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A novel pathway by which the environmental toxin 4-Nonylphenol may promote an inflammatory response in inflammatory bowel disease

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: 4-Nonylphenol is a ubiquitous environmental toxin that is formed as a byproduct in the manufacturing and/or sewage treatment of regular household items. Previous work in our lab has implicated 4-NP in the progression of autoimmune diseases such as inflammatory bowel disease in which macrophages mistakenly attack the intestinal linings, causing chronic inflammation. Several key pro-and anti-inflammatory molecules have been shown to be involved in the manifestation of this disease, including IL-23A, COX-2, IL-8, TLR-4, and IL-10.

Material/Methods: 4-NP's effects on these known mediators of IBD were effectively analyzed using a novel model for IBD, by which 4-NP may promote an inflammatory response. Data were collected using DNA Microarray, RT-PCR, and ELISA, after 48 hour treatment of U937 histiocytic lymphocyte cells and COLO320DM human intestinal epithelial cells with 1 nM and 5 nM concentrations of 4-NP.

Results: Significant dysregulation of the expression of both pro- and anti-inflammatory genes was observed in U937 cells that would promote and prolong inflammation. However, TLR-4, IL-8, and COX-2 gene expressions showed unprecedented effects in COLO320DM cells suggesting that these genes mediate apoptotic processes within the gastrointestinal tract.

Conclusions: Overall, our results suggest that 4-NP administration engenders immune responses linked to apoptotic processes via dysregulation of macrophage signaling. In sum, 4-NP appears to increases the risk of developing inflammatory bowel disease by promoting or prolonging adverse progression of inflammation in the gastrointestinal tract.

Keywords: 4-Nonylphenol (4-NP) • Inflammatory Bowel Disease (IBD) • Toll-Like Receptors (TLR) • Cytokines

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Background

Approximately 80 pathological disorders afflicting over 22 million Americans may be classified as autoimmune in nature [1]. Notably, the unusually high prevalence of autoimmune disorders in female populations [2] strongly indicates an estrogenic mechanistic link in the etiology and progression of pathophysiological processes [3,4]. Furthermore, chronic inflammatory processes may be sustained by activation of estrogen receptors expressed on the surface of lymphocytes [4–6]. From a toxicological perspective, chronic bodily accumulation of xenoestrogenic environmental toxins may represent a potential significant risk underlying the onset and progression of autoimmune disorders in women [7–11].

The present study has focused on potential autoimmune mechanisms linked to administration of the major xenoestrogenic environmental toxin 4-Nonylphenol (4-NP) to cultured human cell lines. 4-NP is the biodegradation product of Nonylphenol Ethoxylate, which is commonly used in the manufacturing of paints, liquid detergents, cosmetics, pesticides and other industrial products [12,13]. 4-NP flows into the environment via sewage effluents and may significantly contaminate drinking water, soil, food, and airborne sources [4,14–17]. Due to widespread and potentially chronic exposure, 4-NP poses a significant biological threat in comparison to other environmental toxins. Additionally, 4-NP has the longest alkyl chain within the alkylphenol family, facilitating its partition into lipophilic fat stores within body compartments [5,8,9,18].

The present study evaluates the role of 4-NP as a potential contributing factor in the etiology of Inflammatory Bowel Disease (IBD). IBD expression is dependent on autoimmune processes driving chronic inflammation of the tissue linings of the gastrointestinal tract [12,16]. Accordingly, we have chosen to model IBD as a severely dysregulated innate immune response directed towards intestinal tissues via dual employment of a human U937 histiocytic cell line as a source of activated pro-macrophages and the human COLO320DM cell line as an *in vitro* intestinal tissue source. Biochemical and molecular dependent measures included hallmark mediators of autoimmunity (COX-2, HSP90b, TLR-4, IL-23A, IL-8, IL-10, IL-4, TNF-α, IL-1β), and apoptotic processes (BAX, BCL-2, and FAS) [14,17,19–25].

