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Upregulation of Androgen-Responsive Genes and Transforming Growth Factor- β 1 Cascade Genes in a Rat Model of Non-bacterial Prostatic Inflammation

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Abstract

Background—Prostatic inflammation is associated with the development of prostatic hyperplasia. We investigated the effects of prostatic inflammation on expression levels of androgen-responsive genes and growth factors in the prostate.

Methods—Prostatic inflammation was induced by formalin injection into bilateral ventral lobes of the prostate of male SD rats. After 28 days, the prostate was harvested for analyses of proinflammatory cytokines, androgen-responsive genes in the epithelium and TGF- β 1 cascade genes in the stroma. Some rats were given a COX-2 inhibitor (celecoxib; 10 mg/kg/day) by oral gavage for 28 days.

Results—The formalin-injected prostate exhibited widespread low-grade inflammation (< 50 leukocytes/10,000 μm^2) along with focal high-grade inflammation (> 100 leukocytes/10,000 μm^2) in limited areas. Compared to control, formalin-injected prostate exhibited a 2.5–6 fold increased protein expression of IL-1 α , IL-1 β , and IL-6. In the low-grade inflammatory regions, 3–9 fold and 2–3 fold upregulations of mRNA levels of androgen receptors/androgen-responsive genes and TGF- β 1 cascade genes were respectively observed in the epithelium and stroma obtained by laser-capture microdissection. Positive staining for androgen receptors in the epithelial nuclei, and TGF- β 1, IL-6 and COX-2 in the stroma was increased in the low-grade inflammation area. COX-2 inhibitor treatment suppressed these upregulations of cytokines, androgen-responsive and TGF- β 1 cascade genes.

Conclusions—Prostatic inflammation induced increased expression of androgen-responsive genes in the epithelium and TGF- β 1 cascade genes in the stroma, which were suppressed by COX-2 inhibitors, suggesting that activation of these genes in the low-grade inflammatory region might be involved in the development of symptomatic BPH.

Keywords

prostate; inflammation; androgen receptor; TGF- β 1; laser capture microdissection

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Conflicts of interest:

The authors have nothing to disclose.

Introduction

Benign prostatic hyperplasia (BPH) is one of the most common disease conditions seen in older males. Although the mechanisms underlying the development of BPH are poorly understood and appear to be complex, an association between chronic prostate inflammation and BPH development has been demonstrated in previous clinical studies (1–3). The REDUCE (Reduction by Dutasteride of prostate Cancer Events) trial indicated a correlation between histological grade of chronic prostatic inflammation and lower urinary tract symptoms (LUTS) (1). Also, in the MTOPS (Medical Therapy of Prostatic Symptoms) trial, patients with acute and chronic inflammation in baseline prostate biopsy were more likely to develop worsening LUTS compared to those without inflammation (4). The upregulation of inflammatory cytokines in BPH tissues has also been described (5–9).

Androgens have been traditionally considered to be important players in the pathogenesis of BPH. The expression levels of androgen receptor (AR) and androgen-responsive genes are elevated in BPH specimens (10), and subjects carrying mutations that impair AR activity or who were castrated before puberty do not develop BPH (11,12). Moreover, existence of a cross-talk between AR activity and inflammatory cytokines is suggested by the activation of AR by cytokines secreted from inflammatory cells in prostate cancer cells (13–15).

Prostate enlargement associated with BPH occurs via prostate tissue remodeling, which is facilitated by tumor growth factor- β 1 (TGF- β 1) and its downstream proteins (16,17). TGF- β 1 modulates stromal cell function either as a paracrine factor generated by either neighboring epithelial cells or macrophages, or as an autocrine factor produced by stromal cells (18,19).

There still exist significant gaps in the understanding of the pathophysiology of BPH including how prostatic inflammation alters the *in vivo* expression of AR, androgen-responsive genes and TGF- β 1 cascade genes in the prostate. In this study, we therefore evaluated whether prostatic inflammation in rats following intraprostatic formalin injection induces the elevated expression of androgen-responsive genes and TGF- β 1 cascade genes in the prostate. We also examined the effects of a cyclooxygenase-2 (COX-2) inhibitor on the changes in androgen and TGF- β 1 signaling following prostatic inflammation.

