

The Pharma Innovation

ISSN (E): 2277- 7695
 ISSN (P): 2349-8242
 NAAS Rating: 5.03
 TPI 2018; 7(8): 73-81
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www.thepharmajournal.com
 Received: 07-06-2018
 Accepted: 10-07-2018

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Phosphatidylcholine attenuates LPS-induced immunomodulatory effect through slc26a3/DRA-mediated signaling in primary chicken intestinal epithelial cells

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Abstract

The present study was aimed to study the protective effect of Phosphatidylcholine (PC) on lipopolysaccharids (LPS)- induced oxidative stress in chicken intestinal epithelial cells and to investigate the possible role of SLC26A3 (the ion exchanger) on immune augmentation. Intestinal epithelial cells from broiler chicks were cultured in Dulbecco's modified Eagle's medium/F12, exposed to PC and LPS. The levels of oxidative damage, antioxidants, cellular proliferation, pro-inflammatory and anti-inflammatory cytokines expression levels were assessed. Further, SLC26A3/DRA mediated signaling was elucidated by silencing DRA *in vitro*. On incubation with LPS, cells showed an increase in MDA levels, with increased pro-inflammatory cytokines including TNF- α , IL-6, IL-1 β , IL-2 and decreased anti-inflammatory cytokines IL-4, IL-10, IL-13, and TGF- β levels compared to control. In contrast, PC treated cells improved the antioxidant levels with increased anti-inflammatory and decreased pro-inflammatory cytokines in intestinal epithelial cells. Mechanistically, siRNA knockdown of DRA showed the decreased anti-inflammatory cytokines ($p<0.01$) and increased pro-inflammatory cytokine ($p<0.01$) in cells compared to control. These results demonstrate that PC exerts protection in primary chicken intestinal epithelial cells from oxidative damage and stimulating anti-inflammatory cytokines mainly through DRA activation suggest that PC might be provided as a feed supplement to improve anti-inflammatory ability of chicken.

Keywords: Phosphatidylcholine, lipopolysaccharids, chicken intestinal epithelial cells

1. Introduction

Phospholipids especially, phosphatidylcholine (PC) derived metabolites demonstrates anti-inflammatory properties in various stress conditions in intestinal cells. Among the lipids in the biological system, phosphatidylcholine occupies as major lipid components of the gastrointestinal mucus layer thus enhance intestinal absorption and has been shown to be effective in number of gut related injuries (Feige *et al.*, 2010; Lordan *et al.*, 2017; Dunjic *et al.*, 1993; Wu and Nakanishi, 2011) [10, 18, 8, 29]. Evidences showing the promise of PC on dietary supplementation in alleviating the mucosal injury of gut in trinitrobenzenesulfonic acid induced colitis in experimental animals through decreased expression of cytokine-mediated progression of inflammatory events and by preserving the microvascular structure in large intestine (Kovacs *et al.*, 2012) [13]. In another study, the neuroprotective properties of orally administered PC was evaluated in rodent systemic inflammation model using LPS showed an induced neuroinflammation with reduced plasma TNF- α levels in the brain tissues (Tokes *et al.*, 2011) [26] and in Caco-2 cells (Treede *et al.*, 2009) [27]. Olson *et al.*, reported that PC supplementation was effective in improving the intestinal barrier defense against *C. difficile* toxin A challenge (Olson *et al.*, 2014) [20]. In this respect, it has been reported that PC content is decreased in the mucus of patients with ulcerative colitis and that local increases in mucus PC levels are accompanied by decreased inflammatory activity by augmenting the antioxidant enzymes (Ishihara *et al.*, 2009) [11].