Material and Methods

Cell culture

U937 histiocytic lymphocyte cell line and COLO320DM human intestinal epithelial cell line (ATCC, USA) were cultured in RPMI 1640 media (Invitrogen, USA) supplemented with 10% fetal bovine serum (Invitrogen, USA), and incubated in a 37°C incubator (NAPCO) supplemented with 5% CO_2.

RNA isolation

After treatment with 4-NP (Sigma-Aldrich, St. Louis, MO) at 1nM, and 5nM., U937 cells were harvested by centrifugation, whereas COLO320DM cells were first detach from the flask using trypsin (Invitrogen, USA) and then pelleted by centrifugation. RNA isolation was carried by the Tri-reagent method (following manufacturer's protocol, (Molecular Bioproducts).

Microarray analysis

The isolated RNA was analyzed on a model 2100 bioanalyzer (Agilent, Santa Clara, CA) using a total RNA nanochip following the manufacturer's protocol. Then, using the Applied Biosystems Chemiluminescent RT-IVT Labeling Kit version 2.0 and manufacturer's protocol, Digoxigenin-UTP labeled cRNA was generated and linearly amplified from 1 µg of total mRNA. Six chips, one for each group per cell line, each used 15µg of labeled cRNA and then hybridized at 55°C for 19 hours using the Applied Biosystems Chemiluminescence Detection Kit and Applied Biosystems 1700 Chemiluminescent Microarray Analyzer (following manufacturer's protocol). Following hybridization, Applied Biosystems 1700 Expression System software was utilized to extract chemiluminescence values from each of the three microarray images [26].

Data analysis

Data from the six microarray whole-genome chips were analyzed using the Spotfire software from TIBCO (Palo Alto, CA). The fold changes were measured by dividing the signals of the two variables by the control signal. The control signal served as the normalizing baseline for each of the other two chips. A fold change of ±3 or more was considered significant dysregulation (Figure 1).

Gene expression

In order to assess the accuracy of the magnitude of microarray data that was collected, conventional Reverse Transcription–Polymerase Chain Reaction (RT-PCR) was performed for the reference gene beta actin. Total RNA (3.5 ug) from treated and untreated cells was reverse-transcribed using random primers (Invitrogen, Carlsbad, CA). RNA was denatured at 95°C and reverse transcribed at 40°C for 1 hr using Superscript III Rnase H-Reverse Transcriptase (Invitrogen, Carlsbad, CA). For the conventional PCR analysis of the various genes, specific forward primer, and reverse primer were used. Each PCR reaction required 10 µL of cDNA. Electrophoresis of the PCR products was done on a 2% agarose gel and then analyzed using GelPro Analysis software (Media Cybernetics, USA). The data obtained by the GelPro Analysis software was then analyzed in an ANOVA test followed by post-HOC Scheffe test ($p<0.05$), in order to determine significance.

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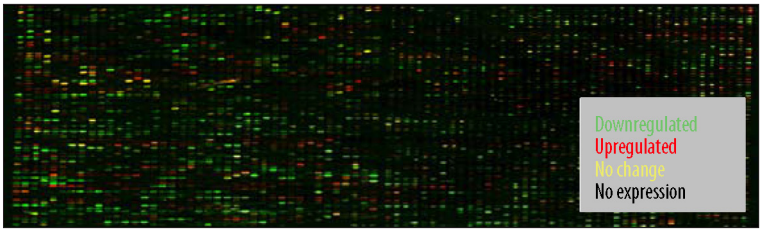

Figure 1. DNAoligo information. Presence of a gene is determined by the intensity of the chemiluminescence. Genes shown in green were down regulated, genes shown in red were up regulated and genes shown in yellow showed no changes in the gene expression compared to the control. Genes not expressed in the cells were left as blanks.

Table 1. DNA microarray analysis in U-937 and COLO320DM cells of cytokines with standard deviation. Treatment groups: Control, 4-NP 1nM, and 4-NP 5nM. Units are represented in Chemiluminescence. Significant differences were determined using a one-way ANOVA test followed by a post-hoc Scheffe test with P<0.05.

