Original Research

Protective antioxidative and anti-inflammatory actions of β-caryophyllene against sulfasalazine-induced nephrotoxicity in rat

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Impact Statement

To our knowledge, the current report is the first to demonstrate the association between NF-κB, TGF-β, Nrf2, AMPK, and AKT with the pathogenesis of sulfasalazine-induced renal injury and oxidative stress. In addition, this study is the first to report nephroprotective effects for β-caryophyllene against SFZ-induced nephrotoxicity. Hence, we suggest that SFZ and BCP co-therapy could offer an alternative better therapeutic approach for rheumatoid arthritis and IBD by enhanced anti-inflammatory actions as well as by preventing SFZ-induced renal injury.

Abstract

The pathogenesis of sulfasalazine (SFZ)-induced nephrotoxicity is unclear. Moreover, there are no reports on the protective effects of β-caryophyllene (BCP) against SFZ-induced renal injury. Hence, in this study, we measured several oxidative stress and inflammatory regulatory molecules alongside the effects of BCP in SFZ-intoxicated rats. Male rats ($n = 48$) were distributed to six equal groups as follows: negative control (NC), normal rats treated with low (N-LD; 200 mg/kg/day) and high (N-HD; 400 mg/kg/day) BCP doses, and animals treated with SFZ individually (PC; 600 mg/kg/day) or combined with BCP low (P-LD) and high (P-HD) doses. All drugs were administrated for 14 consecutive days. The NC, N-LD, and N-HD groups showed comparable renal histology and biochemistry. In contrast, abnormal histology, and increased creatinine and urea alongside oliguria and proteinuria were detected in the PC group. Renal specimens from the PC group revealed increased levels of nuclear factor-kappa B (NF-κB), transforming growth factor (TGF)-β with kidney injury molecule (KIM)-1, while the levels of nuclear factor erythroid 2-related factor 2 (Nrf2), AMP-activated protein kinase (AMPK), and protein kinase B (AKT) declined, relative to controls. The PC renal tissue also had markedly higher levels of inflammatory cytokines (tumor necrosis factor [TNF]-α/interleukin [IL]-1β/IL-6) and pro-oxidants (malondialdehyde [MDA]/H_2O_2/protein carbonyls), whereas those of antioxidants (glutathione [GSH]/glutathione peroxidase [GPx]/superoxide dismutase-1 [SOD1]/catalase [CAT]) and IL-10 decreased and were associated with marked apoptosis. Both BCP regimens ameliorated renal functions and histology, and reduced NF-κB, TGF-β, and KIM-1 levels in addition to those of oxidative stress and inflammation markers. Both protocols also augmented Nrf2, AMPK, AKT, antioxidants, and IL-10. However, P-HD showed better alleviating effects than the N-HD group. In conclusion, this study is the first to link NF-κB, TGF-β, Nrf2, AMPK, and AKT with SFZ-induced nephrotoxicity. In addition, this is the first report to reveal antioxidative and anti-inflammatory effects for BCP against SFZ-associated nephropathy.

Keywords: Nuclear factor-kappa B, transforming growth factor-β, nuclear factor erythroid 2-related factor 2, AMP-activated protein kinase, protein kinase B, kidney injury molecule-1

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Introduction

Sulfasalazine (SFZ) is an anti-inflammatory prodrug widely used for treating rheumatoid arthritis and inflammatory bowel disease (IBD).[1,2] Sulfapyridine (SP) and 5-aminosalicylic acid (5-ASA) are the main constituents of SFZ, and 5-ASA is the main therapeutic anti-inflammatory ingredient.[1,2] While most of 5-ASA metabolites are excreted in feces, about 25% are acetylated and excreted in urine.[3] Unfortunately, the prolonged use of SFZ is linked with several adverse reactions and many cases of SFZ-induced nephrotoxicity have been recorded, which include glomerulonephritis, interstitial nephritis, and acute renal failure.[3–5]

The pathogenic mechanisms underlying SFZ-induced renal damage remain unclear. Nonetheless, numerous animal studies have underscored the major roles played by oxidative stress and inflammation in the pathogenesis of SFZ-induced nephrotoxicity.[6–9] In this regard, several research groups have suggested that the accumulation of SFZ metabolites induces a disequilibrium between increased production of free radicals with a concurrent deterioration in renal antioxidant capacity following mitochondrial

dysfunction.[6–9] Incessant oxidative stress then triggers renal inflammatory responses and cellular damage.[6–9] However, there are no reports on the key regulatory molecules of cellular redox biology and inflammation in the pathogenesis of SFZ-induced nephropathy, including nuclear factor erythroid 2-related factor 2 (Nrf2), nuclear factor-kappa B (NF-κB), and transforming growth factor (TGF)-β.

Currently, there are no approved drugs for the treatment of SFZ-related renal impairment and the clinical management comprises discontinuing SFZ and administering corticosteroids.[10,11] Hence, it has been suggested that using natural products with antioxidant and anti-inflammatory activities may offer a protective approach against SFZ-induced renal injury.[6–9] β-caryophyllene (BCP) is a major volatile constituent of the essential oils of a wide variety of plants and is mainly used for food flavoring.[12–14] Although BCP exhibits potent antioxidative and anti-inflammatory renoprotective actions,[15,16] no prior reports have explored its preventive or alleviatory effects against SFZ-induced renal toxicity.

Therefore, this study was conducted to explore the roles of Nrf2, NF-κB and TGF-β alongside adenosine monophosphate-activated protein kinase (AMPK), protein kinase B (AKT), and kidney injury molecule (KIM)-1 in the pathogenesis of SFZ-related renal injury. Furthermore, this study examined the renoprotective effects of BCP against SFZ-induced nephrotoxicity in relation to oxidative stress and inflammation.