Release of lipopolysaccharide (LPS) from the bacterial cell wall is the major recognizing factor for the stimulation of systemic inflammatory and immune response (Pearson and Hartland, 2014; Wideman *et al.*, 2004) [22, 28]. Extensive reports available on LPS stimulated cellular release of free radicals, proinflammatory cytokines and chemokines, that regulate different metabolic response, induces fever, inflammation and cachexia. Studies on the effects

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of LPS on poultry showed significant physiological and behavioral changes, which included the elevation of cloacal temperature, depression, lethargy, diarrhea, and avoidance of feed, within a few hours of injection finally leads to death in severe cases (Xie *et al.*, 2000) [30]. However birds exert resistance against bacterial endotoxin to some extent than mammals, it is speculated that the innate immunity levels in the birds are mainly responsible for its resistance. Notably, recent studies have demonstrated the anti-inflammatory potential for PC and its metabolites in various conditions linked to endotoxin-induced injuries. SLC26A3 (the ion exchanger) or Downregulated in adenoma (DRA) is known to play important role in the apical Cl⁻/HCO₃⁻ exchange process in the intestinal cells. Decreased expression of DRA shown to have its functional impairment associated with various diarrheal disorders. Further, it was reported that decreased gene expression of DRA is related to the decreased proinflammatory cytokines IL-1 β , interferon- γ (IFN- γ) in ulcerative colitis (Singh *et al.*, 2014) [24]. Considering that SLC26A3/DRA expression is suppressed in various intestinal disorders, the data showing a relationship of this molecule in LPS or PC mediated mechanisms would be intriguing. Based on the above facts, we aimed to evaluate the potential effects of PC on LPS induced oxidative stress and to investigate the role of DRA in immune augmentation in primary chicken intestinal epithelial cells.

2. Materials and methods

2.1. Reagents

Phosphatidylcholine, Dulbecco's modified Eagle's F12 Ham medium, antibiotics, *Salmonella typhimurium* lipopolysaccharide (LPS), Propidium iodide (PI) and DAPI were purchased from Sigma Aldrich (St. Louis, MO, USA). Trizol reagent, RT-PCR kit, and HRP-conjugated secondary antibody were purchased from GeNei (Bangalore, India). Gene-specific primers were purchased from Ocum Biosolutions Inc., Netherlands. Lipofectamine 2000 from (Invitrogen, Grand Island, NY). Primary antibodies, goat polyclonal mouse anti-SLC26A3 (DRA), GAPDH and secondary antibodies Rabbit anti-goat IgG-HRP and Goat anti-rabbit IgG-FITC conjugate were purchased from Santa Cruz Biotechnology (San Diego, USA). All other chemicals used were of reagent grade.

2.2. Chicken intestinal epithelial cells culture

Chicken intestinal epithelial cells isolated from broiler chick's intestine were grown in Dulbecco's modified Eagle's medium/F12 supplemented with 10% chicken serum at 37°C in a humidified atmosphere of 5% CO₂ in air. The medium was changed every 2 days to maintain the logarithmic growth. Cell concentration was adjusted to 1 × 10⁵ cells/mL and wells of 6-well plates were seeded with 2 mL medium.

2.3. Cell Treatment

Cells were seeded in 6-well plates (1 × 10⁵ per well) and incubated for 24 h. Cells were exposed to PC (freshly diluted in the growth media at 0, 10, 100, or 200 μM) for 24 h, with 6 replicates for each treatment. After incubation, cells were washed twice with phosphate buffer and then exposed to fresh media containing 10ng/ml of LPS for an additional 24 h. Cells without any treatment were used as controls and cells only treated with 10ng/ml of LPS were used as positive controls. After treatment, the cells were collected for further analyses.

2.4. Cell proliferation analysis

Cells were seeded in 96-well plates at 2 × 10³ cells/well and treated with PC and then LPS as described above. At the completion of LPS treatment, About 20 μl of MTT (5 mg/ml) was added

to cells of each well containing 100 μl of medium. After 4 h of incubation at 37°C, the medium was removed and formazan crystals were dissolved in 150 μl DMSO, and the absorbance was measured at 570 nm using a micro plate reader. Cell proliferation was measured in the control and AGEs-treated cells from the reduction of MTT to formazan product by the mitochondrial dehydrogenase. The cells were collected by trypsinization and the viability of cells (1 × 10⁶) was determined by stained with propidium iodide. Cells were incubated for 10 minutes in the dark on ice and analysed on the flowcytometer (MoFlo XDP, Cell Sorter, Beckman Coulter)

2.5. Antioxidant assays

After completion of treatments, the media was removed and cells were harvested by trypsinization, washed twice with PBS, and then lysed by sonication in 1 mL of PBS in an ice bath and the sample was centrifuged at 1000 × g for 5 min. Malondialdehyde (MDA) level, antioxidant enzymes such as SOD, catalase, GSH were measured using Abcam assay kits as per the manufacturer's instructions.