	Microarray analysis of genes				
	Results (units of chemiluminescence)				
	IL-1β	IL-8	IL-23A	IL-10	IL-4
U-937 cells					
Control	867.81±43.3	20103.25±1005.163	370.4±18.5	1243.4±62.1	1175.14±58.7
4-NP 1nM	2236.75±111.8	57577.77±2878.8	1406.81±70.3405	783.8±39.1	643.17±32.1
4-NP 5nM	2994.66±149.7	102153.28±5107.6	1930.02±96.501	318.63±15.9315	212.51±10.6255
COLO320DM cells					
Control	18252.9±912.6	6600.6±330.03	82.8±4.14	860.1±43	1530.4±76.5
4-NP 1nM	32009.81±1600.5	3800.5±190.1	159.9±7.99	620.5±31.02	970.5±48.5
4-NP 5nM	45836.87±2291.8	2100.1±105	274.7±13.7	290.1±14.5	440.9±22

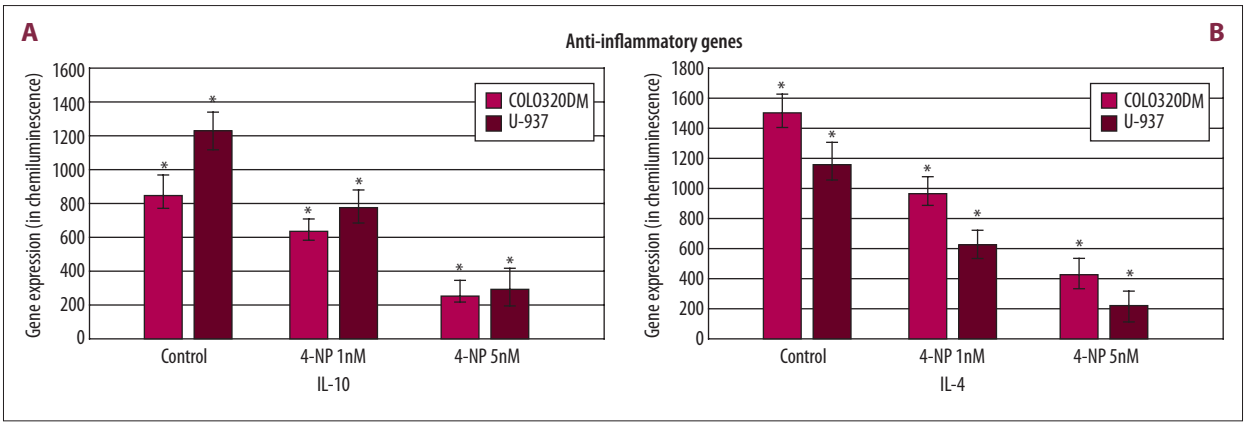

Figure 2. DNA Microarray Analysis in COLO320DM (Blue) and U-937 (Red) cells. Treatment groups: Control, 4-NP 1nM, and 4-NP 5nM. ((**A**) IL-10, (**B**) IL-4) Significant Differences were determined using a One-way ANOVA test followed by a post-hoc Scheffe test with P<0.05 and are labeled by the asterisk.

Protein analysis

Supernatant fluids from both U937 and COLO320DM human intestinal epithelial cell lines were collected and stored at –70°C. The cell pellets were reconstituted in PBS and placed into a –70°C freezer. The freeze and thaw method was carried out to lyse the cells and then harvested the cell lysate. Protein concentration for the various cytokines (IL-10, IL-1β, and TNF-α) was determined by Enzyme-Linked Immunosorbent Assay (ELISA) with the supernatant and lysate from the COLO320DM Cells and U-937 Cells. The ELISA plates were analyzed in the Microplate Reader (BIO-RAD®, USA) and Microplate Manager 4.0 software (BIO-RAD®, USA). The data was further analyzed in Microsoft Excel and SPSS. Finally, an ANOVA test followed