Materials and methods

Chemicals and reagents

BCP solution (0.9 g/mL) and SFZ powder of ⩾98% purity were from Sigma-Aldrich Chemical Co. (MO, USA). While enzyme-linked immunosorbent assay (ELISA) kits for malondialdehyde (MDA), protein carbonyl groups, hydrogen peroxide (H_2O_2), glutathione (GSH), superoxide dismutase-1 (SOD1), catalase (CAT), and glutathione peroxidase (GPx1) were from Cell Biolabs, Inc. (CA, USA), rat tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and IL-10 kits were from Cloud-Clone Corp. (TX, USA). All primary antibodies, apoptosis kit, and polymerase chain reaction (PCR) reagents were purchased from Thermo Fisher Scientific (CA, USA).

Study design and therapeutic regimens

Forty-eight male Wistar rats with body weight ranging from 180 to 200 g and of eight weeks of age each were housed at a regulated temperature (24 ± 1°C temperature with a12-h light/dark cycle). The rats were fed with standard food and water *ad libitum* throughout the study and were distributed following a week of acclimatization into (n = 8 rats/group): the negative controls (NCs), normal animals treated with low (N-LD) and high (N-HD) doses of BCP groups, the positive controls (PCs), which only received SFZ, and two groups that simultaneously received SFZ with either low (P-LD) or high (P-HD) BCP doses. While the NC rats received normal saline as vehicle, SFZ (600 mg/kg/day) and/or the BCP low (200 mg/kg/day) and high (400 mg/kg/day) doses were freshly prepared and delivered to the designated groups for 14 consecutive days by oral gavage as previously reported.[8,17] The Committee for the Care and Use of Laboratory Animals at Umm Al-Qura University, KSA approved all experiments.

Types of samples

At day-14, each rat was housed in metabolic cages (Braintree Scientific Inc., MA, USA) and 24-h urine (24 h-U) samples were collected. Euthanasia was then performed on day-15 by cervical dislocation under anesthesia as previously reported.[18] Blood samples were obtained, and serum was kept at −20°C following centrifugation. Both kidneys were removed from each rat, and a specimen was used for histopathological experiments. Another renal tissue specimen (500 mg) was lysed in RIPA buffer with protease inhibitors. The extracted total protein concentrations were measured by a BCA protein assay (Thermo Fisher Scientific). Deionized water was used to dilute each total protein sample (1000 µg/mL) to be processed by ELISA. The residual tissues were kept at −80°C in RNA*Later* (Thermo Fisher Scientific).

Renal biochemical parameters

Quantification of serum creatinine (Cr), urea, total protein, and albumin alongside the amounts of 24 h-U Cr and total protein was done with Cobas e411 (Roche Diagnostics, Mannheim, Germany).

The calculation of 24 h-U flow and Cr clearance (Cr-Cl) was done as follows

$$\text{Urine flow}\,(\mu\text{L/min}) = \left[\frac{\text{24-h urine volume (mL)}}{(60\,\text{min} \times 24\,\text{h} = 1440)}\right] \times 1000$$

$$\text{Cr-Cl(mL/min)} = \frac{\left[\text{Urine Cr (mg/dL)} \times \text{urine flow (mL/min)}\right]}{\text{Serum Cr (mg/dL)}}$$

Quantitative reverse transcription polymerase chain reaction

RNA was extracted with a Paris kit and cDNA synthesis was achieved with a high-capacity Reverse Transcription (RT) kit. Each sample was processed in triplicate wells for each targeted gene. Forty PCR amplification cycles (95°C/15 s and 60°C/1 min) were performed on a QuantStudio™ 3 Real-Time PCR System (Thermo Fisher Scientific). Each well included 25 ng cDNA (1 µL), DNase/RNase free water (2 µL), SYBR Green master mix (5 µL), and 5 pmol (1 µL) of forward and reverse primer sets (Supplementary Table 1). NCs included a minus-template PCR by substituting the cDNA with nuclease free water in addition to another minus-RT control from the RT step. Normalization was done by *GAPDH* gene and the $2^{-\Delta\Delta Ct}$ method was applied to relatively measure rat caspase (*Casp)-3*, *Nfkb1*, *TGF-β*, *KIM1*, *AMPK*, *Nrf2*, and *AKT1* gene expressions.

Immunohistochemistry

NF-κB p50, TGF-β1, AMPK-α, AKT1, and Nrf2 were localized by polyclonal rabbit IgG antibodies, while KIM-1 was detected by polyclonal goat IgG antibodies, in renal tissues. Briefly, the sections were incubated at 4°C with the primary antibodies (1:200 concentration). All sections were washed

Table 1. Serum and 24–h urine concentrations (mean ± SD) of renal biochemical markers in the study groups.

