Effect of Cernitin Pollen-Extract on Experimental Nonbacterial Prostatitis in Rats

Toshiyuki Kamijo,[1*] Shiegeru Sato, [2] and Tadaichi Kitamura[1]

[1] *Department of Urology, Faculty of Medicine, University of Tokyo, Japan*

[2] *Ohme Research Laboratories, Tobishi Pharmaceutical Co., Ltd., Tokyo, Japan*

BACKGROUND

The treatment for chronic nonbacterial prostatitis (NBP) has not been established. Cernitin pollen extract (CN-009) is reported to have therapeutic effects for NBP. The effects and mechanisms of CN-009 were investigated.

METHODS

Ten-month-old rats were used with administration of estradiol after castration, which were similar to human NBP histologically. Since CN-009 consists of T-60 and GBX, these drugs were administered, respectively. The prostate was evaluated histopathologically including glandular damage (epithelial score), stromal ratio and immunohistochemical assays for epithelial function (PAP), stromal evaluation (Vimentin), cell proliferation (PCNA) and apoptosis (deoxyuridine triphosphate biotin nick end-labeling (TUNEL)).

RESULTS

Controls revealed severe acinar gland atrophy and stromal proliferation. CN-009 showed diminished these damages. Epithelial score was better ($P<0.01$) and PAP positive materials were more abundant in CN-009 and GBX than in Controls. The stromal ratio was lower in CN-009 ($P<0.01$) and T-60 ($P<0.05$). There was no difference for PCNA positive cells in the epithelium and stroma, and TUNEL positive cells in the epithelium. While, the number of TUNEL positive cells in the stroma of CN-009 and T-60 increased ($P<0.01$).

CONCLUSIONS

These findings suggest that CN-009 protects acinar epithelial cells mainly by GBX and also inhibits stromal proliferation in association with enhanced apoptosis mainly by T-60. *Prostate 49: 122-131, 2001.* © 2001 Wiley-Liss, Inc.

KEY WORDS: Cernitin pollen-extract; apoptosis; chronic prostatitis; sex-hormone-induced prostatitis

INTRODUCTION

Three chronic prostatitis syndromes have been recognized; chronic bacterial prostatitis (CBP), chronic nonbacterial prostatitis (NBP) and prostatodynia. NBP is the most frequent disorder of 64% in these three diseases [1]. The etiology of NBP is unknown. A number of organisms or other factors have been reported to be the possible causes for NBP. They are *Trichomonas vaginalis*, *Chlamydia trachomatis*, genital mycoplasmas, staphylococci, coryneforms, genital viruses [2], biofilms [3], stagnation of prostatic secretion, autoimmune disease, allergy, disorder of sex hormone and psychological effects [4, 5]. For the treatment of CBP or NBP, antibiotics of new-quinolone or tetracycline have been administered. However, many cases resist these treatments [6].

Cn-009 is a pollen extract, which contains 20:1 ratio of powdered aqueous and organic extract. It is essentially a microbial digest of a standardized mixture of eight plant species grown at the Scania area in southern Sweden. The active ingredients consist of water-soluble (t-60) and fat-soluble (GBX) fractions [7, 8]. It was reported that CN-009 showed urine discharge action [9.10], anti-prostatic hypertrophic action [7] and anti-inflammatory effects to the prostate [11] in a preliminary study. Since Ask-Upmark [12] Reported CN-009 showed an efficacy to prostatitis, it has been used for the treatment of chronic prostatitis with high therapeutic effects. However, the mechanisms for these effects remain unknown.

To assess the mechanisms of the anti-prostatitis effect by CN-009, the present study was performed using a nonbacterial prostatitis rat model [13, 14] induced by 17b -estradiol administrations and castration.

