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The Effect of Tamoxifen on Bladder Functions and Histology, and the Role of Estrogen Receptor β in a Rat Chemical Cystitis Model

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Aims: The purpose of this study was to investigate the effect of tamoxifen citrate on bladder functions and histology, and also to investigate the role of estrogen receptor β (ER β) in a rat chemical cystitis model. **Methods:** The study included 37 female Sprague–Dawley rats. Chemical cystitis was induced by intravesical instillation of hydrochloric acid in 32 rats, and the treatment group (n = 15) received daily 0.4 mg/kg of tamoxifen citrate with orogastric tube, and the control group (n = 17) received no treatment. The sham group consisted of five rats having no acid instillation and no treatment. Cystometric studies were performed in all rats at the beginning and end of the experiment. The rats were euthanized at 2 months. The bladders were removed and examined histologically for mast cells, inflammatory cells, and ER β. **Results:** The mean maximal bladder volume increased by 73.6% ± 25.2 in the treatment group and decreased by 7.2% ± 10.8 in the control group, revealing a significant difference ($P = 0.007$). The mean bladder compliance increased by 81.2% ± 25.2 in the treatment group and decreased by 4.8% ± 12.7 in the control group, revealing a significant difference between the two groups ($P = 0.005$). ER β positive cells were significantly lower in the bladders with chronic cystitis than in the sham group ($P = 0.038$). **Conclusions:** Tamoxifen citrate may be an alternative choice, as easy, to other treatment options in the treatment of chronic inflammatory condition to improve deteriorated bladder function. In addition, ER β may have a role on chronic bladder inflammation in a rat chemical cystitis model. *Neurourol. Urodynam. 26:309–316, 2007.* © 2006 Wiley-Liss, Inc.

Key words: bladder; bladder capacity; chronic cystitis; estrogen receptor β; tamoxifen; urodynamics

INTRODUCTION

Chronic inflammatory disease of the urinary bladder is generally characterized by urinary frequency, urgency, and pelvic pain with bladder distention. Interstitial cystitis is one of the most commonly seen chronic inflammatory diseases of the urinary bladder [Gillenwater and Wein, 1998]. Although several theories have been proposed to explain the pathogenesis of interstitial cystitis, it remains unknown [Ratliff et al., 1994].

Severe chronic inflammation of the bladder is associated with reduced bladder capacity and compliance. Although the precise cellular mechanisms of impaired bladder capacity and compliance remain undefined, clinical experience has demonstrated that poor bladder capacity and compliance can be dramatically improved by surgical augmentation of bladder volume utilizing intestinal segments, botulinum toxin injections, or anticholinergic drug therapy to decrease detrusor tonicity [Barbalias et al., 2000; Hohenfellner et al., 2000; Çayan et al., 2002, 2003]. The goal of treatment modalities is the creation of low-pressure bladder storage function with increased bladder capacity or inactivation of bladder sensory nerve endings. However, the high incidence of systemic anticholinergic side effects with medical treatment, and time-dependent improvement and requirement of repeated injections with botulinum toxin and short- and long-term complications of surgical treatment may limit long-term patient compliance [Awad et al., 1998; Schwantes and Topfmeier, 1999].

Tamoxifen citrate is a nonsteroidal nonselective high affinity estrogen receptor blocker, and has been used in the treatment of various diseases including breast cancer, male infertility, and gynecomastia [Kadioglu et al., 1999; Khan et al., 2004; Veronesi et al., 2005]. However, to our knowledge,

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no study has investigated the effect of tamoxifen in the treatment of chronic inflammatory bladder diseases such as chronic cystitis and interstitial cystitis. Thus, we hypothesized that tamoxifen citrate would be an attractive alternative treatment to improve deteriorated bladder capacity and compliance in a diseased bladder.

Estrogen receptors α (ER α) and β (ER β) are usually localized in many tissues including urogenital organs [Makela et al., 2000]. The predominant isoform of estrogen receptors in the nuclei of epithelial and smooth muscle cells of bladder is ER β [Saunders et al., 1997; Taylor and Al-Azzawi, 2000]. The aim of this study was to investigate the effect of tamoxifen citrate on bladder function and histology in a rat chemical cystitis model, and also to investigate the role of ER β in these bladders.