		NC group	N-LD group	N-HD group	PC group	P-LD group	P-HD group
Serum	Creatinine (mg/dL)*	0.48 ± 0.07	0.49 ± 0.06	0.48 ± 0.06	1.1 ± 0.19[b,d,f]	0.73 ± 0.13[b,d,f,h]	0.46 ± 0.07[h,j]
	Urea (mg/dL)**	35.2 ± 5.6	34.9 ± 3.8	34.6 ± 4.1	67.9 ± 9.4[b,d,f]	54.1 ± 5.2[b,d,f,h]	43.4 ± 4.9[a,c,e,h,i]
	Total protein (g/dL)**	7.3 ± 0.97	7.1 ± 0.95	7.3 ± 0.74	5.5 ± 0.5[b,d,f]	5.9 ± 0.6[b,d,f]	6.8 ± 0.7[g]
	Albumin (g/dL)**	4.6 ± 0.5	4.7 ± 0.4	4.6 ± 0.4	3.2 ± 0.3[b,d,f]	3.3 ± 0.3[b,d,f]	4.2 ± 0.4[h,j]
24 h Urine	Urine volume (mL)**	9.4 ± 1.2	9.6 ± 1.4	9.3 ± 1.4	3.7 ± 0.6[b,d,f]	4.7 ± 0.8[b,d,f]	7.9 ± 0.9[h,j]
	Urine flow (μL/min)**	6.5 ± 0.8	6.6 ± 1	6.4 ± 1	2.6 ± 0.4[b,d,f]	3.3 ± 0.6[b,d,f]	5.4 ± 0.4[h,j]
	Creatinine (mg/dL)**	46.2 ± 5.1	45.7 ± 4.7	46.2 ± 4.6	25.8 ± 4.1[b,d,f]	35.3 ± 3.1[b,d,f,h]	41.3 ± 3.9[h]
	Creatinine clearance (mL/min)*	0.63 ± 0.15	0.62 ± 0.11	0.63 ± 0.16	0.06 ± 0.01[b,d,f]	0.16 ± 0.03[b,d,f,h]	0.5 ± 0.12[h,j]
	Total protein (mg/dL)*	4 ± 0.7	3.8 ± 0.7	3.9 ± 0.6	13.7 ± 3.1[b,d,f]	10.5 ± 2[b,d,f]	6.2 ± 1.2[b,d,f,h,j]

NC: negative control group; N-LD: negative control + low-dose β-caryophyllene group; N-HD: negative control + high-dose β-caryophyllene group; PC: positive control group; P-LD: positive control + low-dose β-caryophyllene group; P-HD: positive control + high-dose β-caryophyllene group.
[a]$P < 0.05$ compared with NC group.
[b]$P < 0.01$ compared with NC group.
[c]$P < 0.05$ compared with N-LD group.
[d]$P < 0.01$ compared with N-LD group.
[e]$P < 0.05$ compared with N-HD group.
[f]$P < 0.01$ compared with N-HD group.
[g]$P < 0.05$ compared with PC group.
[h]$P < 0.01$ compared with PC group.
[i]$P < 0.05$ compared with P-LD group.
[j]$P < 0.01$ compared with P-LD group.
*Games–Howell's post hoc test was used following ANOVA to compare between the groups.
**Tukey's HSD post hoc test was used following ANOVA to compare between the groups.

twice in the following day and treated with ImmPRESS® HRP Horse AntiRabbit or antiGoat IgG Plus Polymer Peroxidase Kits (Vector Laboratories Inc., CA, USA) by following the manufacturer's protocols. The NCs included renal sections that were identically processed, but in which primary isotype goat or rabbit IgG antibodies (Santa-Cruz Biotechnology Inc., TX, USA) were used as substitutes for the primary antibodies. The slides were examined on a Leica DMi8 microscope (Leica Microsystems, Wetzlar, Germany). Digital images were captured with 40× objective from 10 non-overlapping fields/slide. The ImageJ software (https://imagej.nih.gov/ij/) was utilized to quantify the immunostain intensity as described earlier.[19]

Terminal deoxynucleotidyl transferase-dUTP nick end labeling assay

Renal cell apoptosis/necrosis was detected by a Click-iT™ Terminal deoxynucleotidyl transferase-dUTP nick end labeling (TUNEL) Alexa Fluor™ 488 Imaging Assay (Thermo Fisher Scientific). Active Casp-3 was then co-localized with the apoptotic bodies by incubating the sections for 3 h with anticleaved Casp-3 mouse monoclonal IgG antibodies (1:100 concentration). Subsequently, the sections were incubated for 30 min with secondary donkey antimouse IgG antibodies tagged with Alexa Fluor™ 555. The sections were counterstaining with DAPI (Thermo Fisher Scientific) and observed with 40× objective under a Leica DMi8 fluorescent microscope. The numbers of apoptotic/necrotic cells were counted in 15 different fields/slide to calculate apoptosis index as reported earlier.[19]

ELISA

TNF-α, IL-1β, IL-6, IL-10, GSH, SOD1, CAT, GPx1, MDA, protein carbonyl groups, and H_2O_2 concentrations in renal tissues were measured with an automated ELISA apparatus (Human Diagnostics, Wiesbaden, Germany). Each sample was processed in duplicate wells.

Statistical analysis

All variables were analyzed by SPSS version 25, and normality was assessed by the Kolmogorov and Smirnov's test, whereas the Levene test was applied to assess data homogeneity. According to variance equality, Tukey's honestly significant difference (HSD) or Games–Howell's post hoc analyses were applied following one-way analysis of variance (ANOVA) test for comparing between the study groups. Significance was indicated by a P-value < 0.05.

Results

Serum and urine renal biochemical parameters

Serum Cr, urea, total protein and albumin alongside the 24 h-U volume, 24 h-U flow, and urine Cr and total protein concentrations in the normal rats treated with low and high doses of BCP were equal to those of the NC group (Table 1). In contrast, the PC rats demonstrated significantly higher serum Cr and urea levels alongside marked proteinuria and oliguria, which coincided with a substantial decrease in 24 h-U Cr clearance relative to the NC, N-LD, and N-HD groups. Although the BCP low and high treatment doses in the SFZ-intoxicated rats revealed marked declines in serum Cr and urea along with improved 24 h-U Cr levels and Cr-Cl compared with the PC rats, the ameliorative effects were markedly more prominent in the P-HD animals (Table 1). While all the tested serum and urine renal biochemical parameters were similar in the P-HD and the NC groups, serum and 24 h-U total protein concentrations remained significantly abnormal in the earlier group (Table 1).