MATERIALS AND METHODS

Sex Hormone-Induced Nonbacterial Prostatitis Model

Table 1. The structure of the Experiment

Group	No. of animals	Inflammatory agent	Drug treatment
Sham-ope.	5	No-treatment	No-treatment
Control	6	17 b-estradiol 0.25 mg/kg (s.c.)	1% HCO-60 (p.o.)
CN-009 630	5	17 b-estradiol 0.25 mg/kg (s.c.)	CN-009 630 mg/kg (p.o.)
CN-009 1260	6	17 b-estradiol 0.25 mg/kg (s.c.)	CN-009 1260 mg/kg (p.o.)
T-60	5	17 b-estradiol 0.25 mg/kg (s.c.)	T-60 1200 mg/kg (p.o.)
GBX	6	17 b-estradiol 0.25 mg/kg (s.c.)	GBX 60 mg/kg (p.o.)
TS	5	17 b-estradiol 0.25 mg/kg (s.c.)	Testosterone 2.5 mg/kg (s.c)

Each parenthesis represents the route of administration. s.c, subcutaneous injection; p.o., oral administration

Ten-month-old Wistar aged male rats were purchased from Japan Slc Co. (Tokyo, Japan).

The rats were housed in a climatised environment with a 12-hr light/dark cycle, 40-70% humidity. Food and water were supplied ad libitum. The rats were castrated under ether anesthesia, and then 17b-estradiol (Sigma, MI) 0.25 mg/ 2ml/kg diluted by sesame oil, as an inducer for prostatitis, was subcutaneously injected into the back of rats for 30 days from 1 day after castration [13,14].

Experimental Structure and Schedule

CN-009was suspended for 630 or 1,260 mg/5ml with 1% HCO-60 (Japan Surfactant, Tokyo, Japan). T-60 and GBX were similarly prepared for 1,200 and 60-mg/5 ml, respectively. Testosterone (TS) (Wako Chemicals, Tokyo, Japan), as a positive control, was diluted for 2.5-mg/2 ml with corn oil (Yuro Chemical, Tokyo, Japan).

The experimental structure is shown in Table I and the experimental schedule is illustrated in Figure 1. The rats were divided into seven groups consisting of Sham-operation (Sham-ope), Control, CN-009 630, CN-009 1260, T-60, GBX and TS with five or six animals in each group.

In the Sham-ope group, the rats were treated with only Sham-castration and without any drugs. In the Control group, the rats were injected subcutaneously environment with a 12-hr light/dark cycle, 40-70% humidity. Food and water were supplied ad libitum. The rats were castrated under ether anesthesia, and then 17b-estradiol (Sigma, MI) 0.25 mg/ 2ml/kg diluted by sesame oil, as an inducer for prostatitis, was subcutaneously injected into the back of rats for 30 days from 1 day after castration [13,14].

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In the Sham-ope group, the rats were treated with only Sham-castration and without any drugs. In the Control group, the rats were injected subcutaneously With 17b-estradiol for 30 days from the day following castration and administered orally with only 1% HCO- 60 5ml/kg for 14 days from Day 17. In the CN-009 630, CN-009 1260, T-60 1200 and GBX groups, similar protocols were performed with oral administration of CN-009 630, CN-009 1260, T-60 1200 and GBX 60 mg/kg, respectively. Also in the TS group, the rats were subconsciously with 17b-estradiol for 30 days from the next day of castration. After 14 days, TS 2.5 mg/kg was injected subconsciously for 14days. All studies were conducted in accordance with institutional guidelines of animal care in accordance with Japanese Governmental Animal Protection and Management Law.

Prostate Weights and Histopathological Evaluation

The rats were sacrificed on the day following the final administration. The prostate was extirpated and weighed. Relative prostatic weight was calculated from body weight and absolute weight.

After fixation in 10% neutral buffered formalin, each prostate was cut into coronal blocks. The tissue samples were dehydrated and embedded in paraffin. Sections (3-4 mm thickness) were stained with Hemotoxyline-Eosin (HE), Periodic acid Shiff (PAS) and Masson’s tri-chrome. The specimens were evaluated histopathologically.