2.6. Real-Time Quantitative RT-PCR

For the expression analysis, total RNA was extracted using RNeasy plus mini-kit. First-strand cDNA was synthesized from 1 μg of total RNA in a final volume of 20 μl using RT² First Strand kit. Real-time quantitative RT-PCR (qPCR) was done in using the primers for TNF- α , IL-6, IL-4, IL-10 and GAPDH are TNF- α , 57F-

CTTCTGAGGCATTGGAAAGC; 407R-

ACTGGGCAGTCATAGAACAG, IL-6, 545F-

ATCCGGCAGATGGTGATAAAA; 707R-

CCCTCACGGTCTTCTCCATA, IL-4, 99F-

GGAGAGCATCCGGATAGTGA; 284R-

TGACGCATGTTGAGGAAGAG, IL-10, 249F-

GCTGCGCTTCTACACAGATG; 451R-

TCCCCTTCTCATCCATCTTC, GAPDH, 343F-

GACGTGCAGCAGGAACACTA; 370R-

TCTCCATGGTGGTGAAGACA. PCR amplifications (in triplicates) were carried out in a 20 μl reaction volume using RT2 real-time SYBR Green/ROX PCR Master Mix. The reaction conditions were 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min; this was followed by 1 cycle at 95°C for 1 min, 52°C for 30 s, and 95°C for 30 s for the dissociation curve. The reaction mixture without template cDNA was used as a negative control. Threshold cycle numbers (CT) were determined with standard calculation by CT comparative method. The mRNA expression was normalized to expression values of GAPDH (endogenous control) within each sample and relative to positive and negative controls. After PCR amplification, a melting curve of each amplicon was determined to verify its accuracy.

2.7. Ex Vivo Cytokine release

For the measurement of cytokine release, cells (1 × 10⁶ cells/mL) were cultured in for 48 h at 37 °C and 5% CO₂ with or without PC and LPS. After incubation, cells were centrifuged for 10 min at 1000 rpm and supernatants collected

and stored at -80 °C until analyses. Concentrations of cytokines interleukin TNF- α , IL-6, IL-1 β , IL-2, IL-4, IL-10, IL-13 and TGF- β were measured by commercial ELISA kits according to the manufacturer's instructions (ABclonal, Inc, MA, USA). Cytokine concentrations were quantified using a microplate reader and all measurements were conducted in duplicates.

2.8. Immunofluorescence analysis

Chicken intestinal epithelial cells grown in cover slips treated with LPS and PC were fixed with 4% paraformaldehyde and permeabilized with 1% triton X-100. Monolayers were then incubated with anti-mouse IL-10 antibody (1:50) for 2 h followed by 3 washes for 5 min with 1× PBS and incubated with goat anti-mouse IgG FITC secondary antibody at 1:250 dilution for 60 min at room temperature and nuclear staining was done with DAPI (1:20000 dilution). Images were obtained on Carl Zeiss LSM 700 laser scanning confocal microscope equipped with a $\times 20$ objective. The quantitative assessment was done by Image J software.

2.9. siRNA silencing

Expression of DRA (Gene ID: 417700) was selectively silenced utilizing predesigned siRNAs (Applied Biosystems, CA, USA). Scrambled siRNA was used as a non-targeting control. Chicken intestinal epithelial cells were transiently transfected with 100 pmol of siRNA duplexes (either Dharmacon SMARTpool or RNAxs-designed siRNA) for 48 h using Lipofectamine RNAiMAX Transfection Reagent. Silencing was validated by Western blotting and RT-PCR (F-CTTTGGCAAACGTTGGGAGG; R-CGCTTGTGCGTTCCCTCA; length 194 bp; Tm, 59). The expression of mRNA was normalized with GAPDH as internal control. The intensity of the bands visualized in 2% agarose gel was quantified using image density analysis software. After the silencing was confirmed the levels of cytokines released by the cells was observed using ELISA as mentioned above.