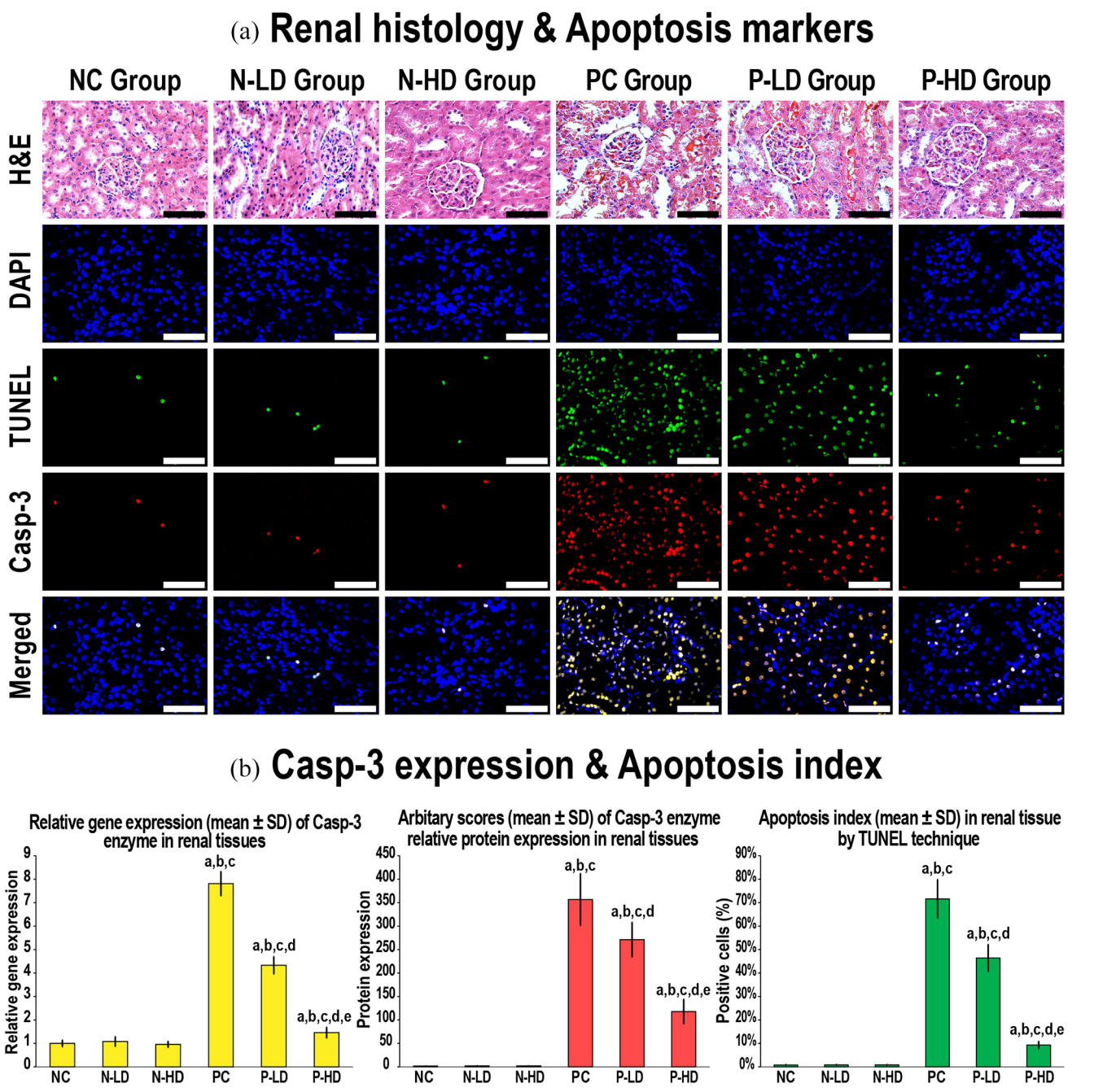

Figure 1. (a) Renal H&E histological characteristics and co-detection of apoptotic bodies by immunofluorescence TUNEL's method (green) with cleaved Casp-3 (red) followed by DAPI counterstain in renal tissues from all the study groups (scale bar = 10 µm). Moreover, (b) Casp-3 relative mRNA and protein expressions of in addition to apoptosis index in the renal tissues are shown as mean ± SD. (A color version of this figure is available in the online journal.)
[a]$P < 0.05$ compared with the NC group.
[b]$P < 0.05$ compared with the N-LD group.
[c]$P < 0.05$ compared with the N-HD group.
[d]$P < 0.05$ compared with the PC group.
[e]$P < 0.05$ compared with the P-LD group.

Renal histology

The renal specimens from the NC, N-LD, and N-HD groups displayed normal features by H&E staining, and Casp-3 protein together with the apoptotic bodies' numbers were minimal in the three groups (Figure 1). In contrast, the PC group demonstrated marked leukocytic infiltration, glomerular damage, interstitial necrosis, widening of tubular lumens, and cellular protrusion in addition to significantly higher Casp-3 with increased apoptosis index. Concordant with renal biochemistry, both the low and doses of BCP in the SFZ-treated rats ameliorated renal histology and decreased Casp-3 with apoptosis index in comparison with the PC animals (Figure 1). Although the P-HD group also showed significantly better histological features alongside lower Casp-3 expression and apoptosis than the P-LD rats, the results were markedly elevated relative to the NC group.