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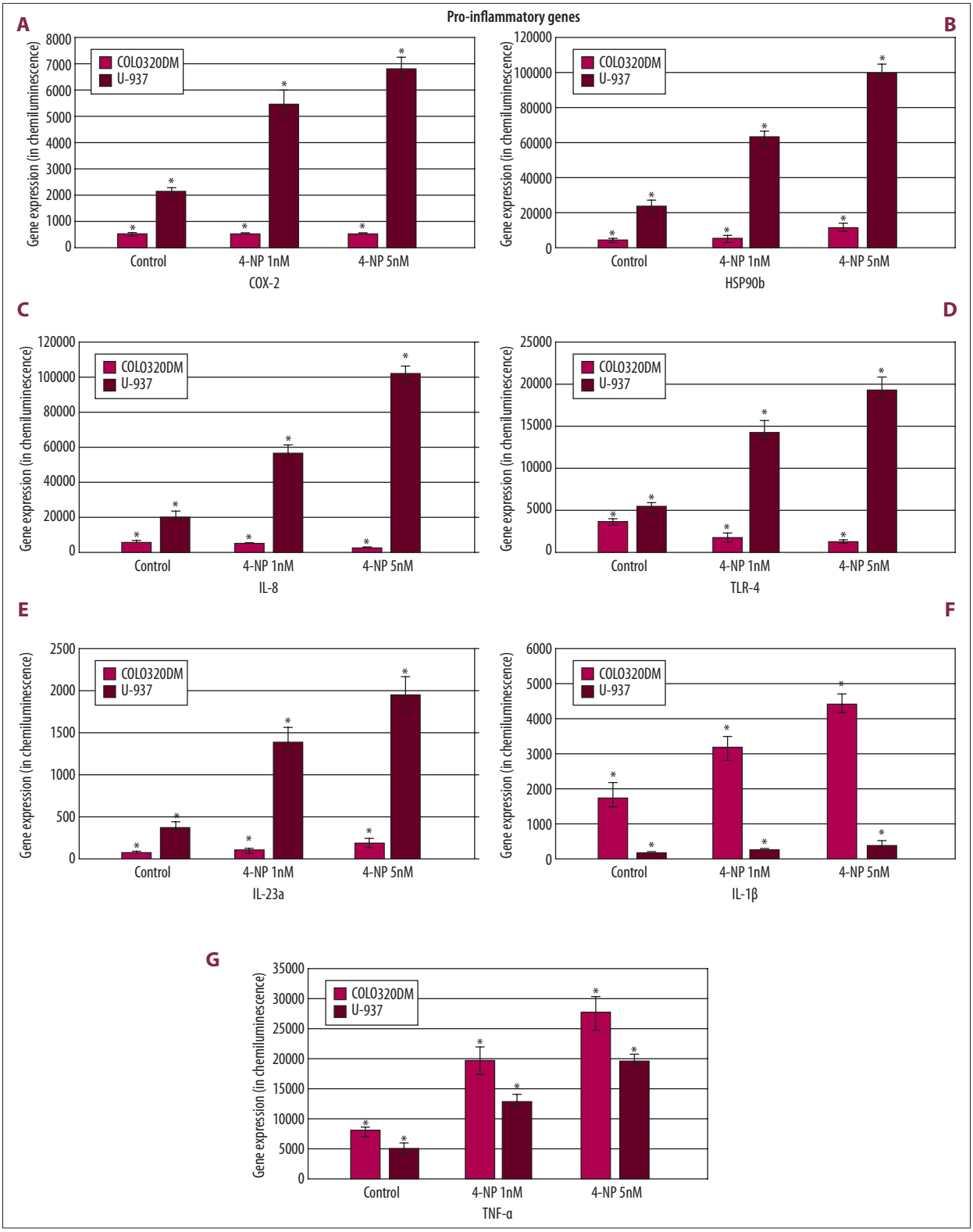

Figure 3. RT PCR Analysis of COLO320DM and U-937 cells RNA. Treatment groups: Control, 4-NP 1nM, and 4-NP 5nM, 48 hrs. ((**A**) COX-2, (**B**) HSP90b, (**C**) IL-8, (**D**) TLR-4, (**E**) IL-23a, (**F**) IL-1β, G. TNF-α) Significant Differences were determined using a One-way ANOVA test followed by a post-hoc Scheffe test with $P<0.05$ and are labeled by the asterisk.