2.10. Cell lysates and Western blotting

Chicken intestinal epithelial cells grown to confluence in 12-well cell culture plates were serum starved overnight and treated with LPS and PC as mentioned earlier. Cells were washed with ice-cold PBS three times and lysed in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton-X 100, 1 mM EDTA, 1 mM EGTA, and 1× complete protease inhibitor cocktail. The cells were sonicated and the lysate was centrifuged at 5,000 g for 5 min at 4°C, the extracted protein sample was quantified using Bradford reagent. Proteins were separated on 10% SDS-PAGE gels and the proteins were transferred to a nitrocellulose membrane, blocked in 5% fat-free dry milk in Tris-Tween buffered saline (TBS; 20 mM Tris/150 mM NaCl, pH 7.5, and 0.1% Tween-20) for 1 h and then were incubated with the primary antibodies overnight at 4°C with and with secondary antibodies after washing. The bands were visualized using enhanced chemiluminescence detection reagents. The expression measurements were normalized to GAPDH.

2.11. Statistical analysis

The results are expressed as mean \pm S.E. Statistical significance was evaluated by one-way analysis of variance using GraphPad Prism Software (GraphPad Software, San Diego, CA). The *p* value of < 0.05 was considered significant.

3. Results and Discussion

Chickens are vulnerable to various endotoxins and infections, specifically because of feed derived oxidant stress and insults (Roque *et al.*, 2015) [23]. It has been stated that poor nutritional status impairs immunity and predisposes to infections, but the immune response to an infection can also by itself impair nutritional status and alter body composition affirm that there is a bidirectional interaction between nutrition, infection and immunity (Calder, 2013) [5]. The immune enhancing function of cytokine adjuvants (Awate *et al.*, 2013) [2] has been well proven in various experimental studies, amongst, phospholipids plays a major role in stimulating the protective immune status of the cells (Mauerhofer *et al.*, 2016; Bretscher *et al.*, 2015) [19, 4]. Phospholipids and its derivatives are the major components in the cellular system rendering protection to the cells apart from maintaining the integrity of the cellular structure (Dial *et al.*, 2008; Li *et al.*, 2015) [7, 16].

Thus the present study was attempted to explore the protective role of Phosphatidylcholine (PC) on LPS endotoxin mediated detrimental effects in primary intestinal epithelial cells from chicken. On incubation with LPS, a dose dependent decrease ($P < 0.05$) in cell proliferation was observed (data not shown) in chicken intestinal epithelial cells, while PC pretreated cells maintained the growth even after LPS exposure in time dependent manner. In addition, the flow cytometry analysis of cell viability also demonstrates the decrease in cell viability in LPS treatment, while cells exposed to PC improved the cell growth (Figure 1). Further, cells exposed to LPS exhibited a 2-fold ($p < 0.01$) increase in MDA levels, while the levels of SOD, Catalase, and GSH were significantly ($p < 0.01$) decreased in cells compared to control. Surprisingly, cells pretreated with PC before LPS stimulation, exhibited an increase ($p < 0.01$) in SOD, Catalase, GSH, GPx levels with attenuated MDA levels (Supplementary figure. 1). The observed results are in correlation with a report showing that PC has a therapeutic potential against oxaliplatin-induced peripheral neuropathy due to its antioxidant property and modulation of microglial activities (Barrios and Lichtenberger, 2000) [3]. Moreover, the protective effect of PC is also extensively proved against cisplatin-induced tubular degeneration and hypertrophy of glomeruli in nephrotoxic in rats via enhancing antioxidant enzyme activity (Lee *et al.*, 2013) [15] and also by suppressing the MDA levels (Karaman *et al.*, 2003) [12].

