Journal of Andrology, Vol. 21, No. 5, September/October 2000

Oxidative Stress in Prostatic Fluid of Patients With Chronic Pelvic Pain Syndrome: Correlation With Gram Positive Bacterial Growth and Treatment Response

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Abstract: The etiology of chronic pelvic pain syndrome (CPPS)/chronic prostatitis category III remains unknown. Whereas a subset of men respond to antimicrobial therapy, Gram positive bacteria isolated from expressed prostatic secretions (EPS) are often considered to be commensal rather than pathogenic. We wished to study oxidative stress as a marker of tissue injury and response in EPS of men with CPPS to determine whether infection with Gram positive bacteria is associated with increased oxidative stress. A total of 300 EPS specimens from 100 men with CPPS were collected for microscopy, culture, and biochemical and molecular assays. Oxidant injury was measured by 8-isoprostane F2α (IsoP) levels and total antioxidant capacity as Trolox equivalents. Total RNA from EPS was used for gene expression of heme oxygenase-1 (HO-1) and granzyme B. The only bacteria found in EPS were Gram positive. For our analysis, these men were classified as having chronic bacterial prostatitis (category II). IsoP levels (pg/mL) were highest in men with category II prostatitis (7315 ± 1428) followed by nonbacterial prostatitis (category IIIa, 2043 ± 561), prostatodynia (category IIIb, 319 ± 81), and asymptomatic controls (298 ± 99). IsoP levels decreased significantly after successful treatment with antibiotics or an antioxidant supplement (Prosta-Q). Antioxidant capacity was detected in 11 out of 18, 4 out of 16, and 1 out of 16 men tested with category II, IIIa, and IIIb prostatitis, respectively. No correlation was observed between IsoP levels and the number of white blood cells in EPS. HO-1 and granzyme B expression was highest in men with category II prostatitis than in men with either category III prostatitis or asymptomatic controls. On the basis of elevated oxidative stress, clinical response to antibiotics, and post-treatment reduction in oxidative stress, we conclude that Gram positive bacteria in some men with CPPS may be pathogens. It is speculated that oxidative stress may be a key pathway in some men with CPPS that can be targeted with antioxidant therapy.

Key words: Chronic prostatitis, inflammation, gene expression, isoprostane, Quercetin.

J Androl 2000;21:669–675

The chronic prostatitis syndromes are among the most poorly understood and difficult to treat in urology. Chronic bacterial prostatitis (classified as category II prostatitis by the National Institutes of Health [NIH]) is diagnosed when positive bacterial cultures localize to the prostate (Nickel et al, 1999). Many researchers hold the opinion that true category II prostatitis must be caused by established uropathogens and accompanied by recurrent lower urinary tract infection; however, this description fits less than 5% of men with chronic pelvic pain syndrome (CPPS). More commonly, men with CPPS have positive cultures for Gram positive bacteria that may nevertheless localize to the prostate (Shoskes and Zeitlin 1999). By the classical definition, these patients would be classified as having category IIIa prostatitis if inflammation is present in expressed prostatic secretions (EPS) and category IIIb prostatitis if no inflammation is observed.

Supported by National Institutes of Health grant RO1 DK53738.

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Received for publication December 8, 1999; accepted for publication March 23, 2000.

While it has been established that Gram positive bacteria may colonize the prostate, particularly in a protective biofilm, it is controversial whether they may be true pathogens or just commensal organisms or contaminants. It is clear that many men with category III CPPS respond to antimicrobial-based therapy, which may be due to undetected bacteria. In fact, molecular studies suggest a wide variety of organisms are present in EPS from these men (Tanner et al, 1999). What defines infection as opposed to colonization is the presence of tissue injury caused by bacteria and a documented injury response of the body's defenses. One of the best reflections of injury and subsequent injury response in a given tissue is the tissue's state of oxidant stress (Halliwell, 1987). Oxidant stress is the net balance of reactive oxygen species (ROS), such as superoxide anion ($O_2\cdot$), hydrogen peroxide (H_2O_2), and the hydroxyl free radical ($HO\cdot$) in the local environment. ROS are highly reactive and can inflict damage to cellular proteins, DNA, cell membranes, and organelles via lipid peroxidation. Cellular antioxidants (superoxide dismutase, glutathione, catalase, etc) can normally scavenge and inactivate ROS, thereby reducing their toxic effects. Therefore, oxidant stress may represent either an excess