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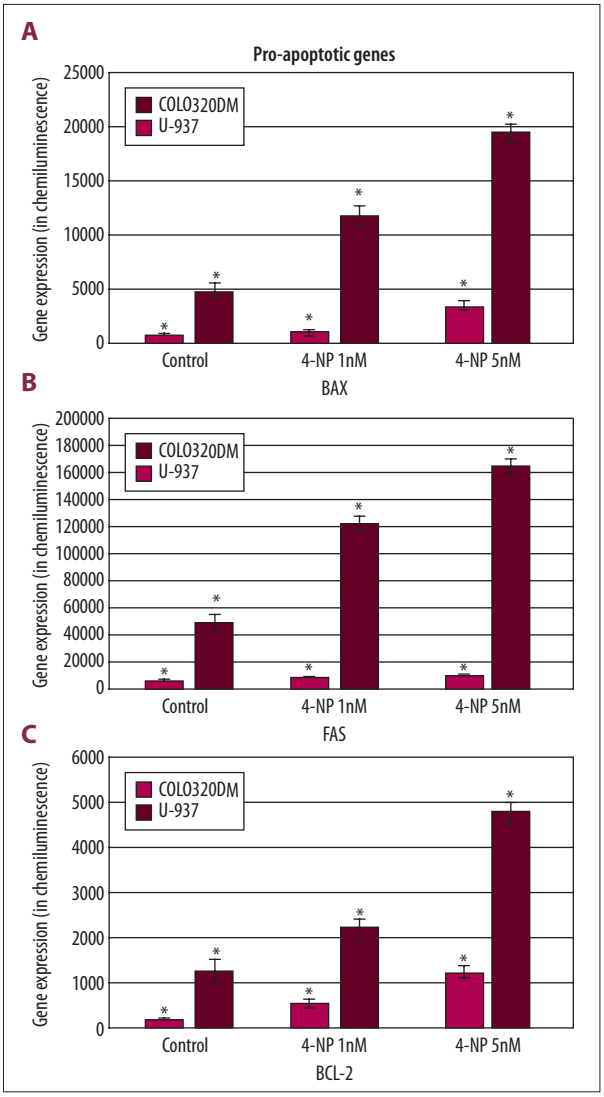

Figure 4. DNA Microarray Analysis in COLO320DM and U-937 cells. Treatment groups: Control, 4-NP 1nM, and 4-NP 5nM. ((**A**) BAX, (**B**) FAS, (**C**) BCL-2) Significant Differences were determined using a One-way ANOVA test followed by a post-hoc Scheffe test with P<0.05 and are labeled by the asterisk.

by post-HOC Scheffe test ($p<0.05$) was carried out to look for statistical significance.

Results

DNA microarray analyses indicated that 4-NP administration significantly increased the expression of the IL-1β, IL-8, IL-23A pro-inflammatory genes, and decreased the expression of the anti-inflammatory genes IL-10 and IL-4 in both U937 and COLO320DM human cells lines (Table 1; Figure 2A, 2B). Conventional RT-PCR analysis demonstrated decreased expression of COX-2, IL-8, and TLR-4 genes in the COLO320DM cells treated with 5nM 4-NP

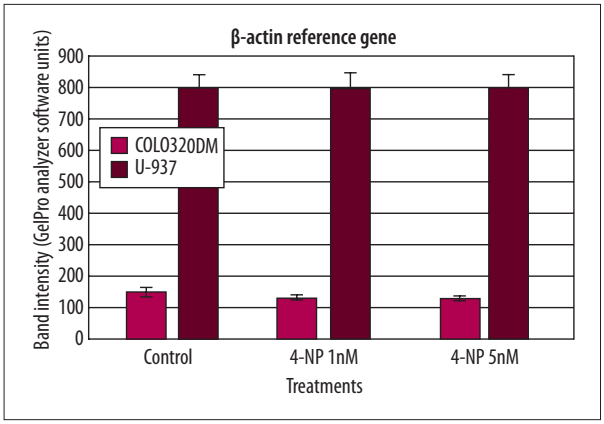

Figure 5. RT PCR Analysis of the Beta actin Reference Gene with N=3 in COLO320DM and U-937 Cells. The x-axis represents the 3 Treatment groups: Control, 4-NP 1nM, and 4-NP 5nM. The y-axis represents the gene expression of Beta actin, as determined by Band Intensity from the gel analysis software. There was no significant difference as the Standard deviation lines overlapped at each level of treatment.