Numerous reports are available using LPS to mimic the immunological stress in cells characterized by symptoms of bacterial infection (Liu *et al.*, 2015; Elson and Alexander, 2015) [17, 9]. Chronic inflammatory changes with the production of reactive species and compromised antioxidant status are described as a feature of acute and chronic stages of infection (Awad *et al.*, 2017) [1]. Moreover, the dysregulation of IL-1 β , IL-6, and TNF- α has been shown to induce inflammation in chickens (Chappell *et al.*, 2009; Parlesak *et al.*, 2007) [6, 21]. Hence, in the present study, the possible effect of PC in minimizing the immunological stress was elucidated by analyzing the expression of pro and anti-inflammatory cytokines. The relative gene expression of pro inflammatory cytokines exhibited significantly higher levels after LPS stimulation which is in concordance with the results obtained with PCR, Western blotting and ELISA method (Figure 2, 3). The *ex vivo* stimulation of LPS in cells pretreated with PC demonstrated attenuated ($P < 0.01$) levels of pro-inflammatory cytokines such as TNF- α , IL-6, IL-1 β , IL-2. The Anti-

inflammatory cytokines IL-4, IL-10, IL-13 and TGF- β were found to be significantly ($P<0.01$) upregulated in these cells suggest that PC exerts protection in LPS mediated effects by inhibiting the ROS induction processes are suggested to have direct link by either suppressing pro-inflammatory or compromised defense mechanism/improving the self-defense of the cells.

SLC26A3 or DRA is the key mediator gene for intestinal Cl $^-$ /HCO $^{3-}$ exchange. It was reported that DRA expression was decreased in experimental models of inflammation as well as in patients with ulcerative colitis, diarrhea. For instance, intestinal epithelial cells caused a significant reduction in DRA levels on enteropathogenic *E. coli* infection (Kumar *et al.*, 2014) [14], while probiotics supplementation improved its expression. Inflammatory mediators including IL-1 β and IFN- γ suppressed transcription of SLC26A3 in Caco-2 cells by which the IFN- γ signalled through JAK1 and JAK2 kinases to activate cytosolic STAT1 and promoted the nuclear translocation in SLC26A3 promoter (Singla *et al.*, 2010) [25]. Hence in the present study, to elucidate the signaling mechanism that PC protects the cells from LPS endotoxin exposure mediated immune suppression, the protein estimation of DRA was done using Western blot analysis (Figure 4A & B). The DRA levels was found to be significantly reduced in LPS exposed cells, while the PC

treatment improved the protein levels of DRA. With the above observation, we further determined to elucidate the effect of DRA silencing on the expression levels of pro and anti-inflammatory cytokines. Interestingly, siRNA knockdown of DRA decreases the anti-inflammatory cytokines level and increased pro-inflammatory cytokine in cells compared to control. Moreover, DRA silenced cells exposed to PC did not show any change compared to PC unexposed cells suggest that endotoxin play a major role in suppressing the anion exchanger. While the PC treatment improved the DRA expression imply that there must be a signal that directly binds with the promoter region of the SLC26A3 transcription. Thus the results of the present study on the potential beneficial role of PC might be stimulation of apical Cl $^-$ /OH $^-$ exchange activity and by increasing the surface levels of DRA in intestinal epithelial cells.

In summary, this study shows that treatment with PC significantly attenuated cellular levels of TNF- α and IL-6 in LPS-exposed chicken intestinal epithelial cells. Further, the improved antioxidant status, anti-inflammatory cytokines with improved DRA expression suggested that PC exerts anti-inflammatory activities in the LPS challenged cells though DRA mediated pathway. This implies that PC may be therapeutically or prophylactically useful for the treatment of inflammatory diseases in chickens in near future.