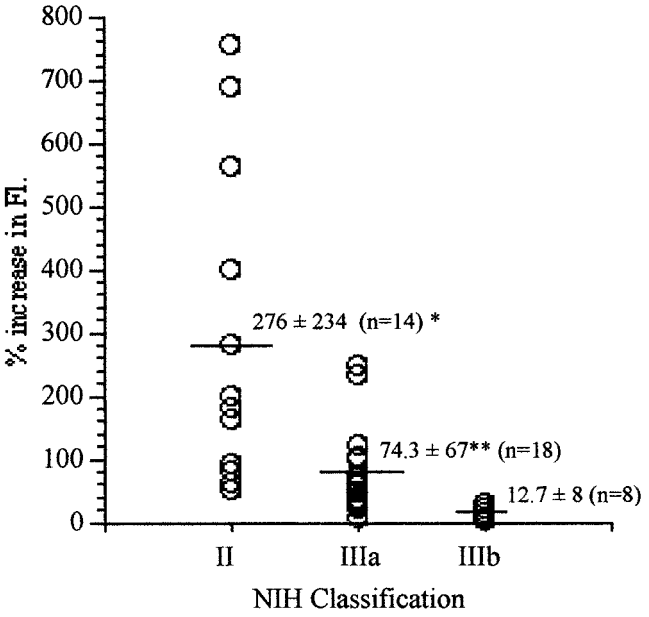

Figure 1. DCF levels in EPS. Percent increase in DCF fluorecence was measured as described in *Materials and Methods.*

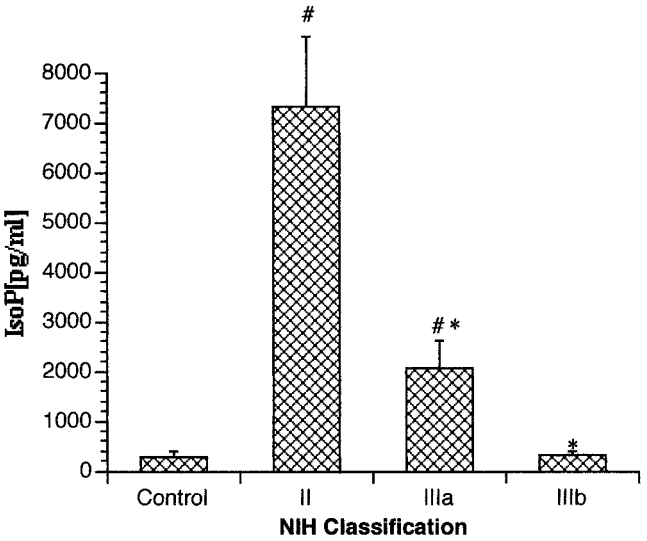

Figure 2. IsoP levels in EPS. IsoP levels (mean ± SEM) were measured by enzyme immunoassay as described in *Materials and Methods.* Men were classified on the basis of NIH criteria (category II prostatitis, n = 22; categories IIIa and IIIb prostatitis, n = 18 each; controls, n = 6); * indicates significantly different from category II; and #, significantly different from control.

production of ROS, a decreased production of antioxidants, or a combination of the two. Inflammatory cells can also produce ROS at the site of inflammation, infection, or injury. Therefore, elevated oxidative stress in EPS may reflect bacterial injury, the effects of an injury response, or both.

It is difficult to directly measure ROS because they are highly reactive and short lived. Thus, oxidant stress in a clinical sample is often assessed by indirect measures of ROS action. Recently, 8-isoprostane F-2α (IsoP) has been reported as a novel and clinically reliable marker of ROS-catalyzed tissue injury (Morrow and Roberts, 1997). IsoP is generated by a noncyclo-oxygenase free radical–mediated mechanism involving peroxidation of arachidonic acid (Awad et al, 1993). IsoP is stable and can be measured by immunoassay or gas chromatography/mass spectrometry (GC/MS) methods (Basu, 1998).

Another potential marker of oxidative stress is the upregulation of the heme oxygenase-1 (HO-1) gene. HO-1 is the inducible form of the microsomal rate-limiting enzyme, heme oxygenase, which catalyzes heme breakdown. Oxidative stress and many other noxious stimuli induce HO-1 expression (Choi and Alam, 1996; Vogt et al, 1996; Niess et al, 1999; Mosley et al, 1998; Keyse et al, 1990). Expression of HO-1 is considered to be a protective cellular response to these stresses and serves as a marker of oxidative stress.