Materials and Methods

Animal surgery

All animal experiments were performed in accordance with institutional guidelines and with an approval from the University of Pittsburgh Institutional Animal Care and Use Committee. Male Sprague-Dawley rats weighing 250–320 g were anesthetized with isoflurane. Following an abdominal incision, formalin (5% in saline) or saline (control) was injected into each ventral lobe of the prostate (50 μ l per lobe) to produce chemically induced prostatic inflammation. Ventral lobes of the prostate were excised 28 days after injection. For COX-2 inhibitor treatment, some formalin-injected rats received 10 mg/kg/day of celecoxib by oral gavage for 28 days starting from the day of intraprostatic formalin injection through post-injection day 27.

Histological analyses

A part of the prostate injected with either formalin or saline ($n = 6$ per group) was embedded in OCT Tissue-Tek compound (Sakura Finetek U.S.A, Torrance, CA), frozen on dry ice, and kept at -80°C until use. Samples were serially sectioned at 8 μm thickness and stained with hematoxylin and eosin.

For immunohistochemical staining, prostate sections were mounted directly on coated slides and fixed with 4% paraformaldehyde for 10 min, rinsed with phosphate-buffered saline (PBS), transferred to 3% hydrogen peroxide for 10 min to block peroxidase activity, and rinsed with distilled water and PBS. Next, the sections were blocked with 10% normal goat serum (Invitrogen, Grand Island, NY) for 30 min. Without rinsing, the tissues were reacted with antibody for AR (1:1000), TGF- β 1 (1:250), IL-6 (1:2000), and COX-2 (1:250) (Abcam, Cambridge, MA) for 1 h at room temperature, and washed with PBS. The tissues were then incubated for 30 min with HRP-labeled polymer anti-rabbit antibody (Dako, Carpinteria, CA) at room temperature and rinsed with PBS. For streptavidin–horseradish peroxidase detection, the tissues were stained with avidin–biotin complex reagent (Dako) for 10 min at room temperature. For counterstaining, hematoxylin was used.

Measurement of inflammatory markers

The prostate tissues of rats injected with formalin or saline ($n = 6$ per group) were homogenized using RIPA lysis buffer. The homogenate was centrifuged at 10,000 rpm for 10 min and the supernatants were stored at -80°C until assayed. IL-1 α , IL-1 β , IL-6, and TNF α levels were determined on a Luminex 200 (Bio-Rad, Hercules, CA) using a MILLIPLEX MAP Rat Cytokine/Chemokine Panel (Millipore, Billerica, MA). Myeloperoxidase (MPO) concentration was measured using specific enzyme-linked immunosorbent assay (ELISA) kits (Hycult Biotech, Plymouth Meeting, PA). Protein concentration was determined using a Bio-Rad® kit with bovine serum albumin as the standard. The cytokine and MPO concentrations were standardized relative to tissue protein levels and expressed in pg/mg and ng/mg total protein, respectively.

Laser-capture microdissection

A part of the prostate injected with either formalin or saline ($n = 6$ per group) was embedded in Tissue-Tek OCT compound and stored at -80°C until use. Samples were sectioned at 8 μm thickness, and sections were mounted on PEN membrane slides (Leica Microsystems, Wetzlar, Germany). Prostate specimens were fixed in 70% EtOH for 30s and rinsed with double-distilled H_2O . Sections were stained with Mayer's hematoxylin (Sigma-Aldrich, St. Louis, MO) for 30s, rinsed with double-distilled H_2O and placed in 0.01% eosin (Acros Organics, Geel, Belgium) for 5s. Prostate specimens were dehydrated for 30s twice in 95% ethanol, 30s in 100% ethanol, and 2 min in xylene. The tissue was air-dried, and laser-capture microdissection (LCM) was performed using a Leica LMD6000 (Leica Microsystems). Stromal tissues and the adjacent epithelium in the low-grade (< 50 leukocytes/ $10,000 \mu\text{m}^2$) or high-grade inflammatory area (> 100 leukocytes/ $10,000 \mu\text{m}^2$) were excised separately (Fig. 1D–F). Excised cells were individually captured into the caps of 0.5 ml Eppendorf tubes.