(Figure 3A, 3C, 3D) and decreased expression of IL-10 and IL-4 genes for all treatment groups in both cell lines. HSP90b, IL-23a, TNFα, IL1β, BAX, FAS, and Bcl-2 genes were significantly up regulated for all treatment groups (Figure 3B, 3E–3G; 4A–4C). Furthermore, the β-actin reference gene had relatively no difference at all between the groups (Figure 5). This verifies that the conditions of the various reactions were similar.

Furthermore, data obtained from ELISA analysis showed a significant decreases in IL-10 protein content, and an increase in TNF-α and IL-1β in the lysate and supernatant fluids from both cell lines treated with 4-NP (1nM and 5nM) (Table 2; Figures 6A, 6B and 7A–7D).

Discussion

The purpose of the present study was to evaluate the causal effects of *in vitro* 4-NP administration on the expression of COX-2, HSP90b, IL-1β, TLR-4, IL-8, IL-23A, IL-10, FAS, BAX, BCL-2, IL4 and TNF-α genes in complementary cell lines. In the U937 histiocytic lymphocyte (pro-macrophage) cell line, 4-NP administration engendered a statistically significant up-regulation of pro-inflammatory genes linked to down-regulation of the prominent anti-inflammatory gene IL-10. These data indicate that 4-NP significantly dysregulates the expression and biological balance of several key genes that are responsible for mediating cellular pro-inflammatory and anti-inflammatory responses, with resultant potentiation of deleterious autoimmune processes. We hypothesize that repetitive 4-NP uptake and retention from contaminated sources will chronically promote autoimmune inflammatory processes by up-regulation of pro-inflammatory

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Table 2. Enzyme-linked immunosorbent assay (ELISA) in U-937 and COLO320DM cells of cytokines with standard deviation. Treatment groups: Control, 4-NP 1nM, and 4-NP 5nM. Units are represented in concentration pg/mL. Significant differences were determined using a one-way ANOVA test followed by a post-hoc Scheffe test with P<0.05.

	ELISAs			
	Results (concentration pg/mL)			
	IL-1β Supernatant	IL-10 Supernatant	IL-1β Lysate	IL-10 Lysate
U-937 cells				
Control	232.3±15.8	272.9±18.5	227.7±15.4	27.14±1.8
4-NP 1nM	422.4±28.7	122.7±8.3	395.3±26.8	15.13±1.04
4-NP 5nM	721.6±49.06	72.3±4.9	690.1±46.9	10.2±0.6
COLO320DM cells				
Control	39.4±2.7	19.3±1.3	44.3±2.9	47.9±3.2
4-NP 1nM	88.8±5.9	18.1±1.2	97.1±6.5	27.5±1.8
4-NP 5nM	135.6±9.1	15.1±1.02	153.3±10.3	13.8±0.93

