relation to IVO14 (g) groups. (h) Index of Opn protein expression (0: absent; 1: low; 2: moderate; 3: intense); *($P < 0.05$) *vs.* IVO14-T and IVO14-NT. (a, b, c, e, f and g × 100).

the time interval, the IVO14 groups showed significantly greater gene expression of this cytokine compared to the IVO1 groups (Figure 3a).

In relation to the *Opn* gene expression, all the IVO groups showed a statistically significant increase compared to the sham group (Figure 3b). When analysing the effect of treatment for the same time interval, it was seen that the IVO1-NT group showed higher *Opn* expression compared with the IVO1-T group ($P \leq 0.01$), while among the IVO14 groups, no significant difference in expression values occurred between the treated and untreated groups (Figure 3b). Comparisons between the groups with regard to treatment time revealed a significant difference, with the IVO14 groups showing greater amplification of *Opn* than the IVO1 groups (Figure 3b).

Proteins were differentially expressed in obstructed groups

Regarding TGF-β1 protein expression, all groups showed a statistically significant increase when compared to the sham group (Figure 4a-c). When the IVO groups were compared with each other, no differences were observed with respect to time or treatment. Data are shown in Figure 4d.

On the other hand, Opn protein expression, as assessed by immunohistochemistry, was higher in sham-operated and day 1 IVO groups when compared to day 14 IVO group,

suggesting a decrease in Opn during disease development (Figure 4e–g), and table with data are shown in Figure 4h.

Discussion

The development of the process of bladder fibrosis involves numerous molecular mechanisms that are still poorly elucidated, and their knowledge could be strategic in trying to intervene in the progression of this injury. Currently, numerous clinical and experimental studies seek to clarify each of these pathophysiological mechanisms while focusing on developing new strategies for intervention in this process. Despite such efforts, it is still not possible to prevent the fibrotic process in the urinary bladder or its complications. Regardless of aetiology, the initial injury is followed by a common morphological pattern, with replacement of specialized cells by fibroblasts and collagen deposition, causing alterations in bladder function (Kisseleva & Brenner 2008).

It is well established that tissue fibrosis is usually preceded by inflammation. In agreement with this, the present study verified the presence of inflammatory infiltrate in all the groups that were subjected to experimental bladder outlet obstruction, regardless of treatment. As expected in acute processes, the IVO1 group infiltrate was primarily neutrophilic. In addition, some animals also showed intense haemorrhaging, a reaction that can also occur in such acute events (de Boer *et al.* 1994; Tagaya *et al.* 2009). The IVO14 groups continued to demonstrate inflammatory infiltration, but the cell types were different, consisting mainly of macrophage. Similar results were also reported in several previous studies (de Boer *et al.* 1994; Amaro *et al.* 2005; Bouchelouche *et al.* 2006; Anumanthan *et al.* 2009). What caught our attention in this study is that amitriptyline did not affect the inflammatory infiltration process, which was interesting because studies have shown that tricyclic antidepressants (TCAs) inhibit the release of inflammatory mediators, such as IL-6, IL-1β and TNF-α (Xia *et al.* 1996; Yaron *et al.* 1999; Tai *et al.* 2009). In the case of fluoxetine and amitriptyline, Yaron *et al.* (1999) showed that these drugs inhibit the release of nitric oxide and prostaglandin E2 in synovial cells. Moreover, Tai *et al.* (2009) observed that amitriptyline inhibits the expression of proinflammatory cytokines through increased IL-10, corroborating the concept that these TCAs may act as inflammatory suppressors (Xia *et al.* 1996; Yaron *et al.* 1999; Achar *et al.* 2003; Tai *et al.* 2009). However, studies exist reporting possible cytotoxic effects of amitriptyline (Viola *et al.* 2000; Kitagawa *et al.* 2006; Lirk *et al.* 2006; Moreno-Fernandez *et al.* 2008; Cordero *et al.* 2009), manifested through lipid peroxidation, decreased levels of cytochrome C and other alterations that indicate mitochondrial injury, suggesting that amitriptyline induces oxidative stress, which is known to increase the intensity of the inflammatory response.