Immunohistochemistry

Immunohistochemical studies were performed with anti-prostatic cid phosphatase (PAP), and vimentin. PAP staining was performed for the evalutation of glandular epithelial function. In PAP stained specimens, anti-PAP polyclonal antibody (Chemicon International, New York, NY) was diluted by PBS including 0.1% BSA of a 1:100 ratio, and incubated for 2 hr at 37°C. Biotinylated anti-rabbit IgG and the avidin-biotin peroxidase complex (ABC) method were performed. Unitect rabbit Immunohistochemistry detection systems (Oncogene Science, New York, NY) were reacted by those methods. Vimentin staining was performed for the evaluation of stromal proliferation. An ImmunoCruz staining system (Santa Cruz BioTech, Santa Cruz, CA) for Vimentin Staining was used according tot eh manufacture's instructions.

Cell Proliferation and Apoptosis

Cell proliferation and apoptosis were investigated with proliferating cell nuclear antigen (PCNA) and terminal deoxynucleotidyl transferase mediated deoxyuridine tri phosphate biotin nick end-labeling (TNEL), respectively. PCNA staining was performed with PCNA staining kit (ZYMED Laboratories, South San Francisco, CA). TUNEL method was performed with ApoTag Peroxidase In Situ Apoptosis Detection kit (Intervene, New York, NY). In PCNA and TUNEL specimen, 5,000 cells were countedunder a microscope in glandular epithelial cells and stromal cells respectively.

Acinar Epithelial Score and Stromal Area Ratio

To evaluate glandular damage, acinar epithelial cells were classified and scored, as follows; columnar (2 points), cuboidal (1 point), squamous-like (0 point) shape. Three different pathologists without any information judged the score. Using this scoring evaluation, 20 acinar glands of each specimen were investigated. To assess stromal proliferation, all areas of the specimen and the glandular area were measured using a digitizer (Graph Tech, Tokyo, Japan) with photomicrographs. Using these findings, the stromal ratio was calculated.

Statistical Analysis

All experiments were repeated at least twice. Each value was demonstrated as the mean±SD. Dunnett's test if in equal variance, or non-parametric Dunnett's test if in unequal variance between treatment groups and Control group was performed after 1-way ANOVA followed by Bartlett variance analysis test. Mann-Whitney U test was performed between the Sham-ope and Control groups.

RESULTS

Body and Prostate Weights

(Fig. 2)

In the Sham-ope group, the prostate was larger than in other groups. Acinar glands were roundly shaped. The acinar lumen was filled with eosinophilic materials. Acinar epithelial cells were cylindrical with a normally situated nucleus and the supranuclear spaces of these cells contained secretory materials, which were strongly stained with PAP antibody. A few fibrous tissues were found in the stroma (Figs. 3A and 4A). Vimentin positive cells were few, and the Vimentin positive was small (data not shown).

In the Control group, the prostate was atrophic acinar glands were irregularly shaped. The acinar lumen was poor with pale stained eosinophilic materials and filled with inflammatory cell infiltrations mainly characterized by neutrophils. Acinar epithelial cells were flattened similar to a squamos cell. A few secretory materials in the epithelial cells were poorly reacted with PAP antibody. The stroma showed severe proliferation with many lymphocyte and monocyte infiltrations and marked fibrosis with fibroblasts (Figs. 3B and 4B). The stroma was stained very strongle with Vimentin. The Vimentin positive area was significantly increased (data not shown). In the CN-009 630 group, the findings were basically identical with the Control group (data not shown).

In the CN-009 1260 group, acinar glands were more roundly shaped than in the Control group. Acinar epithelial cells were cuboidal, and the supranuclear spaces contained secretory materials stained with anti-PAP that were much more abundant than the in control group. Inflammatory cell infiltrations into the acinar lumen were diminished. The stroma showed mild proliferation with few lymphocytes, monocytes, and mild fibrosis with fibroblasts (Figs. 3C and 4C). The Vimentin positive area was much less than that of the Control group (data not shown).

In the GBW group, acinar epithelial cells were more cuboidal than in the Control group. Epithelial cells contained secretory materials stained with anti-PAP, which was basically identical with the CN-009 1260 group. Diminished call infiltration into the lumen was found (Fig. 3E). However, the stroma showed a proliferative condition with many lymphocyte and monocyte infiltrations and marked fibrosis with many fibroblasts. The stroma was stained strongly with Vimentin, and the positive area was markedly increased (data not shown).