MATERIALS AND METHODS

Animals and Study Design

The experimental protocol was approved by the Committee on Animal Research at the University of Mersin School of Medicine. In this study, 37 female Sprague–Dawley rats, 200–300 g, were used: 15 in the treatment group, 17 in the control group, and 5 rats with no acid instillation and no treatment as a sham group. In all of the treatment and control groups, chemical cystitis was induced by intravesical instillation of HCl, and this was repeated monthly to maintain chronic inflammation. One week after HCl instillation, all rats underwent urodynamic study to determine baseline bladder pressures, capacity, and compliance before the treatment. After performing baseline urodynamic studies, the rats in the treatment group received tamoxifen citrate 0.4 mg/kg daily via orogastric tube, and the rats in the control group received no treatment. No anesthesia was used for the treatment with tamoxifen citrate. Cystometric studies were repeated in all rats before euthanasia. The timing of the interventions and evaluation was not standardized with respect to the reproductive cycle of the rats. The rats were euthanized 1 week after the last acid instillation at 2 months (15 treated, 17 control, and 5 sham). The bladders were removed and examined histologically for mast cells, inflammatory changes and ER β activity.

Induction of Chemical Cystitis

Chemical cystitis was induced by intravesical instillation of hydrochloric acid (0.2 ml of 0.4 N HCl), as described by Rivas et al. [1997] and also as used in our previous studies [Çayan et al., 2002, 2003, 2006]. Rats were anesthetized with ketamine (50 mg/kg) and, under sterile conditions, a 22 G catheter was inserted transurethrally. After all the urine was aspirated, HCl was instilled into the bladder lumen for 4 min. This instillation was repeated monthly to maintain chronic inflammation. The treatment and control groups received intravesical instillation of HCl three times. At catheter insertion, the rats received ciprofloxacin (20 mg/kg i.m.) to prevent urinary infection.

Treatment Protocol

One week after the initial HCl instillation the rats were anesthetized with ketamine (50 mg/kg). The rats received a single 20-mg/kg dose of ciprofloxacin. After performing urodynamic study, the treatment group received (0.4 mg/kg) of tamoxifen citrate dissolved in water via orogastric tube daily (Nolvadex®, Astra-Zeneca, London, UK), giving no anesthesia. No treatment was given to the rats in the control group.

Functional Evaluation

Urodynamic studies were performed before the treatment and just before the euthanasia, as we previously described [Çayan et al., 2002, 2003, 2006]. All rats underwent cystometry under ketamine anesthesia (50 mg/kg i.m.). A 22 G catheter was inserted transurethrally and connected by a polyethylene tube to a pressure transducer using urodynamic equipment (Life-Tech, Inc., Houston, TX) and a computer program (Urolab Primolus, Life-Tech, Inc., Houston, TX). After the measurement of residual urine volume at the time of catheter insertion, each rat underwent cystometric measurements with an infusion of warmed (37°C) normal saline solution at 0.1 ml/min (Abbott infusion pump). During the study, the baseline pressure (empty bladder), opening pressure (at first leakage) peak pressure (maximal pressure during voiding), and maximal bladder capacity were recorded. Bladder compliance (ml/cm H_20) was calculated according to the following formula: Compliance = Maximal bladder volume/Opening pressure − Baseline pressure.

Histological Evaluation

The bladder was removed through a lower midline abdominal incision. After the removal of the bladder, the rats were euthanized by pentobarbital overdose (200 mg/kg) and bilateral thoracotomy. After both ureters were ligated, the bladder was filled with 10% formalin solution through a catheter transurethrally and kept distended overnight. The specimen was split longitudinally, and routine tissue processing for light microscopy was performed.

Bladder tissue was embedded in paraffin. Sections (4 μm) were cut by microtome and stained with hematoxylin and eosin (H&E) to assess inflammatory changes with the number of leukocytes, and toluidine blue for mast cells. Slides were examined by an Olympus BX50 light microscope and photographed by an Olympus PM10SP photograph system.

Leukocyte infiltration was evaluated to determine the severity of inflammation that resulted from intravesical

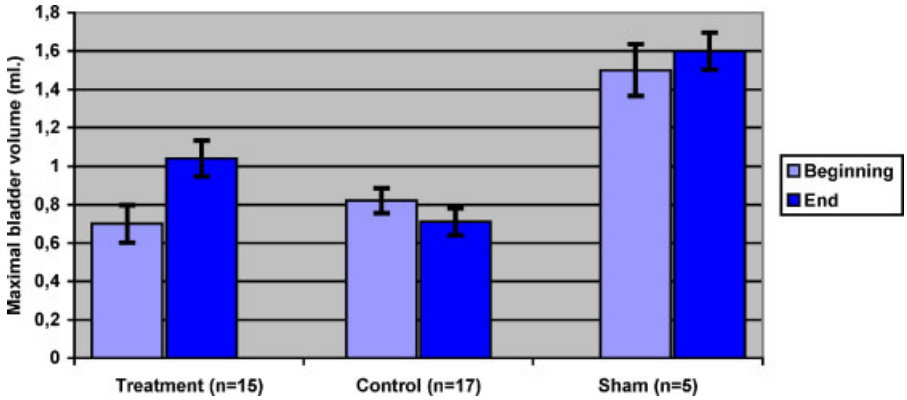

Fig. 1. The mean maximal bladder volume at the beginning and at the end of the study for the treatment, control, and sham groups. The difference in the increase or decrease of the mean maximal bladder volume was statistically significant between the treatment and control groups ($P = 0.02$).