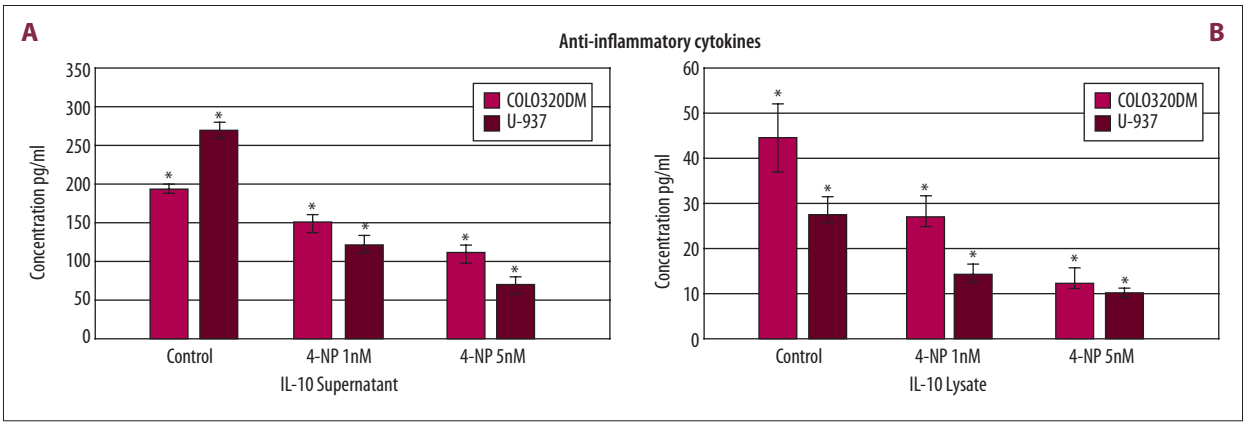

Figure 6. Enzyme-linked Immunosorbent Assay (ELISA) in U-937 and COLO320DM cells of Cytokines with Standard Deviation. Treatment groups: Control, 4-NP 1nM, and 4-NP 5nM. ((**A**) IL-10 Supernatant, (**B**) IL-10 Lysate) Units are represented in Concentration pg/mL. Significant Differences were determined using a One-way ANOVA test followed by a post-hoc Scheffe test with P<0.05.

genes linked to coordinate down-regulation of anti-inflammatory genes, i.e., IL-10. This presents 4-NP as a potential significant causal element in the pathogenesis of autoimmunity [9].

In the COLO320DM cell line, however, 4-NP administration produced a more complex pattern of change. Whereas 4-NP administration significantly up-regulated the expression of the pro-inflammatory genes HSP90b, IL-23A, IL-1β, and TNF-α, the expression of the major pro-inflammatory genes COX-2, TLR-4, and IL-8 was significantly down-regulated. 4-NP administration was without effect on the expression of the β-actin reference gene. Down-regulation of TLR-4 gene expression means that this key pro-inflammatory regulator is being disrupted by exposure to 4-NP; TLR-4 has been shown to play a role in the development and progression in some autoimmune diseases [11,15,19,24,27]. This suggests that the immune system is made more vulnerable to autoimmune dysfunctions and other immune pathologies because there will be an incoherent immune response due to the disruption of multiple key genes involved in the immune-response-reaction-process, as may be the case in IBD. Even more importantly, these phenomena have been observed in intestinal epithelial cells, which line the intestines. The intestines serve home to many viable bacteria which have the precise ability to cause infections, and especially in a disrupted immune state, infection is more likely to occur, causing tissue damage and inflammation, and potentially leading to the more conducive IBD [27,28].

Interestingly, previous published work indicates a causal association of down-regulated COX-2 gene expression and cellular apoptosis [25,29,30], thereby suggesting that 4-NP may directly promote gastrointestinal tissue damage via convergent