The purpose of this study was to measure oxidative stress in EPS of men with CPPS (NIH category III) and determine whether men with positive localizing cultures of Gram positive bacteria showed evidence of tissue injury and an inflammatory response in contrast to those with negative bacterial cultures, with or without inflammation. We also hypothesized that oxidant stress may play a role in the pathophysiology of the symptoms observed in CPPS, regardless of the etiology of their generation. We wished, therefore, to study levels of oxidant stress, antioxidant capacity, and the expression of HO-1 and granzyme B in EPS of men with CPPS.

Materials and Methods

Patients

A total of 300 EPS samples were obtained from 100 men attending the Harbor-UCLA Medical Center Chronic Prostatitis Clinic between November 1997 and July 1999. This group included 34 men enrolled in the NIH Chronic Prostatitis Collaborative Network study. All patients suffered from chronic pelvic pain for at least 3 months of the previous 6 months and did not have an active urinary tract infection. In addition, 8 men with no clinical evidence of chronic prostatitis or CPPS consented to prostatic massage and use of their EPS for research (4 men had benign prostatic hyperplasia, 1 was subsequently found to have prostate cancer, and 3 had no urinary or prostatic symptoms). EPS samples were placed into cryovials and stored at −80°C until use. Because of the small volume of EPS obtained, it was not possible to perform every test described in this paper in all samples or patients. The number of men for each test in each category are provided in the "Results" section. The clinical outcome of a subset of these men was previously reported (Shoskes and Zeitlin, 1999) and oxidant stress data on 4 of these men was also previously published (Shoskes et al, 1999).

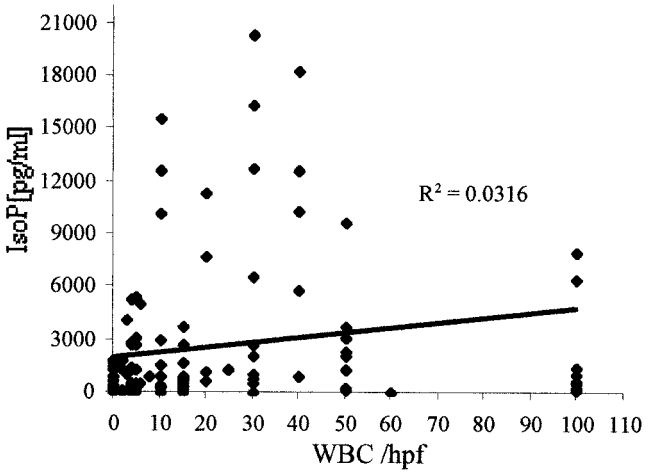

Figure 3. Correlation of IsoP levels with white blood cell (WBC) count in EPS. WBCs in EPS were counted as described in *Materials and Methods.* IsoP levels from all EPS samples were used.

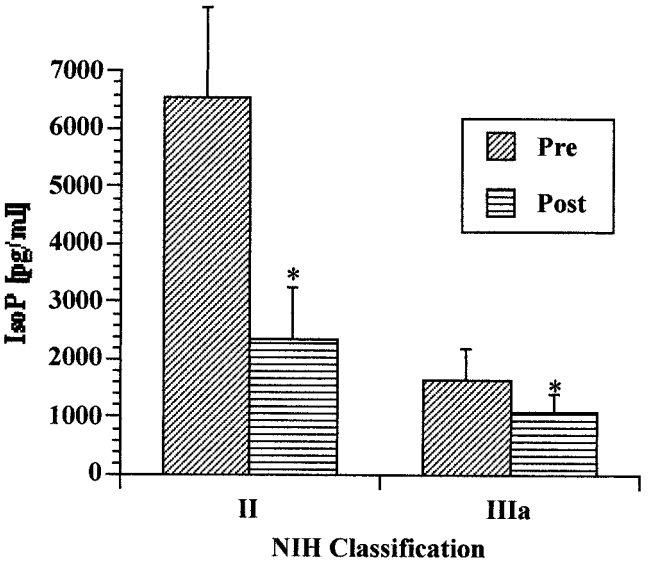

Figure 4. Pretreatment and post-treatment IsoP levels (mean ± SEM) were measured in EPS samples collected from men at their first visit and again after treatment. Category II, n = 18; category III, n = 13.