instillation [Çayan et al., 2002, 2003, 2006]. Each section was divided into 10 subsections, and leukocytic infiltration was examined in each of the subsections with the following scale: 0—no extravascular leukocytes; 1—<20 leukocytes/high power field (HPF); 2—20 to 45 leukocytes/HPF; 3—>45 leukocytes/HPF. The total score for all subsections was divided by the maximal possible score (30 in 10 subsections of the section) and multiplied by 100.

The total number of mast cells was counted in 10 random sections of the bladder from each rat. The average number of mast cells was used for comparison [Çayan et al., 2002, 2003, 2006].

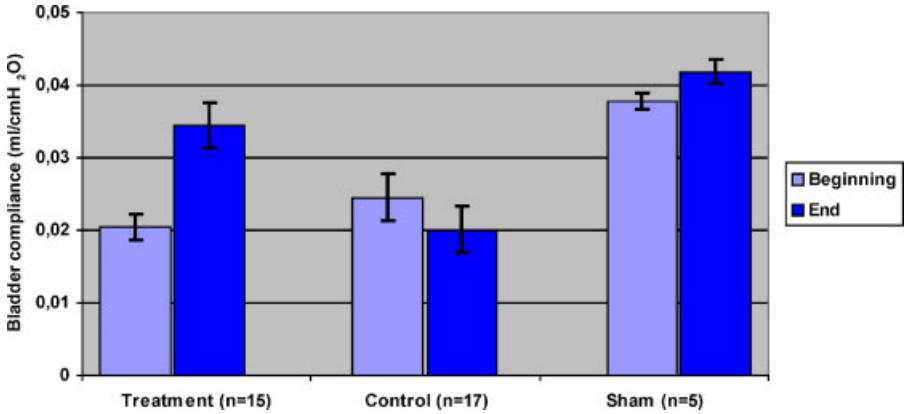

Fig. 2. The mean bladder compliance at the beginning and at the end of the study for treatment, control, and sham groups. The difference in the increase or decrease of the maximal bladder compliance was statistically significant between the treatment and control groups ($P = 0.01$).

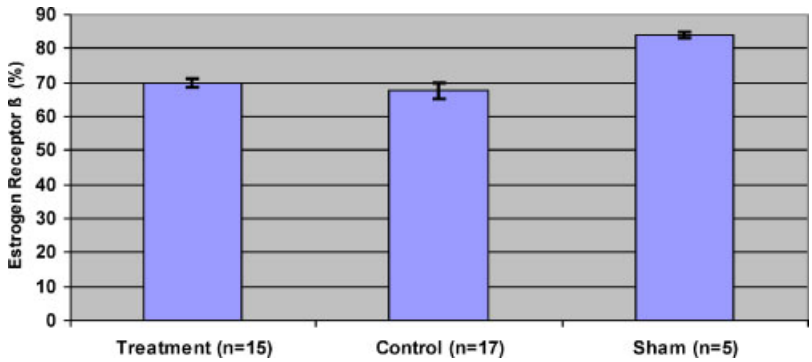

Fig. 3. ER β in the three groups. Note that the difference in ER β was statistically significant in the treatment and control groups as compared with the sham group ($P = 0.038$, $P = 0.049$, respectively).

Immune Histochemical Evaluation

Paraffin sections were dewaxed (xylene 3 × 5min) and rehydrated by passing through graded alcohol series and rinsed in water. Sections were tripsinized and endogenous peroxidase activity was blocked by 3% H_2O_2 for 10 min. To block nonspecific antibody bindings, sections were treated with 2% diluted normal goat serum in phosphate buffered saline (PBS) for 1 hr. One per three hundred diluted polyclonal rabbit antiestrogen primary antibody (Santa Cruz Biotechnology, sc-8974, Santa Cruz, CA) was dropped on the sections and incubated overnight in a humidity chamber at +4°C. The following day, sections were incubated with biotinylated secondary goat antirabbit antibody (Jackson Immunoresearch Laboratories, West Grove PA) at room temperature for 1 hr. 3,3′-diaminobenzidine tetra-hydrochloride (DAB) was used for visualization of antibody binding. The sections were counterstained with hematoxylin. PBS including 0.5% bovine serum albumin (BSA) and no primary antibody was dropped on the sections separated for negative control. Images were captured using the same equipment above. For quantitative evaluation, in each section, starting from a randomized area, positive and negative totally 100 epithelial cell nuclei were counted. Positive/negative cell nuclei ratio was calculated. In addition, we examined ER β in the smooth muscle, however, cytoplasmic ERB was expressed diffusely and intensely in smooth muscle, and no different staining pattern was observed. Therefore, in light of these findings, we thought that comparing epithelial ER β expression is more useful than smooth muscle cells.