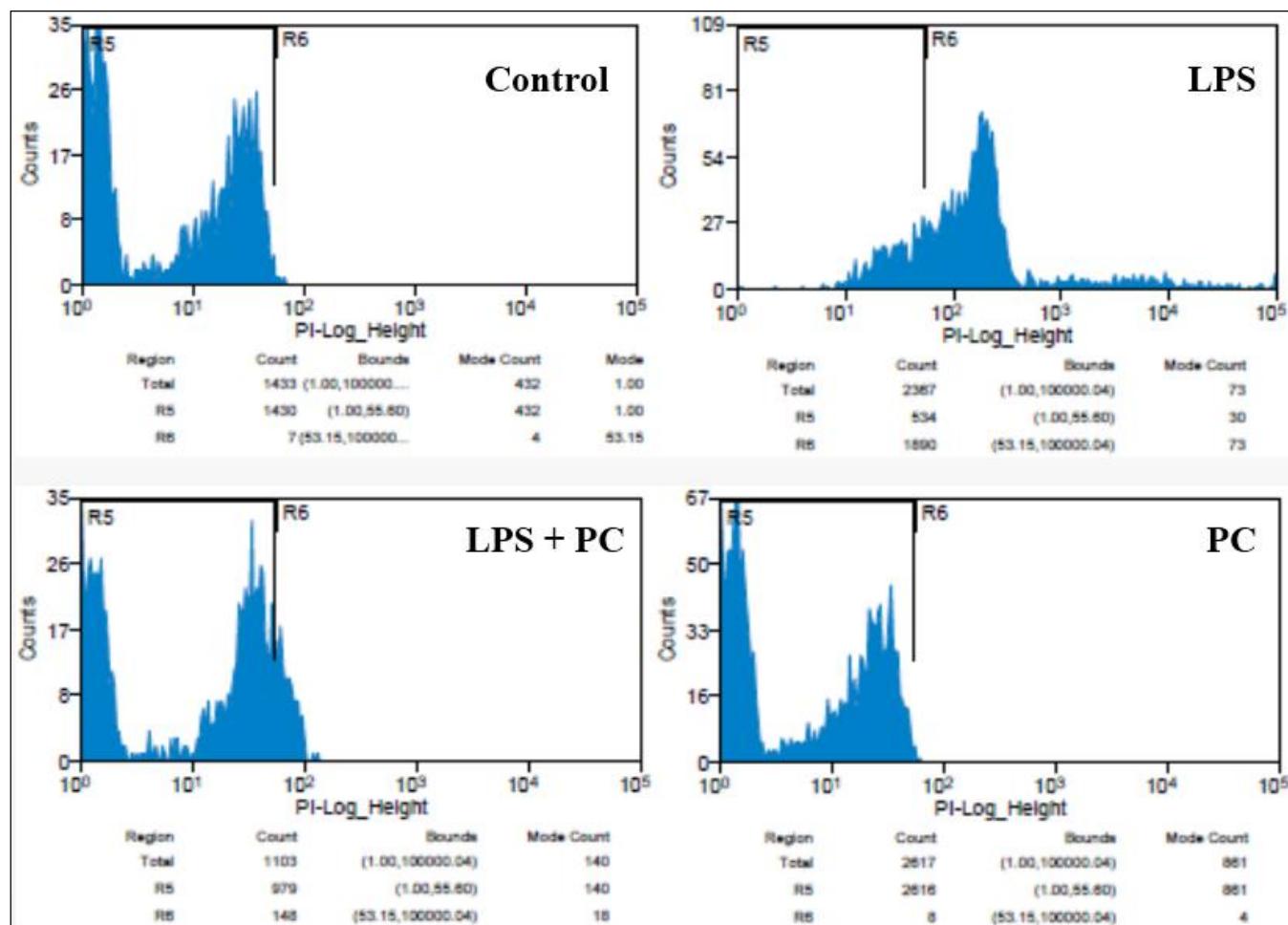


Fig 1: Chicken intestinal cell proliferation was determined by flow cytometric analysis as described in ‘‘Materials and Methods.’’ ** $p<0.01$, compared to control, \$ $p<0.05$ compared to LPS group, & $p<0.01$ compared to LPS group.