All samples from the urethra (VB1), mid-stream urine (VB2), EPS, and postmassage urine (VB3) if EPS was not obtained at the first visit, were cultured for aerobic bacteria for 5 days and any growth was reported. The white blood cell (WBC) count in EPS was estimated using a wet mount under 400× total magnification and reported as the mean of 4 separate fields. An arbitrary cutoff of 10 WBCs per hpf or greater was considered positive for inflammation. Cultures were considered positive and localized to the prostate if the bacteria found in EPS or VB3 were unique to those specimens or, if also present in VB1, represented at least a 2-log increase when compared with VB1.

ROS Generation

ROS generation in EPS was measured by the oxidation of 2′,7′dichlorofluorescin diacetate (DCF; Molecular Probes, Inc, Eugene, Ore). DCF (2 mg/mL) was dissolved in methanol and diluted with Krebs-Ringer buffer to a final concentration of 20 μg/mL. EPS samples (2–10 μL) were added to 50 μL Krebs-Ringer buffer in a 96-well plate and 100 μL of DCF solution was rapidly added to each well. Fluorescence measurements (Ex = 484, Em = 530) were taken at intervals of 1, 15, 30, and 60 minutes using a Victor 1420 multilabel counter (EG&G Wallac, Gaithersburg, Md). At sensitivity setting 4, a relative fluorescence of 1000 represented 440 pmol of DCF oxidized as determined using a standard curve for oxidized DCF. Results are expressed as percent increase/μL of EPS.

Measurement of Total Antioxidant Capacity

The reaction mixture consisted of 10 μL of EPS, 300 μL of 5 mM 2,2′-azinobis 3-ethylbenzothiazoline 6-sulphonate (ABTS, Sigma Chemical Co, St Louis, Mo) and 36 μL of 70 μM metmyoglobin (Sigma) and 167 μL of 450 μM hydrogen peroxide in a total volume of 1 mL (Miller et al, 1993). Reaction was started by adding hydrogen peroxide and samples were incubated for 6 minutes at 30°C in a shaking water bath and absorbance was measured at 730 μm. A standard curve using 0.5 to 2.5 mM Trolox (Aldrich Chemical Company, St Louis, Mo) was run simultaneously. Trolox is an antioxidant that inhibits the absorption of the ABTS radical formed in the presence of metmyoglobin and hydrogen peroxide. The antioxidant capacity of EPS is thus measured as Trolox equivalent antioxidant capacity (TEAC), on the basis of the curve.

Determination of IsoP in EPS

IsoP levels were measured by using an enzyme immunoassay kit (Caymen Chemicals Company, Ann Arbor, Mich) according to the manufacturer's directions. EPS was centrifuged at 5000 × *g* for 5 minutes to remove cells and other debris, and an aliquot (5–10 μL) was assayed in duplicates. An IsoP standard curve (3.9–500 pg/mL) was simultaneously run on the same enzyme immunoassay plate. EPS samples were not purified before the assay because of the small sample volume (average 100 μL); however, we verified our results by adding a known amount of standard IsoP to some of the EPS samples.

RNA Extraction and Reverse Transcription-Polymerase Chain Reaction of HO-1 and Granzyme B

Total RNA (tRNA) from fresh or frozen (−80°C) EPS samples was isolated by the Trizol method as per the manufacturer's directions (Fisher Scientific, Tustin, Calif). One μg of tRNA was used for complementary DNA (cDNA) synthesis. Polymerase chain reaction (PCR) was performed using 1 μL of cDNA in a total volume of 25 μL and gene-specific primers. The primers for HO-1 were 5′-CAGGCAGAGAATGCTGAGTTC-3′ and 5′-GCTTCACATAGC GCTGCA-3′ (350 bp); and for granzyme B, 5′-TGCAGGAAGA TCGAAAGTGCG-3′ and 5′-GAGGCATGCCATTGTTTCGTC-3′ (188 bp). The PCR cycles were performed at 94°C (1 min), 60°C (1 min), and 72°C for 32 cycles, with a final extension cycle for 7 minutes at 72°C. Expression of β-actin was used to normalize the band density. PCR products were separated on 2% agarose gels and band density was measured on a UVP gel documentation system (UVP Inc, Upland, Calif).

Quercetin Treatment

We used a supplement containing the bioflavonoid, quercetin (Prosta-Q, Farr Laboratories, Santa Clarita, Calif) as therapy for category III CPPS (Shoskes et al, 1999). In 8 men from whom both a pretreatment and post-treatment EPS sample was available, IsoP levels were measured as described earlier.