Statistical Analysis

Statistical analyses were performed using the Anova test to compare body weight, differences in cystometric findings among the treatment, control, and sham groups, the "paired *t*-test" to compare cystometric findings from pretreatment to post treatment for each group, the "independent *t*-test" to compare differences in cystometric findings at the beginning and end of each experiment between the treatment and control groups, body weights between the two groups, degree of leukocytic infiltration, number of mast cells, and expression of ER β between the two groups. In addition, one "Spearman correlation test" was performed to investigate the correlation of ER β with the number of mast cells, and leukocyte infiltration. Data are presented as mean ± Standard Error (SE) for all findings. Probability values of <0.05 were considered statistically significant.

RESULTS

Functional Study Findings

No significant differences in the mean body weight were observed between the treatment, control, and sham groups ($P = 0.622$).

In all rats, no significant differences were noted at the beginning and at the end of the experiment in the residual urine volume, baseline pressure and maximal voiding pressure between the treatment, control, and sham groups ($P = 0.064$, $P = 0.371$, and $P = 0.342$, respectively). Figures 1 and 2 show the mean maximal bladder volume and bladder compliance at the beginning and end of the experiment in the treatment,

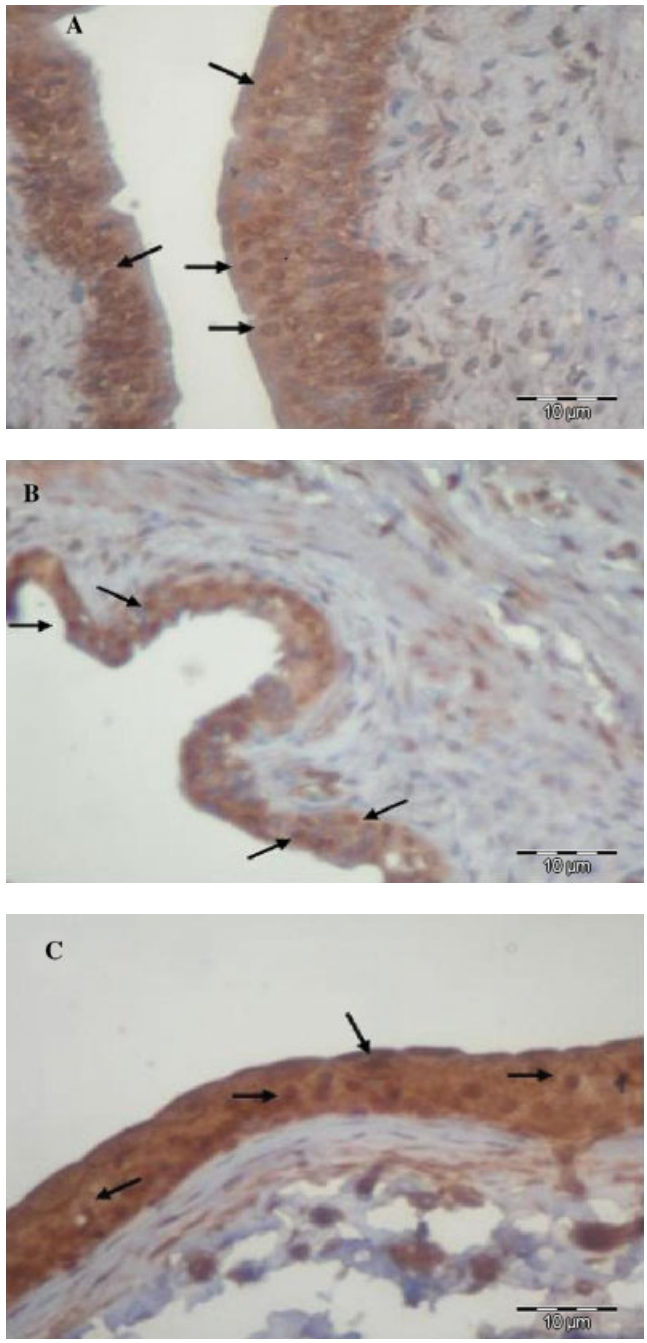

Fig. 4. Immunohistochemical staining of ER β in the bladder of each rat in the treatment (**A**), control (**B**), and sham groups (**C**). Arrows show ER β (+) staining nuclei of the epithelium. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