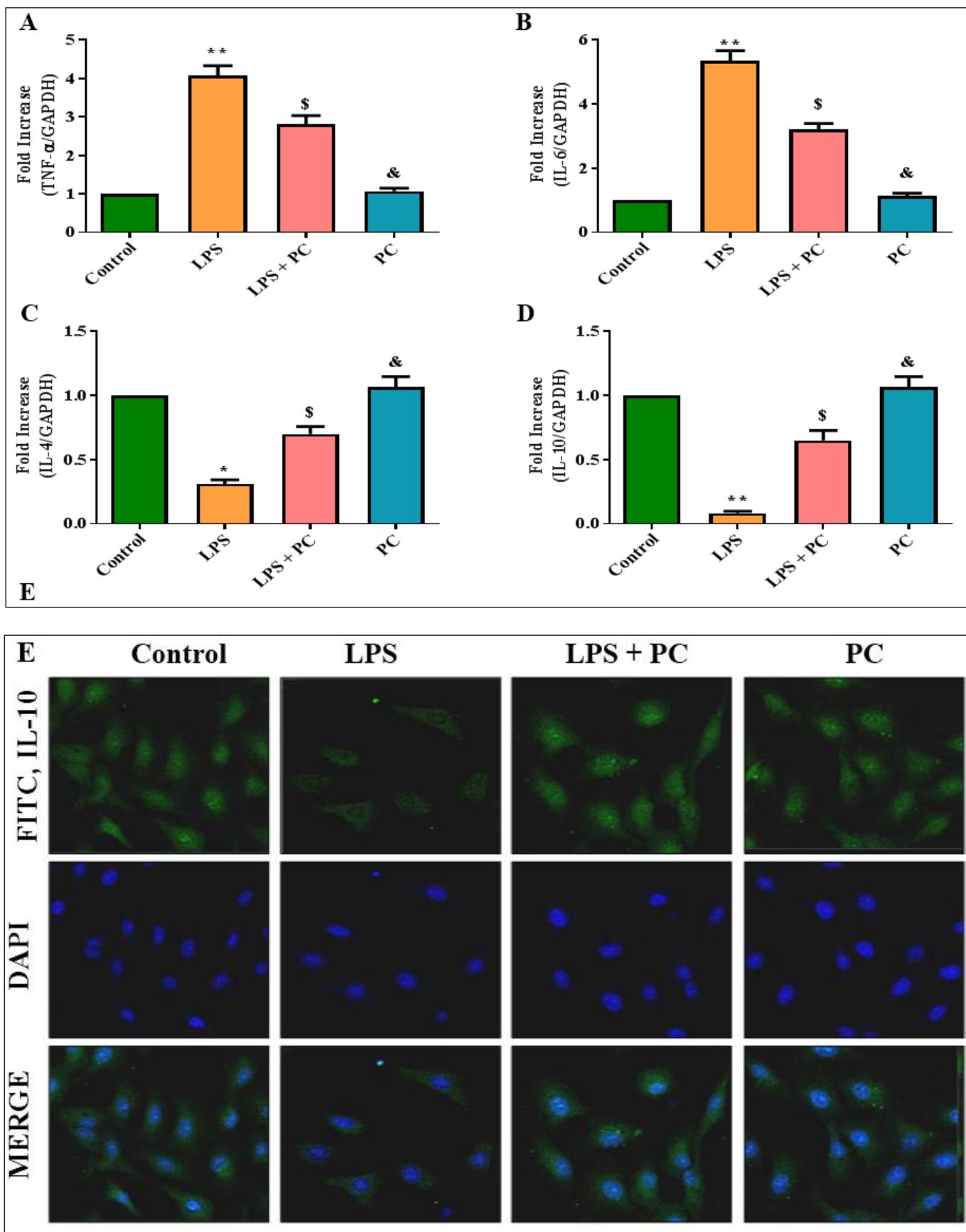


Figure 2: Expression analysis of cytokine marker genes in the LPS and PC treated chicken intestinal epithelial cells. A-D) represents the mRNA expression of TNF- α , IL-6, IL-4 and IL-10 in cells normalized to GAPDH expression. Values are expressed as means \pm S.E (n = 6). Statistical significance expressed as *p < 0.05, **p < 0.01 compared to control, \$p < 0.05 compared to LPS group, &p < 0.01 compared to LPS group. E) Representative immunofluorescence analysis of chicken intestinal epithelial cells stained with anti-mouse IL-10 primary antibody and FITC-conjugated secondary antibody (green) and counterstained with DAPI (blue). The details of the experiments were given in methodology section. Results shown are the representative of three independent experiments. Cells were observed at 20X magnification.