Statistical Analysis

Data are presented as mean ± SEM. Data were analyzed using either analysis of variance and Student's *t*-test or nonparametric analysis with the Mann-Whitney *U* test and Kruskal Wallis test, where applicable. Statistical significance was set at $P < .05$.

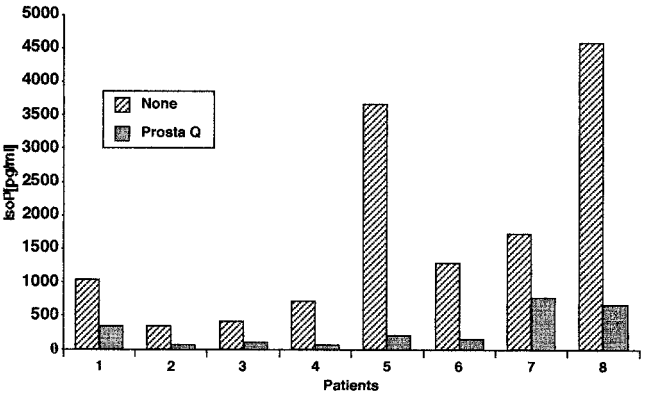

Figure 5. EPS IsoP levels before and after Prosta-Q treatment. Eight men took Prosta-Q twice daily for 1 month.

Results

Patient Demographics

The 100 men studied had a mean age of 44 years (23–72 years) and a median symptom duration of 3 years (3 months–30 years). The men had been previously treated for prostatitis by a median of 4 doctors. No patient fulfilled the classical definition of having chronic bacterial prostatitis, with high counts of Gram negative bacteria localizing to the prostate and recurrent urinary tract infection. Nevertheless, 36 men had positive EPS cultures for Gram positive bacteria that localized to the prostate. In the present study, these men were classified as having NIH category II prostatitis for the purpose of our analyses. The most common genera or Gram positive bacteria were staphylococci, corynebacteria, and enteroccoci. An additional 38 men had EPS inflammation with negative cultures (category IIIa, nonbacterial prostatitis). The remaining 26 men had negative cultures and no inflammation (category IIIb, prostatodynia).

Total Oxidant Levels and Antioxidants in EPS

The hallmark of pathogenic infection, as opposed to commensal growth, is tissue injury, including increased oxidant stress and induction of tissue antioxidant enzymes. We measured total oxidant levels in EPS by measuring DCF oxidation, and the data, percent increase in DCF florescence, are shown in Figure 1. The oxidant stress level in EPS of men with category II prostatitis (276 ± 234, n = 14) with positive localized cultures for Gram positive bacteria was significantly higher than in men with category IIIa (74.3 ± 67, n = 18) and category IIIb (12.7 ± 8, n = 8) prostatitis combined ($P = .0006$ and 0.0001, respectively). In addition, oxidant stress level in men with category IIIa prostatitis was significantly different than it was in those with category IIIb prostatitis ($P = .0004$).

Men with Gram positive bacteria in EPS had a significantly higher proportion of samples with detectable total antioxidant capacity. Antioxidant capacity was detected in 11 out of 18 (61%) men with category II prostatitis, whereas only 4 out of 16 (25%) with category IIIa and 1 out of 16 (6%) with category IIIb had measurable levels ($P < .05$). The TEAC values ranged from 0.3 to 3.74 mM in category II, and 0.24 to 1.95 mM in category IIIa.

IsoP Levels in EPS

IsoP was detected in EPS of all men studied, suggesting that this marker of oxidative stress is generated in EPS. As seen in Figure 2, EPS IsoP levels (pg/mL) were significantly higher in 22 men with category II prostatitis (7315 ± 1428, $P < .008$) than in the 18 men with category IIIa (2055 ± 561), the 18 with category IIIb (319 ± 81, $P < .004$), or the asymptomatic controls (298 ± 99, $P < .05$) There was no simple linear relationship between WBC count and IsoP levels (Figure 3). A significant decrease in IsoP levels was detected in EPS following clinically successful treatment either with antimicrobial or anti-inflammatory therapy, both in men with category II (64%) and category IIIa (32%) prostatitis (Figure 4). Duration of therapy varied from 2 to 12 weeks in these men and included antibiotics of several classes, on the basis of culture sensitivities (eg, quinolones, macrolides, tetracyclins, etc) and anti-inflammatories with different mechanisms (eg, Cox-2 inhibitors, phytotherapy).