In the TS group, acinar glands were roundly shaped. The acinar lumen was filled with eosinophilic materials with a few cell infiltrations. Acinar epithelial cells were cylindrical and the supranuclear spaces contained many secretory materials with reactive anti-PAP. However, the stroma was stained strongly with Vimentin and showed mild proliferation with fibroblasts (data not shown).

Cell Proliferation and Apoptosis

(PCNA and TUNEL Positive Cell Counts (Fig. 5))

No significant differences among the groups were observed in the PCNA positive cell counts in epithelial cells (Fig. 6) or in stromal calles (Fig. 7). In the Sham-ope group, a few TUNEL positive cells were found (Fig. 5A). The findings of the Control group were basically identical with the Sham-ope group (Fig. 5B). In the CN-009 1260 group, TUNEL positive cells in the stroma were more abundant than in the Sham-ope and Control groups (Fig. 5C). In the TUNEL positive cell counts, no significant differences were observed in acinar epithelial cells (Fig. 8). However, in the stroma, TUNEL positive cells were significantly ($P< 0.05$)

Fig. 3. HE staining of the prostate in the experimental nonbacterial prostatitis rat. (**A**) Sham-ope group: The acinar lumen is filled with eosinophilic materials without any cells. Acinar epithelial cells are cylindrical. A few fibrous tissues are found in the stroma. (**B**) Control group: The acinar lumen is filled with induced inflammatory cells mainly characterized by neutrophilis. Acinar epithelial cells are flattened similar to squamous cells. The stroma shows severe proliferation with many lymphocyte and monocyte infiltrations and remarkable fibrosis with fibroblasts. (**C**) CN-009 1260 group: Acinar epithelial cells are cuboidal. Inflammatory cell infiltrations into the acinar lumen are diminished. The stroma shows mild proliferation with a few lymphocytes, monocytes, and fibroblasts. (**D**) T-60 group: Stromal proliferation is relatively mild without severe inflammatory cells. (**E**) GBX group: Acinar epithelial cells are cuboidal, and diminished inflammatory cell infiltrations are shown. 400 The bar indicates 10 mm.

Increased in the CN-009 1260 group or T-60 group compared with the control group (Fig. 9).

Acinar Epithelial Score (Fig. 10)

In the Control group, acinar epithelial score was significantly lower ($P > 0.01$) than that of the

Sham-ope group. In comparison with the Control group (Fig. 10), the acinar epithelial score was significantly higher (P < 0.01) in the CN-009 1260, GBX and TS groups.

Stromal Area Ratio (Fig. 11)

In the Control group, the stroma area ratio was significantly higher (P < 0.01) than that of Sham-ope group.

Fig. 4. Immunohistochemical findings (PAP Staining) of the prostate in experimental nonbacterial prostatitis rats. (A) Sham-ope group: Supranuclear spaces of acinar epithelial cells contain secretory materials which are stained with anti-PAP. (B) Control group: Acinar epithelial cells are flattened similar to squamous cell. Secretory materials are poorly reactive with anti-PAP. (C) CN-009 1260 group: Supranuclear spaces contained secretory materials with PAP staining, which are significantly more abundant than in the Control group. ´400 the bar indicates 10 mm.

In comparison with the Control group (Fig. 11) the stromal area ratio of the CN-009 1260 was significantly (P < 0.01) lower. The T-60 group was also significantly (P < 0.05) lower than the Control group. However, there was no difference between other groups.

Fig. 5. Immunohisochemical findings (TUNEL) of the prostate in rats. (A) Sham-ope group: A few TUNEL positive cells are shown. (B) Control group: The findings are basically identical to these of the Sham-ope group. (C) CN-009 1260 group: TUNEL positive cells in the stroma are more abundant compared with the Sham-ope and Control groups. **´400 the bar indicates 10 mm**

Discussion

Although chronic prostatitis is a common disease, it is very difficult to treat effectively. Typical clinical findings are decreased potentia, perineal or scrotal pain, urethral discharge and lower urinary tract irritative symptoms. The prostate gland is irregularly indurated and the numbers of leukocytes in expressed prostatic secretion are increased [15]. Pathological findings of this disease are chronic inflammation characterized by aggregates of lymphocytes in the stroma and acute inflammation characterized by the presence of neutrphic polymorphonnuclear leukocytes in the lumen of acinar glands [15 – 17]. Pathological definition of chronic prostatitis is different from the clinical definition for the urologists. Clinical definition has been the combination of a clinical symptom and inflammatory cells in expressed prostatic secretion. The pathological inflammation of the prostate was repoted to be not frequent in the patients with symptoms of chronic prostatitis/chronic pelvic pain symdrome [16].