RNA isolation and real-Time PCR analysis

Captured individual tissue specimens were lysed, and RNA isolation, reverse transcription, and real-time PCR were performed using a Cells Direct™ One-Step qRT-PCR kit (Invitrogen, Carlsbad, CA). Gene-specific primers and TaqMan probes crossing exon/exon junctions were designed using the Primer 3 Software (Primer 3, Totowa, NJ). Probes contained FAM fluorophore and TAMRA quencher. The primer and probe combination were optimized within suitable ranges for efficiency and correlation coefficient using standard curve dilutions and data output on an ABI Step-One Plus thermocycler (Applied Biosystems, Foster City, CA). Amplification of cDNA was performed under the following conditions: one cycle of 50°C for 15 min, 95°C for 2 min, followed by 40 cycles of 15 s at 95°C , 45 s at 60°C . The reactions were analyzed in triplicate and normalized relative to GAPDH. Real-time PCR data were analyzed by the DCp (crossing point) method as $R =$

$2^{-(C_p \text{ sample} - C_p \text{ control})}$ to generate the relative expression ratio (R) of each target gene relative to that of GAPDH.

In epithelial cells, we measured AR and six androgen-responsive genes including U19/Eaf2, ELL2, FKBP5, calreticulin and ornithine decarboxylase. In stromal cells, we analyzed AR, TGF- β 1, Hic-5, and fibronectin (Table 1). To determine the specificity of the primer/probe, we performed electrophoresis to verify that the primers only amplified cDNA and not the genomic DNA; the optical band was amplified without nonspecific bands. We also determined specificity to cDNA using real-time PCR to verify that our primer/probe sets did not amplify genomic DNA. All of our primer/probe combinations showed efficiencies of 95–105% (Table 1).

Statistics

The unpaired Student's t-test was used to compare two values. All tests were two-sided, and p values < 0.05 were considered statistically significant. All statistical analyses were performed using SPSS software (SPSS Inc., Chicago, IL).

Results

Histopathology

Hematoxylin and eosin staining demonstrated that intraprostatic formalin injection induced widespread low-grade inflammation along with focal high-grade inflammation in limited areas of the prostate (Fig. 1D–F). We defined the low-grade or high-grade inflammatory areas as those with less than 50 leukocytes/10,000 μm^2 in the slightly expanded stroma and the normal appearance of epithelium or with more than 100 leukocytes/10,000 μm^2 in the highly edematous stroma and the atrophic epithelium, respectively.

Measurement of inflammatory markers

The protein levels of IL-1 α , IL-1 β , IL-6, and TNF α expression were significantly increased by 2–3 fold in the formalin-injected prostate compared to saline-injected controls (Table 2). In contrast, the treatment with COX-2 inhibitor restored the expression of proinflammatory cytokines in formalin-injected rats to levels that were comparable to controls.

The level of neutrophil infiltration in the prostate can be estimated by MPO activity as MPO is released from neutrophils during tissue inflammation. MPO activity was also significantly increased in formalin-injected rats compared to vehicle-injected controls whereas there was no significant difference in MPO activity between control and formalin-injected rats with COX-2 inhibitor treatment.

Androgen-responsive genes in the epithelium and TGF- β 1 cascade genes in the stroma

AR and androgen-responsive genes such as Eaf2, ELL2, FKBP5, calreticulin and ornithine decarboxylase were upregulated by 2–9 fold in laser-captured epithelial cells obtained from low-grade inflammatory regions of the formalin-injected prostate, whereas these genes were significantly downregulated in the high-grade inflammatory regions (Fig. 2). The treatment with a COX-2 inhibitor suppressed the upregulation of these genes in epithelial cells obtained from the low-grade inflammatory regions of the formalin-injected prostate.

In the stroma, AR and TGF- β 1 cascade genes such as TGF- β 1, Hic-5, and fibronectin were upregulated by 2–3 fold in the low-grade inflammatory regions, whereas only fibronectin was significantly increased in the high-grade inflammatory regions (Fig. 3) but not AR, TGF- β 1 or Hic-5. However, increased levels of AR, TGF- β 1, Hic-5 and fibronectin were absent in the formalin-injected prostate with COX-2 inhibitor treatment.