control, and sham groups. The mean maximal bladder capacity increased from 0.7 ± 0.1 to 1.04 ± 0.1 ml in the treatment group, and decreased from 0.82 ± 0.07 to 0.71 ± 0.07 ml in the control group (Fig. 1). The difference in the increase or decrease of the mean maximal bladder volume was statistically significant between the two groups ($P = 0.02$). As shown in Figure 2, the mean bladder compliance increased from 0.0204 ± 0.0020 to 0.0344 ± 0.0032 ml/cm H_2O in the treatment group and decreased from 0.0245 ± 0.0034 to 0.0201 ± 0.0029 ml/cm H_2O in the control group, revealing statistical significance between the two groups ($P = 0.01$). There was no significant difference according to the mean maximal bladder volume and compliance changes in the sham group ($P = 0.44$ and $P = 0.21$, respectively). However, when compared with the control group, the differences in the maximal bladder volume and compliance were statistically significant in the sham group ($P = 0.01$ and $P = 0.02$, respectively).

As a percentage, the mean maximal bladder volume increased 73.6% ± 25.2 in the treatment group and decreased 7.2% ± 10.8 in the control group, revealing significant difference ($P = 0.007$). The mean bladder compliance increased 81.2% ± 25.2 in the treatment group and decreased 4.8% ± 12.7 in the control group, revealing significant difference between the two groups ($P = 0.005$). In the sham group, mean maximal bladder volume and compliance changes were 8.9% ± 5 and 10.7% ± 4.2, and these changes were not significant ($P = 0.440$, $P = 0.212$, respectively).

Estrogen Receptor β and Bladder Capacity

The percentage of ER β positive cells was 84% ± 1.16 in the sham group, 67.7% ± 3.5 in control the group, and 69.67% ± 1.9 in the treatment group (Fig. 3). The difference in ER β was statistically significant in the treatment and control groups compared with the sham group ($P = 0.038$, $P = 0.049$, respectively) (Fig. 4A–C).

In all rats, the relationship between ER β and bladder function was noted. In all rats, at baseline, the mean maximal bladder volume was 0.67 ± 0.06 in the presence of ER β of ≤70% and 1.15 ± 0.19 ml in the presence of ER β of >70%, revealing significant difference between the two groups ($P = 0.017$). As shown in Figure 5, in the presence of ER β of ≤70%, in the treatment group (n = 8), the mean maximal bladder volume increased from 0.53 ± 0.04 to 0.91 ± 0.12 ml after the treatment, revealing significant difference ($P = 0.043$). In the control group (n = 8), the mean maximal bladder volume decreased from 0.85 ± 0.1 to 0.63 ± 0.1 ml at the end of the study, revealing no statistical significance ($P = 0.169$). In the presence of ER β of >70%, in the treatment group (n = 7), the mean maximal bladder volume increased from 1.30 ± 0.15

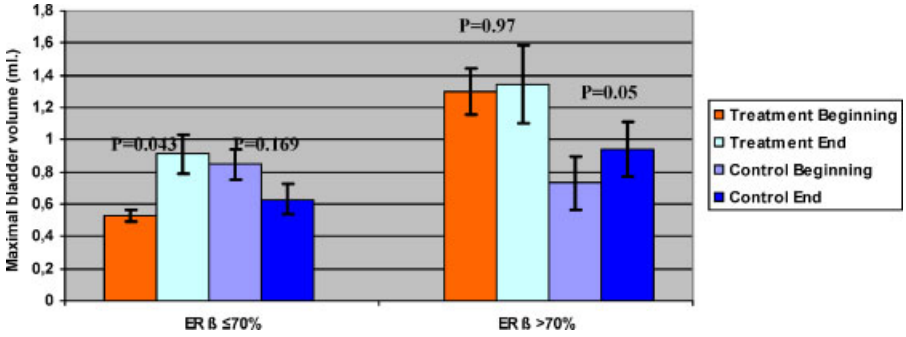

Fig. 5. The difference in the mean maximal bladder volume from the beginning to the end of the study between the treatment and control groups according to ER β of ≤ 70% or >70. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

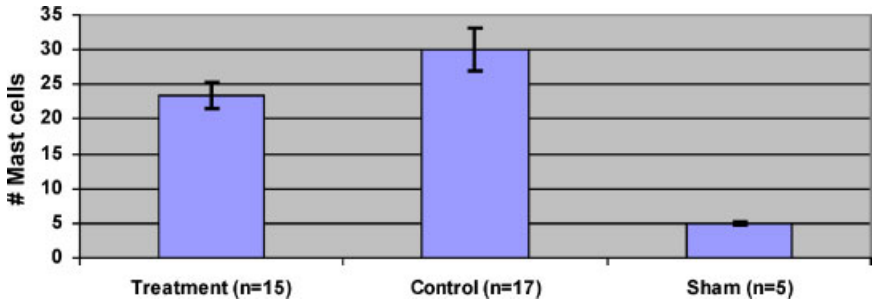

Fig. 6. Mast cell numbers in the treatment, control, and sham groups. There are no significantly differences in the mean number of mast cells between the treatment and control groups ($P = 0.095$). Significantly differences were observed in treatment and control groups compared with the sham group ($P = 0.000$ and $P = 0.000$, respectively). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

to 1.34 ± 0.25 ml after the treatment, revealing no significance ($P = 0.970$). In the control group (n = 9), the mean maximal bladder volume increased from 0.73 ± 0.17 to 0.92 ± 0.17 ml at the end of the study, revealing significant difference ($P = 0.051$).