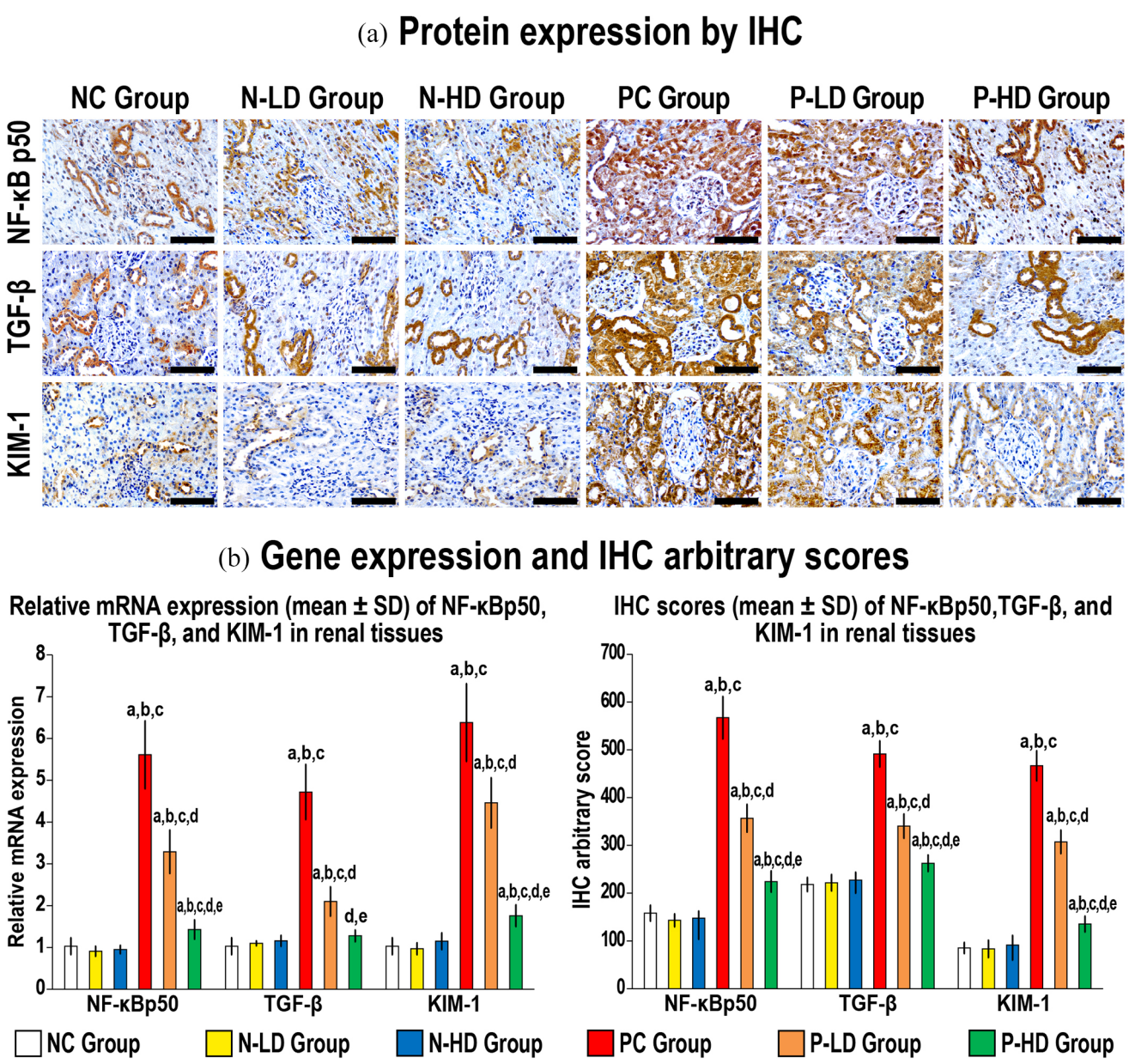

Figure 2. (a) Detection of renal NF-κB p50, TGF-β1, and KIM-1 by immunohistochemistry (IHC; scale bar = 10 μm) in addition to (b) their mRNA expression and IHC scores are expressed as mean ± SD. (A color version of this figure is available in the online journal.)
[a]$P < 0.05$ compared with the NC group.
[b]$P < 0.05$ compared with the N-LD group.
[c]$P < 0.05$ compared with the N-HD group.
[d]$P < 0.05$ compared with the PC group.
[e]$P < 0.05$ compared with the P-LD group.

Expression of pathogenic molecules and damage markers in renal tissues

The gene and protein expressions of NF-κB p50, TGF-β1, and KIM-1 (Figure 2) as well as AMPK-α, AKT1, and Nrf2 (Figure 3) in renal tissues were similar among the NC, N-LD, and N-HD groups. In contrast, the PC renal tissues displayed significant escalations in the NF-κB p50, TGF-β1, and KIM-1 mRNA and protein levels (Figure 2), while the levels of AMPK-α, AKT1, and Nrf2 genes and proteins declined markedly (Figure 3), relative to the NC, N-LD, and N-HD groups. Despite that the genes and proteins of the molecules of interest showed substantial alterations in the P-LD and P-HD groups compared with the PC group, the results were significantly more evident in the P-HD than the P-LD group (Figures 2 and 3). Moreover, the NF-κB p50, TGF-β1, and KIM-1 gene and protein expressions in the P-HD were equivalent to the NC renal tissues (Figure 2), whereas the amounts of AMPK-α, AKT1, and Nrf2 remained markedly lower in the earlier group (Figure 3).