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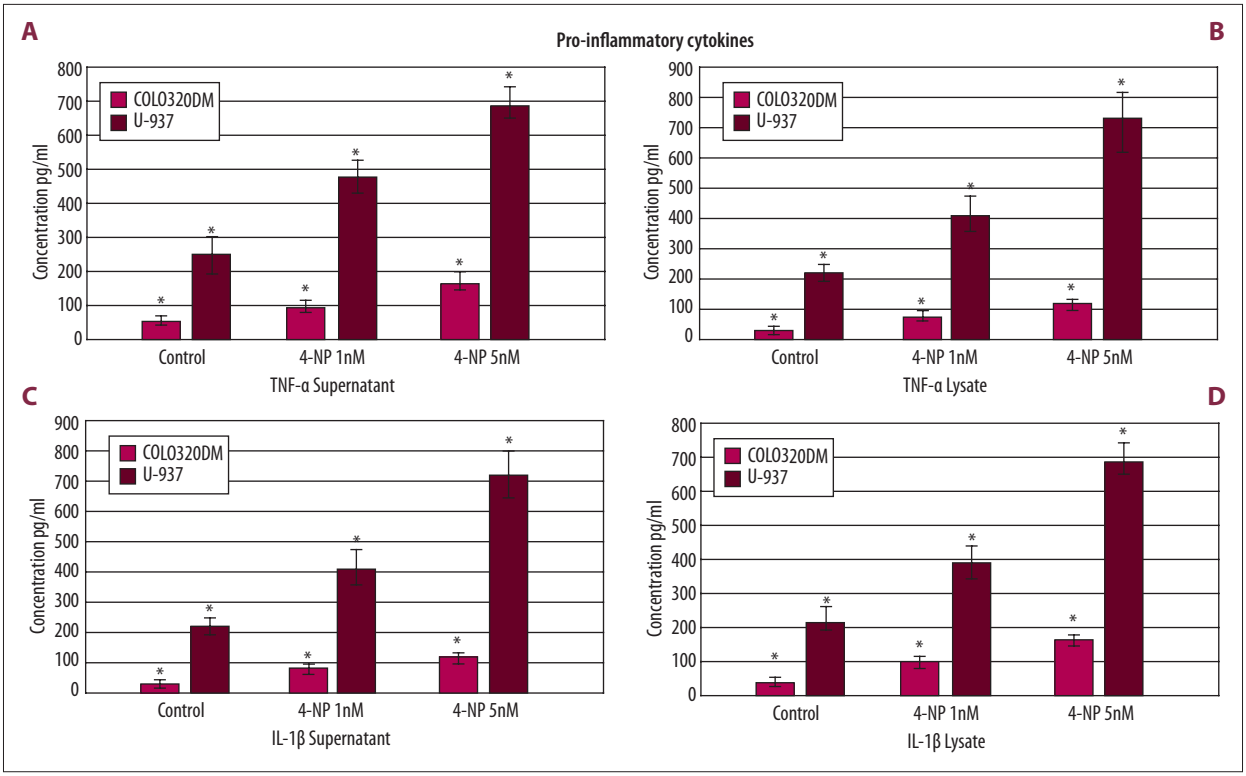

Figure 7. Enzyme-linked Immunosorbent Assay (ELISA) in U-937 and COLO320DM cells of Cytokines with Standard Deviation. Treatment groups: Control, 4-NP 1nM, and 4-NP 5nM. ((**A**) TNF-α Supernatant, (**B**) TNF-α Lysate, (**C**) IL-1β Supernatant, (**D**) IL-1β Lysate) Units are represented in Concentration pg/mL. Significant Differences were determined using a One-way ANOVA test followed by a post-hoc Scheffe test with $P<0.05$.

inflammation linked to induction of apoptotic signaling pathways. Coordinate up-regulation of BAX and FAS gene expression suggests that activation of an apoptotic signaling pathway has been promoted by 4-NP administration to COLO320DM cells. As previously observed, induction of BAX, and FAS gene expression promotes apoptosis whereas TLR-4, IL-8, and COX-2 gene expression is associated with restricted apoptotic processes [27,31,32]. Accordingly, in the present study the observed up-regulation of BAX and FAS gene expression with coordinately down-regulated TLR-4, IL-8, and COX-2 gene expression support a hypothesis whereby 4-NP induces convergent pattern of dysregulated gene expression leading to rampant apoptotic processes within the gastrointestinal epithelium [33,34]. Further amplification of deleterious cellular processes is mediated by activated macrophages that are recruited to phagocytize higher numbers of apoptotic cells [35], thereby chronically triggering the pathophysiological symptoms of IBD. Based on the current findings, 4-NP may disrupt immune processes both directly, by dysregulating genes involved in mediating inflammation in U937 cells, and indirectly, by up-regulating the expression of genes that promote apoptosis in COLO320DM cells, which in turn, promotes amplified inflammatory responses.

Conclusions

In conclusion, 4-NP induces a prolonged, hyper inflammatory reaction in U937 cells and may contribute to the onset of autoimmunity, and also induces prolonged inflammatory response in the COLO cells which may lead to the unregulated apoptosis of intestinal epithelial cells. Future studies could be directed to evaluate the long term effects of 4NP on U937 and COLO cells. This would allow collection of data relevant to real-life exposure to 4-NP. Additionally, apoptotic pathways affected by 4-NP will also need to be studied in depth, as results suggests that various apoptotic genes have the potential to act as possible mediators of IBD.

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