Histological Findings

Figure 6 shows the number of mast cells in the treatment, control, and sham groups. No significant differences were observed in the mean number of mast cells between the treatment and control groups, although lower mast cell counts were seen in the treatment group than in the control group ($P = 0.095$). However, the number of mast cells in the treatment and control groups was significantly higher statistically than in the sham group ($P = 0.000$ and $P = 0.000$, respectively) (Fig. 7–C). ER β and the number of mast cells was not correlated ($P = 0.79$).

Figure 8 shows the degree of leukocyte infiltration in the treatment, control, and sham groups. No significant differences were observed in the degree of leukocyte infiltration between the treatment and control groups ($P = 0.371$). The degree of leukocyte infiltration in both the treatment group and the control group was significantly higher than in the sham group ($P = 0.000$ and $P = 0.000$, respectively). As shown in Figure 9–C leukocyte infiltration was similar in the bladder of each rat in the treatment and control groups. ER β and leukocyte infiltration was positively correlated. The correlation was statistically significant ($P = 0.046$).

DISCUSSION

The current treatment options for decreased bladder capacity, and/or compliance are either intravesical or oral use of anticholinergic agents or botulinum toxin injections [Barbalias et al., 2000; Çayan et al., 2003]. When insufficient efficacy of medical treatment or botulinum toxin injections is seen, surgical options of either autoaugmentation or augmentation with detubularized gastrointestinal segments may be used to improve deteriorated bladder capacity and compliance [Awad et al., 1998; Hohenfellner et al., 2000; Çayan et al., 2002]. However, common systemic side effects of medical treatment, time-dependent improvement, requirement of repeated injections with botulinum toxin, and short- and long-term complications of surgical options, led clinicians to investigate new treatment modalities [Awad et al., 1998; Schwantes and Topfmeier, 1999].

Tamoxifen is a synthetic nonsteroidal antiestrogen with high affinity for estrogen receptors. Most of its effects are elicited via estrogen receptor-independent routes. Tamoxifen has been used in various diseases including breast cancer, male infertility, and gynecomastia modalities [Kadioglu et al., 1999; Khan et al., 2004; Veronesi et al., 2005]. However, to our knowledge, no study has investigated the effect of tamoxifen in the treatment of chronic inflammatory bladder diseases such as chronic cystitis and interstitial cystitis.