Effect of Bioflavonoid Quercetin on IsoP Levels in EPS

We have used oral therapy with the antioxidant dietary supplement, Prosta-Q, successfully in men with CPPS (Shoskes et al, 1999). All 8 men with category III CPPS receiving Prosta-Q showed a significant decrease in IsoP levels following 1 month of therapy (Figure 5). The overall pretreatment and post-treatment IsoP levels were 1243 ± 506 and 153 ± 112, respectively ($P < .007$). IsoP levels decreased in all men whether or not they had a significant reduction in symptoms; 6 patients exhibited fewer symptoms, defined as a greater than 25% reduction

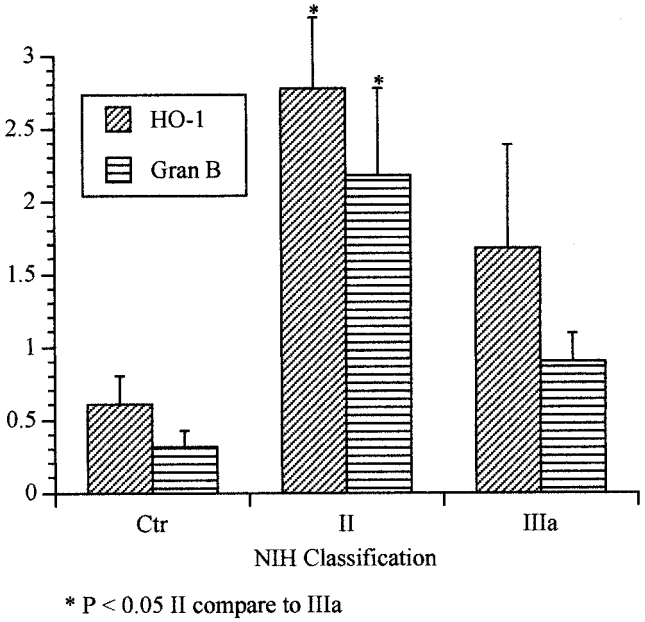

Figure 6. Expression of HO-1 and granzyme B mRNA in EPS. RT-PCR of these genes was performed as described in *Materials and Methods* using gene specific primers.

in NIH–Chronic Prostatitis Symptom Index score (Litwin et al, 1999); 2 men did not.

Expression of HO-1 and Granzyme B mRNA in EPS

In addition to causing local injury, an infection by pathogens should provoke an inflammatory injury response. HO-1 expression is up-regulated by many stimuli, including increased oxidative stress. Induction of HO-1 and granzyme B was detected in all EPS samples tested. The ratios (HO-1 or granzyme B/β-actin) were much higher in men with category II (n = 13) than in men with category III (n = 8) prostatitis (Figure 6). A faint or no band was detected in EPS of men who were asymptomatic (data not shown).

Discussion

The underlying etiology of chronic prostatitis/CPPS remains highly controversial. Arguments have been made for infectious, autoimmune, inflammatory, and neuromuscular etiologies; nevertheless, a single mechanistic explanation or therapeutic option has been elusive. Typically, when localized cultures are propagated with urine and EPS, fewer than 5% of samples exhibit an established uropathogen. Gram positive organisms are commonly present in the distal urethra, and a high incidence of them appear in EPS; however, we have found Gram positive bacteria in EPS that were not cultured from urine or the urethra in roughly 40% of men with CPPS (Shoskes and Zeitlin, 1999). Because these Gram positive bacteria are common and patients rarely develop recurrent cystitis from them, they are not considered to be classic pathogens, and many investigators believe that they are commensal organisms and unrelated to the symptoms of CPPS.

A role for Gram positive bacteria in the pathogenesis of chronic prostatitis has long been suspected (Nickel and Costerton, 1993). We have previously shown that the treatment and eradication of these bacteria leads to a resolution of symptoms in about a third of patients with CPPS (Shoskes and Zeitlin, 1999). Furthermore, we have found diverse and unique Gram positive bacterial species in EPS of men with CPPS by 16S recombinant RNA techniques (Tanner et al, 1999). These observations suggest that these bacteria may be pathogens, or at least, markers of a unique subset of patients with CPPS. Results of the present study appear to be consistent with this suggestion.