Inflammatory and oxidative stress markers in renal tissues

The renal tissue concentrations of TNF-α, IL-1β, IL-6, IL-10, GSH, SOD1, GPx1, CAT, MDA, H_2O_2, and protein carbonyl

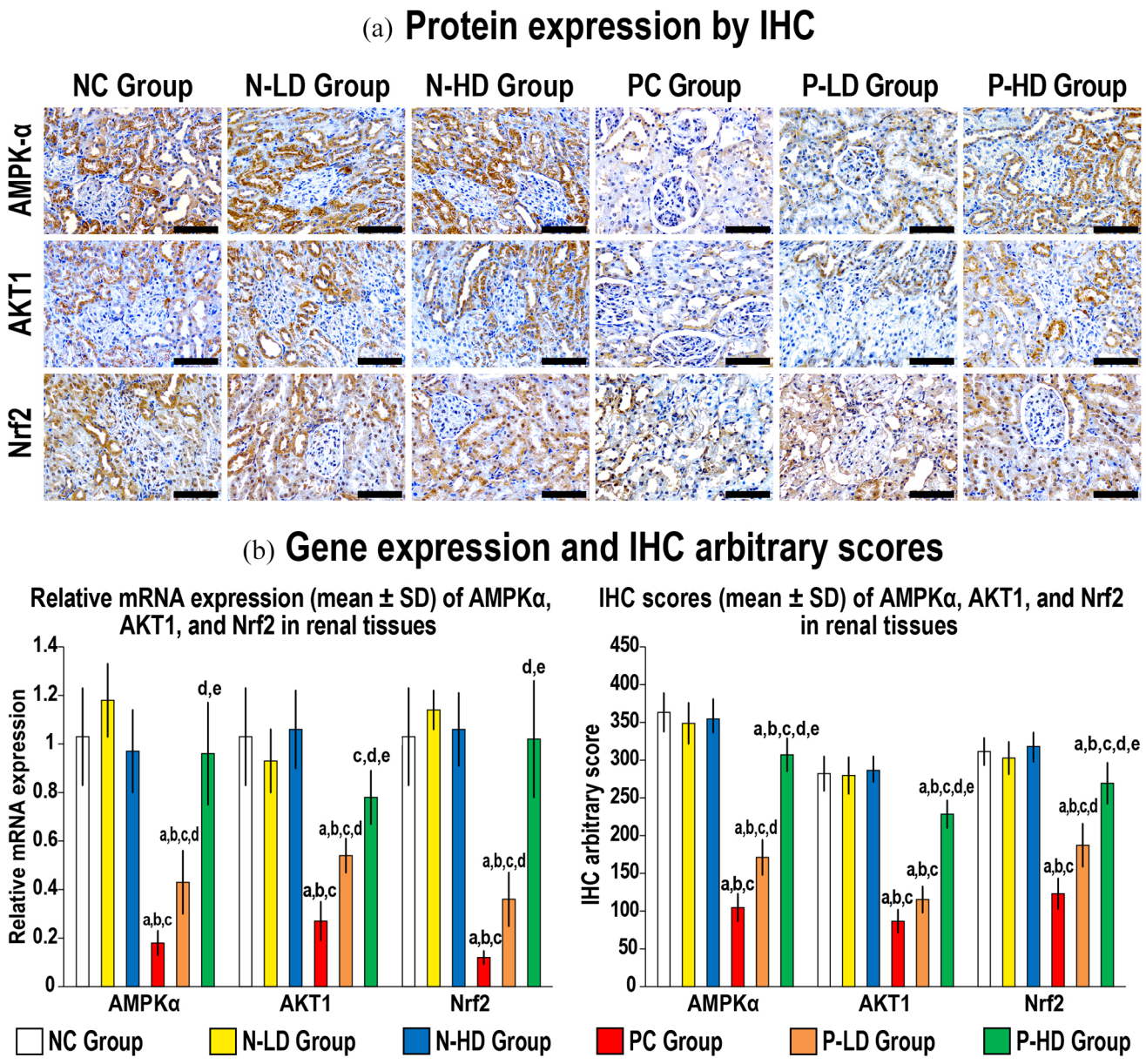

Figure 3. (a) Detection of renal AMPK-α, AKT1, and Nrf2 in renal tissues by immunohistochemistry (IHC; scale bar = 10 μm) in addition to (b) their mRNA expression and IHC scores are expressed as mean ± SD. (A color version of this figure is available in the online journal.)
[a]$P < 0.05$ compared with the NC group.
[b]$P < 0.05$ compared with the N-LD group.
[c]$P < 0.05$ compared with the N-HD group.
[d]$P < 0.05$ compared with the PC group.
[e]$P < 0.05$ compared with the P-LD group.

groups were similar in the NC, N-LD, and N-HD groups (Table 2). However, the levels of TNF-α, IL-1β, IL-6, MDA, H_2O_2, and protein carbonyl groups augmented, whereas IL-10, GSH, SOD1, GPx1, and CAT diminished, markedly in the PC renal specimens than the NC, N-LD, and N-HD groups (Table 2). While the levels of pro-inflammatory and pro-oxidative stress markers weakened, the anti-inflammatory and antioxidative molecules augmented, significantly in the P-LD and P-HD renal tissues compared with the PC group. The P-HD protocol, however, demonstrated significantly more potent effects than the P-LD group on the targeted inflammatory and oxidative stress markers, except for GPx1 that was similar between both groups (Table 2). In addition, the renal concentrations of TNF-α, IL-6, IL-10, GSH, SOD1, GPx1, CAT, and MDA were equal between the P-HD and NC groups, whereas IL-1β, H_2O_2, and protein carbonyl groups remained markedly higher in the former group (Table 2).

Discussion

This study examined both the molecular mechanisms that could contribute to SFZ-induced nephrotoxicity as well as the potential protective actions of BCP in respect to renal oxidative stress and inflammation. The data revealed substantial elevations in serum urea and creatinine, whereas

Table 2. Renal tissue concentrations (mean ± SD) of inflammatory and oxidative stress markers in the study groups.