Immunohistochemistry

In order to confirm the increased protein expression of some of molecules upregulated in the prostate with formalin-induced inflammation, we performed immunohistochemical staining for AR, TGF- β 1, IL-6, and COX-2 expression in the low-grade inflammatory region (Fig. 4). Positive staining for AR was observed in the epithelial nuclei of the vehicle-injected prostate. Staining for AR was upregulated in the epithelium of the formalin-injected prostate and it was weakly positive in the stroma. Staining for TGF- β 1 was positive in the stromal area of controls and was strongly positive in the stromal area of the formalin-injected prostate. Staining for IL-6 was negative in the control, but positive in formalin-injected prostate around the inflammatory cells infiltrating in the stroma. COX-2 expression was negligible in the control, but upregulated in the stroma of the formalin-treated rats. In addition, upregulation of these molecules was suppressed in formalin-injected prostate treated with COX-2 inhibitor.

Discussion

Androgens and age have been traditionally considered the main determinants of prostate enlargement, but increasing evidence over the last years has established a potentially important role of inflammation in the development and maintenance of prostatic hyperplasia and LUTS. Inflammation is characterized by infiltration of activated lymphocytes and macrophages into the prostate mediated by proinflammatory cytokines. Lymphocytes and macrophages play an important role in the induction of COX-2 expression in the prostate epithelium as reported in BPH tissues (20). COX-2 mediates increased cell proliferation possibly, due to overexpression of anti-apoptotic gene Bcl-2 (3,20,21) and it is suppressed by COX-2 inhibitors (22,23). Released cytokines are characterized by pleotropic effects and are known to induce proliferation of both the epithelial and stromal compartments, which leads to a subsequent loss of normal epithelial function and the dedifferentiation of smooth muscle cells in the prostate gland (24). Various reports also indicate that initiation and maintenance of proliferation in the human prostate is primarily influenced by the stromal compartment (9,25,26). Penna et al. demonstrated that human stromal prostate cells obtained from BPH tissue can actively contribute to the inflammation by secreting proinflammatory cytokines and chemokines, which create a positive feedback loop that can amplify inflammation (9). In animal studies, induction of intraprostatic inflammation also reportedly resulted in prostatic epithelial proliferation (24,27).

Recent reports indicate upregulation of TGF- β 1 cascade genes in the stroma and androgen-responsive genes in the epithelium of BPH specimen (17). Coffey et al. showed that nuclear AR protein levels are elevated by 47% in BPH specimens relative to the normal controls (28). Our previous study also found upregulation of AR and androgen-responsive genes in hypertrophied epithelium relative to the adjacent normal epithelium one of the same prostate (10). However, the relationship between the inflammation and upregulation of these genes has not been previously investigated. In this study, we demonstrated that intraprostatic inflammation induces upregulation of androgen-responsive genes and TGF- β 1 cascade genes, which mimics clinically relevant molecular alterations seen in BPH pathophysiology.

Intimate association of androgens with BPH does implicate an important role for the upregulated androgen-responsive gene expression in epithelial cells in BPH pathogenesis. Several androgen-responsive genes, including Eaf2 and calreticulin, are shown to be growth suppressive and/or tumor suppressive and they are downregulated in prostate cancer (29,30). Therefore, upregulated expression of these genes in BPH is likely to prevent epithelial cells from undergoing malignant transformation (29,30). These key differences in the androgen-responsive gene expression for BPH and prostate cancer may explain the benign and malignant prostate growth in BPH and prostate cancer, respectively.

The signaling between stromal and epithelial cells in the prostate is mediated by growth factors such as TGF- β 1, which is also critical for prostatic smooth muscle remodeling (16,17). TGF- β 1 induces trans-differentiation of fibroblasts into smooth muscle cells and myofibroblasts, which secrete distinct extracellular matrix components such as collagen I and fibronectin. Additionally, the transcriptional coregulator and focal adhesion protein Hic-5, which is one of downstream proteins of TGF- β 1 signaling, impacts on the response of many downstream TGF- β 1 targets that have been implicated in tissue homeostasis (31). Hic-5 is also known to be involved in transaction between TGF- β 1 and androgen signaling (31,32). Taken together, since previous clinical studies demonstrated a positive correlation between prostate inflammation and LUTS in BPH patients (1–3), the upregulation of androgen-responsive genes and TGF- β 1 cascade genes associated with low-grade prostatic inflammation found in this study could be a potential underlying mechanism for the development of symptomatic BPH. Thus, rats with non-bacterial prostatic inflammation induced by intraprostatic formalin injection would be a suitable animal model for studying the role of an inflammatory component in BPH pathogenesis. In addition, utilization of LCM techniques can provide the useful information regarding the region-specific changes in androgen- and/or TGF- β 1-related molecules associated with tissue inflammation in the prostate.