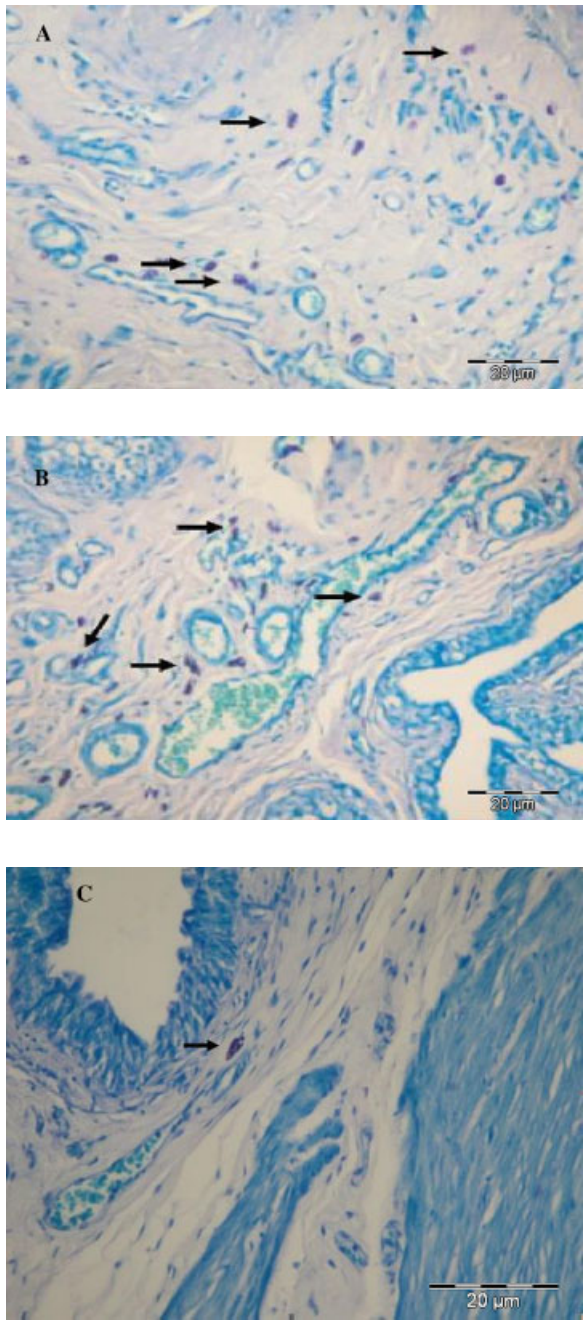

Fig. 7. Toluidine blue staining to show mast cells (arrows). **A**: Mast cells in the bladder of a rat in the treatment group. **B**: Mast cells in the bladder of a rat in the control group. **C**: Mast cells in the bladder of a rat in the sham group. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Thus, tamoxifen might be an attractive alternative treatment to improve deteriorated bladder capacity and compliance in a diseased bladder. In our study, the mean maximal bladder volume and compliance significantly increased after the treatment with tamoxifen in the treatment group and significantly decreased in the control group, revealing a highly significant difference between the treatment and control groups.

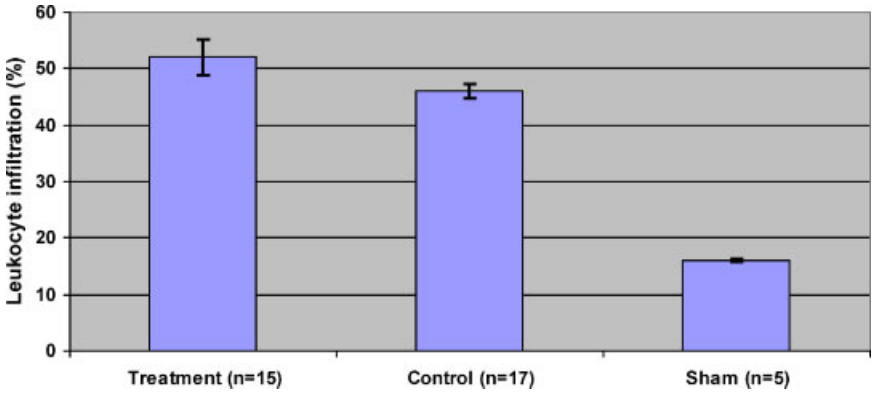

Fig. 8. The degree of leukocyte infiltration in the treatment, control, and sham groups. There were no significantly differences in the degree of leukocyte infiltration between the treatment and control groups ($P = 0.371$). However, significantly differences were observed in treatment and control groups compared with the sham group ($P = 0.000$ and $P = 0.000$, respectively).

Tamoxifen has been reported to inhibit protein kinase C and calmodulin, to protect against oxidative damage and to induce apoptosis [Wiseman, 1995; O'Brian et al., 1985; Mandlekar and Kong, 2001]. In addition to its antiestrogen activity, tamoxifen has also a selective estrogen modulator effect. It has been known that estradiol has a significant impact on immune responsiveness, inflammation, thymic functions, and morphology [Carlsten et al., 1989]. Our previous experimental study showed that bladder functions and histology deteriorated much more with chronic cystitis in ovariectomized rats, and chronic inflammatory bladder improved by estrogen replacement therapy in ovariectomized rats [Çayan et al., 2006]. Estrogen exerts actions via specific nuclear protein receptors that are members of the steroid/thyroid receptor superfamily of transcription factors. Neurogenic inflammation has been suggested to play a role in the pathogenesis of chronic inflammatory diseases such as interstitial cystitis [Bjorling and Wang, 2001]. Estrogen may also modulate neurogenic inflammation by interaction with other substances and cells that participate in the pathogenesis of neurogenic inflammation, including substance P, bradykinin, and mast cells [Bjorling and Wang, 2001]. In addition, there is a strong negative correlation between estrogen and plasma concentrations of substance P, suggesting that estrogen can alter the degradation of substance P.

In our study, the number of mast cells was significantly higher in the treatment and control groups than in the sham group, suggesting that mast cells play a major role in chronic cystitis [Pang et al., 1995; Spanos et al., 1996]. Vliagoftis et al. [1992] reported that tamoxifen might inhibit mast cell secretion in rats. However, in our study, lower mast cell counts were observed in the treatment group than in the control group, although the difference was not statistically significant. Mast cells release cytokines, kinins, histamine, leukotrienes, prostaglandins, and proteases, supporting our findings that the degree of leukocyte infiltration was significantly higher in treatment and control groups than in the sham group. However, in our study, leukocyte infiltration was similar in the bladder of each rat in the treatment and control groups.