	NC group	N-LD group	N-HD group	PC group	P-LD group	P-HD group
TNF-α (pg/mL)*	36.7 ± 6.8	38.1 ± 8.7	35.7 ± 6.3	106.2 ± 14.9[b,d,f]	62.4 ± 19[a,c,e,h]	35.4 ± 7.4[h,i]
IL-1β (pg/mL)*	33.3 ± 7.5	32.3 ± 4.1	32.6 ± 4.9	224.2 ± 33.4[b,d,f]	168.7 ± 25.3[b,d,f,g]	62.2 ± 11.6[b,d,f,h,i]
IL-6 (pg/mL)**	72.7 ± 15.9	70.8 ± 11.9	73.2 ± 14.6	253.4 ± 26.8[b,d,f]	188.9 ± 22.1[b,d,f,h]	91.3 ± 23.7[h,i]
IL-10 (pg/mL)**	50.2 ± 7.3	51.1 ± 6.5	50.3 ± 6.9	11.7 ± 2.8[b,d,f]	26.6 ± 7.9[b,d,f,h]	44.6 ± 5.7[h,i]
GSH (mg/g)**	41.9 ± 6.6	40.3 ± 3.7	40.3 ± 3.1	16.7 ± 4.6[b,d,f]	25.9 ± 4.2[b,d,f,h]	39.4 ± 4.8[h,i]
SOD1 (U/g)**	48.9 ± 6.6	47.8 ± 4.9	48.2 ± 5.3	24.8 ± 5.7[b,d,f]	33.3 ± 4.5[b,d,f,g]	43.1 ± 4.7[h,i]
GPx1 (μg/mg)**	4.8 ± 0.7	4.9 ± 0.7	4.8 ± 0.5	2.4 ± 0.6[b,d,f]	3.3 ± 0.7[b,d,f]	4.3 ± 0.8[h]
CAT (U/mg)**	292.7 ± 23.5	287.8 ± 26.8	291.4 ± 24.2	156.6 ± 23.6[b,d,f]	200.4 ± 20.3[b,d,f,h]	270.1 ± 21.7[h,i]
MDA (nmol/g)**	30.1 ± 4.8	29.7 ± 5.2	30.1 ± 4.6	67.4 ± 6.1[b,d,f]	50.6 ± 6.3[b,d,f,h]	36.2 ± 5.1[h,i]
H_2O_2 (μM/g)*	1.1 ± 0.3	0.9 ± 0.2	1 ± 0.2	51.4 ± 6.9[b,d,f]	36.6 ± 6.3[b,d,f,h]	1.9 ± 0.4[b,d,f,h,i]
Protein carbonyl groups (nmol/g)*	0.4 ± 0.07	0.39 ± 0.06	0.41 ± 0.06	4.7 ± 0.6[b,d,f]	3.1 ± 0.6[b,d,f,h]	1.1 ± 0.3[b,d,f,h,i]

TNF-α: tumor necrosis factor-α; IL-1β: interleukin-1β; IL-6: interleukin-6; IL-10: interleukin-10; GSH: glutathione; SOD1: superoxide dismutase 1; CAT: catalase; GPx1: glutathione peroxidase-1; GR: glutathione reductase; MDA: malondialdehyde; H_2O_2: hydrogen peroxide; NC: negative control group; N-LD: negative control + low-dose β-caryophyllene group; N-HD: negative control + high-dose β-caryophyllene group; PC: positive control group; P-LD: positive control + low-dose β-caryophyllene group; P-HD: positive control + high-dose β-caryophyllene group.
[a]$P < 0.05$ compared with NC group.
[b]$P < 0.01$ compared with NC group.
[c]$P < 0.05$ compared with N-LD group.
[d]$P < 0.01$ compared with N-LD group.
[e]$P < 0.05$ compared with N-HD group.
[f]$P < 0.01$ compared with N-HD group.
[g]$P < 0.05$ compared with PC group.
[h]$P < 0.01$ compared with PC group.
[i]$P < 0.01$ compared with P-LD group.
*Games–Howell's post hoc test was used following ANOVA to compare between the groups.
**Tukey's HSD post hoc test was used following ANOVA to compare between the groups.

serum total protein and albumin declined significantly, and coincided with oliguria, proteinuria, and decreased creatinine clearance in the PC group than the NC group. The PC renal specimens also demonstrated marked increases in the oxidative stress (MDA/H_2O_2/protein carbonyl groups) and inflammatory (TNF-α/IL-1β/IL-6) molecules together with decreases in the antioxidants (GSH/SOD1/GPx1/CAT) and anti-inflammatory (IL-10) markers relative to controls.

SFZ is commonly utilized to treat IBD and rheumatic diseases, but the drug has been disclosed by several clinical reports to induce renal impairment in the form of glomerulonephritis, interstitial nephritis, and acute renal failure.[3–5] Rats treated with SFZ also manifested dose-dependent glomerular dysfunction, tubular damage, marked leukocytic infiltration, and interstitial necrosis along with abnormal serum and urine renal biochemical parameters.[6–9] Proposed mechanisms for SFZ-induced nephrotoxicity involve mitochondrial damage with elevations in cellular free radicals alongside decreases of several antioxidants (e.g. GSH, SOD, GPx1, and CAT), thus causing lipid peroxidation, protein carbonylation, and renal cell injury.[6–9] Concurrently, renal impairment induced by SFZ is also associated with elevated inflammatory cytokines.[6–9] Our results correlate with many earlier reports and further reinforce that oxidative stress and inflammation are major pathogenic mechanisms contributing to SFZ-induced renal damage.[3,6,8,9]