In experimental animals, Lewis, Wistar and Copenhagen rats have a high incidence of spontaneous nonbacterial prostatitis [14]. Administration of exogenous 17b-estradiol can induce 100% of the incidence on prostatitis in old Wistar rats [18] and castration also has a similar effect [13, 18]. Naslund et al. [13] reported that histopathological findings were very similar between spontaneous nonbacterial prostatitis and estradiol-induced prostatitis in rats [13]. These histopathological findings in rat spontaneous age-dependant prostatitis demonstrated several similarities to pathological defined chronic prostatitis in humans [19, 20]. These findings suggested that this rat model would be a useful model for the study of the treatment of human chronic prostatitis. Therefore, we decided to investigate the effects and mechanisms of CN-009 using a nonbacterial prostatitis rat model [13, 14] induced by 17b-estradiol injection and castration.

No differences in the prostate weight were found in CN-009 630, CN-009 1260, T-60 and GBX groups compared with the Control group. Since the weight of the prostate is mostly determined by the amount of residual secretory fluid, these findings may indicate that CN-009 cannot prevent the reduction of secretory prostatic fluid.

In the CN-009 1260 group, we observed roundly shaped acinar glands, cuboidal acinar epithelial cells containing secretory materials with positive PAP staining and diminished cell infiltrations into the lumen compared with the Control group. The acinar epithelial score was significantly increased. CN-009 could protect acinar epithelial function and cell shape against nonbacterial inflammation. GBX had a similar effect to CN-009 in the acinar glands. T-60 was not effective in the acinar epithelial function of this rat model. Therefore, GBX may play a role for the protection of epithelial damage in NBP. The effect of GBX is discriminated from TS effect. In an in vitro study, GBX was reported to inhibit the cyclooxygenases and 5-lipooxygenases in the biosynthesis of the prostaglandins and leucotriens enhance inflammatory cell infiltrations, GBX may protect against inflammation into the acinar lumen by inhibition of these enzymes. Furthermore, CN-009 showed an inhibition on the heat-induced hemolysis, which is correlated to lysosomal membrane stability [11]. CN-009 appears to stabalize a lysosomal membrane, recover cell function and protect against degeneration of the acinar epithelium.

In addition, T-60 was shown to inhibit the growth of an immortal prostate cancer cell line in vitro [22]. However, their mechanisms are unknown. In the present study, the ratio of stromal area was significantly decreased in the CN-009 1260 and T-60 groups. Stromal TUNEL positive cell counts were increased in these groups. Therefore, CN-009 and T-60 may inhibit stromal cell proliferations by enhanced apoptosis. Although the exact mechanism of this process is unclear, several speculations are possible such as the direct effect by the apoptosis of fibroblast, and the indirect effect by the apoptosis of lymphocytes through the inhibition of several cytokines, such as several interleukins. Further laboratory studies are necessary to elucidate the exact mechanisms of this compund.

Since no toxicological effects have been shown even in long-term administration, CN-009 is thought to be a safe drug [6, 23]. Here we reported the effects and mechanisms of CN-009 on rat experimental nonbacterial prostatitis model. CN-009 will also be a safe and effective agent against human nonbacterial chronic prostatitis.

In conclusion, CN-009 can work as a potent anti-inflammatory agent against chronic prostatitis. These present findings suggest that GBX, a fat-soluble fraction of CN-009, protects the function and shape of acinar glandular epithelium and T-60, a water-soluble fraction of CN-009, inhibits stromal cell proliferations in association with enhanced apoptosis.

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