ER α and ER β are usually localized in many tissues including accessory sex glands in male rats and the lower urinary tract of both sexes [Makela et al., 2000]. ER α was seen in kidney, bladder smooth muscle and epithelial cells, Sertoli and Leydig cells and furthermore in the ER β in addition to epididymis, prostate and urethral epithelial cells. However, ER β appears to be the predominant estrogen receptor type in the urinary bladder and urethral epithelium of both sexes [Saunders et al., 1997; Taylor and Al-Azzawi, 2000]. Therefore, we investigated the role of ER β in this experimental chronic cystitis model. In our study, we observed significantly lower ER β in the bladder of chemical cystitis-induced rats than normal rats, suggesting that ER β might be used as a diagnostic parameter. We also observed the relationship between ER β and bladder functions after treatment with tamoxifen. ER β in the bladders with chronic cystitis was significantly lower than the ER β in the normal bladder, and the prognosis was worse in the bladders with ER β of $\leq$70%, but the recovery was also more significant in those bladders. Our findings suggest that ER β may be used in the diagnosis of chronic inflammatory bladder, and might have a role to predict clinical progress. However, further studies are needed to support these findings.

CONCLUSIONS

Tamoxifen citrate may be an alternative choice in the treatment of chronic inflammatory conditions to improve deteriorated bladder function. This is the first study in the literature showing that there is a significant decrease in ER β in the bladders of rats with experimentally induced chemical cystitis compared with the normal bladders. The prognosis was worse in the bladders with ER β of $\leq$70%, but the recovery was also more significant in those bladders. These findings suggest that ER β may have a role in predicting the prognosis of chronic bladder inflammation and also to predict a response

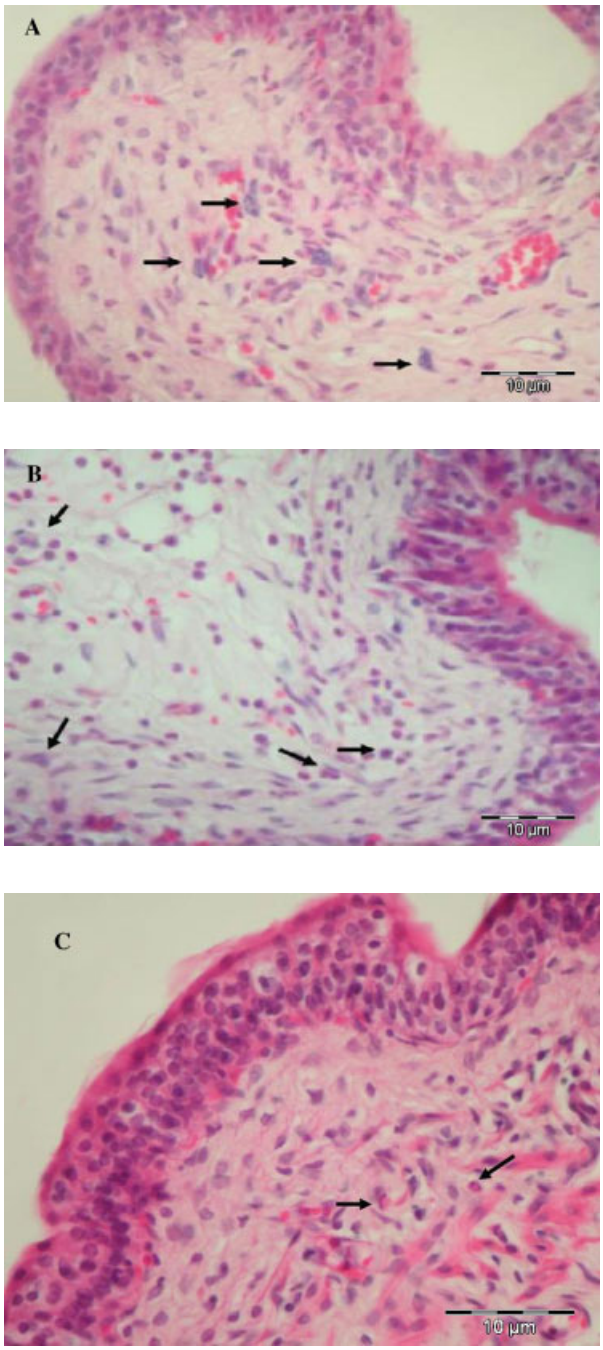

Fig. 9. Histologic appearance of the bladder in a rat of the treatment group (**A**), control group, (**B**) and sham group (**C**) with Hematoxylin and eosin staining. Arrows show leukocyte infiltration. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

to tamoxifen treatment in a rat chemical cystitis model. However, further studies are needed to support these findings.

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