One of the best reflections of injury and subsequent repair in a given tissue is its state of oxidant stress. Results from both the direct measurement of ROS (via the DCF method) or IsoP levels demonstrated that oxidant stress was significantly higher in men with positive, localized EPS cultures than in those with negative cultures or in asymptomatic controls. These levels of oxidant stress were independent of WBC count and therefore did not represent just a marker of the presence of WBCs. Increased oxidant stress is consistent with a local tissue injury. ROS are quite reactive, potentially harmful, and can be produced at the site of injury by inflammatory cells (Zwart et al, 1999). ROS play a significant role as mediators of cellular injury and cell death and are associated with aging and diseases such as atherosclerosis, cancer, and reperfusion injury (Knight, 1995). It is interesting that IsoP has recently been shown to directly cause smooth muscle contraction in the urinary bladder (Tarcan et al, 2000). This oxidative stress byproduct may therefore be part of the pathophysiology of urinary tract dysfunction in men with CPPS.

Increased levels of IsoP have been measured as markers of oxidative stress in many clinical situations such as coronary reperfusion (Delanty et al, 1997), in the cerebrospinal fluid in degenerative neurological disorders (Montine et al, 1999), in broncheoalveolar lavage fluid in interstitial lung disease, and within blood vessels during formation of atherosclerotic lesions (Pratico et al, 1998). Our results also show a decrease in the level of IsoP or oxidative stress in men with category II and category III prostatitis after treatment with either antimicrobials (in category II) or the bioflavonoid preparation, Prosta-Q (in category III). The mechanism of these agents in reducing oxidative stress is not known. Antimicrobials may reduce oxidant stress by eradicating pathogenic bacteria, which

leads to termination of tissue injury, or they may act by direct anti-inflammatory mechanisms (Yoshimura et al, 1996; Galley et al, 1997; Aoki and Kao, 1999). It is suggested that bioflavonoids in Prosta-Q exert their beneficial effect through antioxidant (Yokoo and Kitamura, 1997), anti-inflammatory (Sato et al, 1997), or as-yet-unknown mechanisms.

A third method used to determine oxidant stress in EPS was the expression of HO-1 in the cells present in EPS. HO-1 expression is up-regulated by various stimuli, including oxidative stress, and has been used as the molecular marker of the same. Our results show expression of HO-1 mRNA to be higher in men with category II and III prostatitis than in controls. HO-1 is an inducible enzyme and catalyzes heme breakdown. The products of HO-1 reaction include billiverdin, carbon monoxide, and iron (Vogt et al, 1996; Niess et al, 1999). Billiverdin is subsequently converted into billirubin, a potent antioxidant, by billiverdin reductase. Iron and carbon monoxide may also be indirectly protective. These results also are consistent with increased oxidative stress in EPS of men with CPPS.

Of particular interest was finding elevated granzyme B expression in men with CPPS who had positive EPS cultures. Granzyme B is a marker for activated cytotoxic T cells, a lineage not typically associated with a simple antimicrobial immune response. The presence of activated cytotoxic T cells in EPS suggests an inflammatory reaction, more consistent with autoimmunity or secondary remodeling of tissue injury. Why these T cells would be present primarily in men with positive cultures for Gram positive bacteria rather than other forms of prostatic injury is unknown; however, it is interesting to speculate that bacterial superantigen may be involved. Bacterial superantigen is found on Gram positive bacteria such as *Staphylococcus*, and can directly stimulate the clonal expansion of cytotoxic T cells and lead to autoimmune reactions (Kotb, 1998).

Increased oxidative stress, whether due to infection or some other mechanism, appears to be a common pathway in symptomatic CPPS. This proposition is supported by, 1) increased ROS production, especially with positive cultures; 2) reduced antioxidant capacity in men with category III prostatitis; 3) increased IsoP levels, a potent marker of oxidative stress; 4) up-regulation of HO-1 mRNA, a marker of oxidative stress; and 5) a decrease in IsoP levels after treatment with antimicrobials (category II) or with the antioxidant dietary supplement, Prosta-Q (category III). Furthermore, it is suggested that Gram positive bacteria in EPS of some men with CPPS may represent true pathogens on the basis of clinical response to antibiotics and post-treatment reduction in oxidative stress. Alternatively, it is also possible that these bacteria may be markers of other inflammatory or autoimmune mechanisms, leading to oxidative stress and CPPS. Because these findings do not conform to the current classification of CPPS, a separate category within category II or III chronic prostatitis may be considered.

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