Despite the well-established roles of oxidative stress and inflammation in the pathophysiology of SFZ-associated nephrotoxicity,[3,9] none of the previous studies explored the key regulators of renal redox homeostasis and inflammatory responses in the context of SFZ-induced nephropathy. Indeed, renal redox biology and immune processes are tightly controlled by numerous molecular pathways that could undergo pathological alterations during the pathogenesis of SFZ-induced nephrotoxicity. Under this theme, cellular NF-κB escalates significantly with a variety of renal diseases and promotes the production of inflammatory cytokines.[20,21] TGF-β1 equally plays an important role in cellular inflammation, and the protein level increases substantially with numerous renal diseases, including diabetic nephropathy and acute renal failure.[22,23] Moreover, both NF-κB and TGF-β pathways interact during the pathogenesis of renal diseases, thus aggravating renal inflammation and cell damage that subsequently trigger the main apoptotic molecule, Casp-3.[24–27] In contrast, Nrf2 is a ubiquitous protein that protects against oxidative stress-induced renal damage by promoting the expression of several antioxidant enzymes.[28,29] Likewise, the activation of renal AMPK/AKT pathway attenuates cell injury associated with oxidative stress and inflammation.[30–32]

To the best of our knowledge, this report is the first to unveil substantial increases in NF-κB, TGF-β1, and KIM-1 genes and proteins, while those of AMPK-α, AKT1, and Nrf2 declined, in the PC renal specimens in comparison with the NC rats. In addition, both the percentages of apoptotic bodies and Casp-3 expression increased drastically in the PC renal specimens compared with control rats. Hence, we suggest that the pathogenesis of SFZ-induced renal inflammation and oxidative stress could involve overexpression of NF-κB and TGF-β1 with simultaneous inhibition of AMPK-α, AKT1, and Nrf2 genes and proteins that subsequently initiate apoptosis by elevating Casp-3.[9,25,26,28,30] Moreover, we propose that KIM-1, which is a sensitive and specific marker of tubular damage, could be used to monitor SFZ-induced nephrotoxicity.[33,34] However, additional studies should

further investigate the molecular mechanisms that turn SFZ from an anti-inflammatory drug to an inducer of renal inflammatory reactions to corroborate our propositions.

Clinical management of SFZ-induced renal injury mainly consists of drug discontinuation together with corticosteroids to minimize renal inflammation.[10,11] Alternatively, only a few animal studies have reported nephroprotective effects for several nutraceutical products against SFZ-associated kidney impairment.[6–9] Herein, co-administration of low or high doses of BCP with SFZ preserved renal histology, ameliorated serum and urine renal biochemical parameters, lowered apoptosis index, and reduced Casp-3 relative to the PC animals. Moreover, both BCP protocols diminished the renal levels of oxidative stress and pro-inflammatory markers and augmented the antioxidant and anti-inflammatory molecules relative to the PC renal tissues. Nonetheless, the alleviating effects of the high BCP dose were significantly more pronounced than the low-dose group. In addition, healthy animals treated with the low and high doses of BCP showed comparable histological, biochemical, and apoptosis index to those of the NC group.

Concordant with our observations, many prior studies have reported non-toxic effects for BCP following treatments with similar or higher doses (up to 700 mg/kg/day) that were administrated for equal and/or longer durations of therapy (90 days) in healthy murine.[35,36] Moreover, BCP exhibited potent anti-inflammatory and antioxidative stress activities against a wide variety of pathologies.[12–14] In this context, BCP treatment downregulated NF-κB, induced the activities of antioxidant enzymes, reduced the levels of KIM-1, and inhibited apoptosis, thus alleviated hyperoxaluria-induced renal impairment in rats.[15] Moreover, BCP inhibited NF-κB with several inflammatory cytokines and simultaneously upregulated various Nrf2-dependent antioxidant mechanisms, thus attenuated the adverse effects of high glucose on mesangial cells.[16] Others have likewise shown ameliorative effects for BCP in the treatment of acute lung injury induced by lipopolysaccharide in mice following reductions in several inflammatory markers, including TGF-β.[37] BCP also alleviated cerebral injury induced by ischemia-reperfusion in rat as well as deterred lipid accumulation in human hepatocyte subsequent to enhancing the AMPK/AKT signaling network in the targeted cells.[38,39]

The present data agree with several earlier reports and advocate that BCP could be used as renoprotective agent against SFZ-induced renal injury by concurrently suppressing NF-κB and TGF-β1, and promoting renal antioxidant capacity via Nrf2-dependent pathways.[15,16,37] Furthermore, preclinical studies have also demonstrated remedial effects of BCP for the treatment of IBD and arthritis in rats.[40,41] Therefore, we suggest that SFZ and BCP co-therapy may provide a superlative treatment strategy for rheumatoid arthritis and IBD by enhanced anti-inflammatory actions as well as by preventing SFZ-induced renal injury.[40,41]

In conclusion, this study is the first to reveal that SFZ-induced renal oxidative stress and inflammation may result from pathological alterations in renal NF-κB, TGF-β1, Nrf2, and AMPK/AKT signaling pathways. While both BCP regimens attenuated SFZ-induced renal damage through antioxidative stress and anti-inflammatory actions, the high-dose protocol showed better ameliorative activities. However, further experiments are needed to measure the therapeutic and/or protective activities of BCP and/or SFZ single and dual therapies for treating autoimmune diseases and/or preventing SFZ-associated adverse events.

AUTHORS' CONTRIBUTIONS

Both B.R. and M.E.B. equally contributed to conceptualization, methodology, formal analysis, investigation, and resources and project administration. B.R. wrote original and revised versions of the manuscript and both authors have read and approved all versions.

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SUPPLEMENTAL MATERIAL

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