In the present study, anti-COX-2 treatment suppressed tissue inflammation as well as overexpression of androgen-responsive genes and TGF- β 1 cascade genes. Previous studies have also demonstrated that COX-2 inhibition causes decreases in AR activity of prostate cancer cells (33,34). Moreover, a daily use of non-steroidal anti-inflammatory drugs is reported to prevent increase in prostate volume in population-based cohort study (35). Thus, anti-inflammatory treatments using COX-2 inhibitors could be useful agents in new strategies for BPH prevention and treatment.

We are mindful of several limitations in this study. First, the formalin-induced prostatitis model used in this study does not completely mimic the human condition. Although the pathogenesis of BPH is still unclear, the selection of current model was encouraged by the fact that prostatic inflammation seen in BPH tissues is not associated with bacterial infection. Secondly, we did not confirm epithelial or stromal proliferation in this model as shown in the previous studies using animal models of bacterial prostatitis (27). Therefore, further investigation using animal models of prostatic inflammation including bacterial prostatitis will be needed to identify the long-term effects of prostatic inflammation on epithelial or stromal proliferation due to changes in expression of androgen-responsive and/or TGF- β 1 cascade genes.

Conclusions

Prostatic inflammation causes the changes in expression of androgen-responsive genes in the epithelium and TGF- β 1 cascade genes in the stroma. Activation of androgen-responsive genes and TGF- β 1 cascade genes in the low-grade inflammatory region might contribute to epithelial and stromal proliferation in the development of BPH, respectively. In addition, COX-2 inhibitors, which can suppress changes in these gene expressions, might be beneficial for the treatment of patients with symptomatic BPH.

Acknowledgments

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Key Definitions for Abbreviations

BPH	benign prostatic hyperplasia
LUTS	lower urinary tract symptoms
AR	androgen receptor
TGF-β1	tumor growth factor- β 1
COX-2	cyclooxygenase-2
PBS	phosphate-buffered saline
MPO	myeloperoxidase
ELISA	enzyme-linked immunosorbent assay
LCM	laser-capture microdissection

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Fig. 1. Microscopic findings of the prostate

Representative photomicrographs (Figs. 1A–C) of prostatic sections showing the LCM methods to excise the stroma in low-grade inflammatory regions (Fig. 1B) and the adjacent epithelium (Fig. 1C). Figs. 1A–C show the same prostatic section before (A) and after LCM (B and C). Excised cells were individually captured into the caps of 0.5 ml Eppendorf tubes. In hematoxylin-eosin staining (Fig. 1D–F), compared with the vehicle-injected prostate (Fig. 1D), the formalin-injected prostate sections demonstrated low-grade inflammation in most of the prostate tissue (Fig. 1E) and high-grade inflammation in some areas with a large number of infiltrating leukocytes, expansion of stromal area and atrophic epithelium (Fig. 1F). The images were taken at the magnification of $\times 200$. Scale bars; 100 μm

Fig. 2. Relative expression of androgen-responsive genes in the LCM-dissected epithelium
 Statistically significant upregulation of androgen-responsive gene expression was observed in the LCM-dissected epithelium obtained from the low-grade inflammatory region (light-gray bars). COX-2 inhibitor treatment suppressed the upregulation of these genes. In contrast, statistically significant downregulation was observed in the epithelium obtained from the high-grade inflammatory region (dark-gray bars). COX-2 inhibitor treatment partially restored the downregulation of androgen receptor, but not those of other androgen-responsive genes. Data are expressed as the relative expression ratio of each target gene relative to that of GAPDH. c; control, f; rats with formalin-induced prostatic inflammation, f +CCX; rats with prostatic inflammation treated with celecoxib. Light gray column: low-inflammatory regions. Dark gray column: high-inflammatory regions. N = 6 per group. * $p < 0.05$ compared to the control. # $p < 0.05$ between groups.