In relation to fibrogenesis, consistent with reports in the literature, the rats in the study groups developed fibrosis 14 days after obstruction, independent of treatment (Cortivo *et al.* 1981; Amaro *et al.* 2005; Rubinstein *et al.* 2007). It is noteworthy that more pronounced fibrosis was verified in rats treated with amitriptyline. This trend was not observed in the groups evaluated after 1 day, which leads to speculation that amitriptyline may influence the natural course of fibrosis.

In the obstructed rats, collagen deposition was observed throughout the urinary bladder wall simultaneously with muscular hypertrophy. Observation of hypertrophy of the detrusor tunic was expected, reflecting the compensatory response observed early in chronic bladder outlet obstruction (Zhang *et al.* 2004; Amaro *et al.* 2005; Lepor 2005; Fujita *et al.* 2006). Amitriptyline did not alter the pattern of hypertrophy observed in the experimentally obstructed rats.

In relation to gene and protein expression of cytokines and growth factors that modulate fibrosis, we found that obstructed rats presented higher values of TGF-β1. As it is known that macrophages are the major source of this cytokine in fibrous tissues (Flynn *et al.* 2002; Khan & Sheppard 2006; Kisseleva & Brenner 2008; Anumanthan *et al.* 2009), these findings suggest that such values are directly related to the higher macrophage infiltration observed in the rats, which is consistent with the greater intensity of fibrosis observed. The fact that the rats treated with amitriptyline showed greater macrophage infiltration and increased intensity of fibrosis suggests that this drug may exert deleterious effects in cases of infravesical outlet obstruction. Although no similar work exists in the literature involving amitriptyline, most studies demonstrate altered expression of TGF-β1 in obstructed urinary bladders. Some authors report that TGF-β1 expression shows lower values in the first few hours after bladder outlet obstruction and that these values continue to increase concomitantly with the onset of chronicity of the injury and the onset of tissue fibrosis (de Boer *et al.* 1994; Chen *et al.* 1994; Santarosa *et al.* 1994; Baskin *et al.* 1996; Fujita *et al.* 2006; Ku *et al.* 2006; Tagaya *et al.* 2009).

Regarding Opn protein expression, the higher values observed in the sham and IVO1 groups were probably caused by basal synthesis of this molecule in normal urinary bladders. Arafat *et al.* (2002) and Brown *et al.* (1992) demonstrated the protein expression of this molecule in normal urothelium and the detrusor layer, suggesting that Opn has an important role in the adhesion and communication of luminal epithelial cells and assists in maintaining the impermeability of the urothelium cell. In addition, other studies with different models have reported greater Opn protein expression in the first few days after injury (Diamond *et al.* 1995; Ophascharoensuk *et al.* 1999; Giachelli & Steitz 2000; Achar *et al.* 2009; Wang *et al.* 2009). The most intriguing in our finding concerning the expression of this molecule is the paradox between gene and protein expressions. Once Opn is mainly detected in the urothelium, the decrease in protein expression in obstructed rats could be associated with the lesion of this layer, caused by stretching or ischaemia and reperfusion, which occurs in bladder tissue owing to IVO. In the case of amitriptyline, besides the injury itself, the obstruction could also elicit the mitochondrial

damage and increased oxidative stress that this drug causes among the fibroblasts and may be a factor in the diminished synthesis of Opn (Viola *et al.* 2000; Kitagawa *et al.* 2006; Lirk *et al.* 2006; Moreno-Fernandez *et al.* 2008; Cordero *et al.* 2009). Another aspect that should be emphasized in relation to Opn and fibrosis, which strengthens our findings, is that this molecule appears to have a protective function in the process of tissue regeneration and survival of certain cell types, while its reduction shows deleterious effect on the process (Diamond *et al.* 1995; Ophascharoensuk *et al.* 1999; Denhardt *et al.* 2001).

Thus, under the conditions of the experimental model used in this study, it can be concluded that amitriptyline showed amplifying effects on the process of fibrosis as a whole, reflected by increased intensity in the infiltration of inflammatory cells and collagen deposition. It was not possible to clearly determine the mechanism responsible for these effects in this study, but it seems reasonable to suggest that this was attributable to the modulation of molecules involved in this process. It is clear that further investigation is required to broaden current understanding of the process of bladder fibrosis.

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