Fig. 3. Relative expression of TGF-β1 cascade genes in the LCM-dissected stroma from the low-grade inflammatory region

TGF-β1 cascade genes were significantly upregulated in the LCM-dissected stroma in the low-grade inflammatory region (light-gray bars) whereas only fibronectin was significantly upregulated in the stroma from the high-grade inflammatory region (dark-gray bars). COX-2 inhibitor treatment suppressed these changes. Data are expressed as the relative expression ratio of each target gene relative to that of GAPDH. c; control, f; rats with formalin-induced prostatic inflammation, f+CCX; rats with prostatic inflammation treated with celecoxib. Light gray column: low-inflammatory regions. Dark gray column: high-inflammatory regions. N = 6 per group. * p < 0.05 compared to the control. # p < 0.05 between groups.

Fig. 4. Photomicrographs showing immunohistochemical staining in prostatic sections A–C; anti-androgen receptor, D–F; anti-TGF- β 1, G–I; anti-IL-6, J–L; anti-COX-2. Compared to the respective control (A, D, G and J), androgen receptor, TGF- β 1, IL-6, and COX-2 expression was remarkably enhanced in the formalin-injected rats (B, E, H and K, respectively). These increased expressions were suppressed by celecoxib treatment after formalin injection (C, F, I, and L, respectively). Images were taken at the magnification of $\times 200$. Scale bars; 100 μ m.

Table 1

Sequences of primers and probe

	5' primer	3' primer	Taqman probe	efficacy (%)
androgen receptor	TTGGCGAGAGACAGCTTGTA	ATCTGGTCAATCCACATGCAA	GGTCAAGTGGGCCAAGGCCT	95.3
Eaf 2	CACACCGTGGCTATGACT	CCTTTGAAAACTGTGACTGGTG	GGTTGGCAAAGGTGAACAGGTGACA	98.0
ELL 2	CGCTGGAGACTTACCAGAGC	CATTGAAGGGATCATTTTGG	TCAGTTCCAAGGACTCCAAGGGC	98.5
FKBP 5	GCCGTTTGTCTTTAGCCTTG	GATCTCGCCTTTCTTTCATGG	GGCCAAGTTATCAAAAGCCTGGGA	99.1
calreticulin	ACGAGCCAAAGATTGATGACC	TCCCACTCTCCATCCATCTC	GCCTGAGGACTGGGACAAGCCA	96.3
ornithine decarboxylase	CCTGTGCTGTGAGGAGACAG	CTCGATGTCCTCTGAAACA	TTGACCTTGTGAGAGCTGGCCA	99.8
TGF-β1	TGAGTGGCTGTCTTTTGACG	ACTGAAGCGAAAAGCCCTGTA	TCCGGCAGTGGCTGAACCAA	98.2
Hic 5	AACCTATTGCTGGCAAAGTG	CCTCTGCAAAAGGAAGTGCTC	CCGCCCTGGGTAGAGCCTGG	102.0
fibronectin 1	CAGTACGGGCACCAAGAAAGT	CCAGTCACTGTGTGCTGGT	TTCACCAACAGCGCCAGCAC	102.3
GAPDH	AGACAGCCGCATCTTCTTGT	GATACGGCCAAAATCCGTTC	GCAGTGCCAGCCTCCTCTCA	104.0

Table 2

Cytokine expression and MPO activity

	control	formalin	formalin + celecoxib
IL-1 α	4.5 $\pm$ 1.2	12.3 $\pm$ 3.0 *	7.8 $\pm$ 1.6
IL-1 β	3.3 $\pm$ 1.1	7.4 $\pm$ 0.7 **	5.0 $\pm$ 0.8 #
IL-6	2.4 $\pm$ 0.7	9.4 $\pm$ 1.6 **	4.4 $\pm$ 1.5 #
TNF α	0.45 $\pm$ 0.11	2.43 $\pm$ 0.40 **	1.02 $\pm$ 0.34 #
MPO	251 $\pm$ 49	2119 $\pm$ 1050 *	327 $\pm$ 54

IL-1 α , IL-1 β , IL-6, and TNF α expression levels are expressed as pg/mg total protein and myeloperoxidase (MPO) activity is expressed as ng/mg total protein. N = 6 per group.

* p < 0.05,

**

p < 0.01 compared to control.

p < 0.05 compared to the formalin group. There were no statistically significant differences between control and formalin + celecoxib groups.