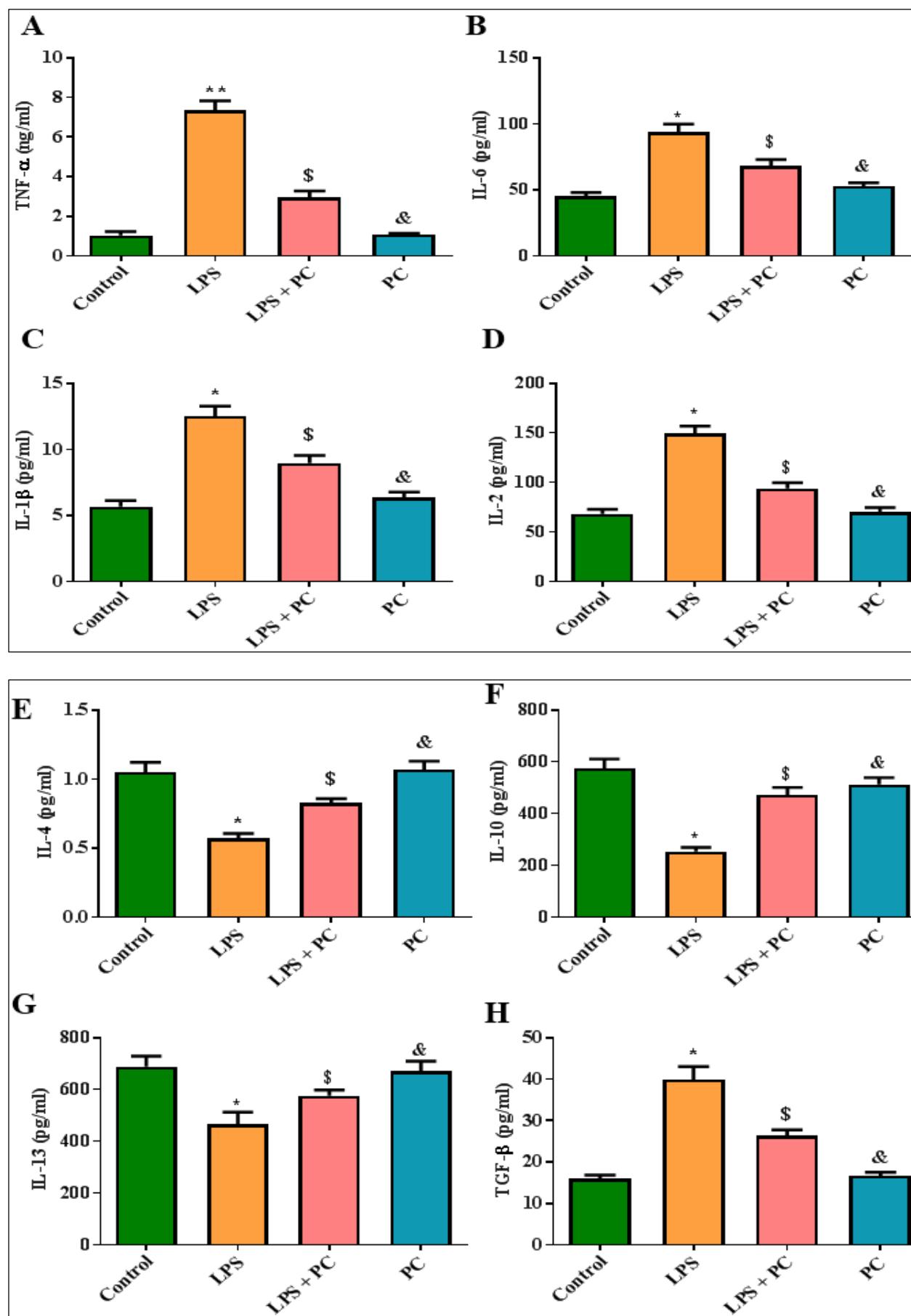


Figure 3: Expression of cytokine marker genes in the LPS and PC treated chicken intestinal epithelial cells. A-D) represents proinflammatory cytokines expression of TNF- α , IL-6, IL-1 β and IL-2 respectively in cells. E-F) represents anti-inflammatory cytokines expression of IL-4, IL-10, IL-13 and TGF- β respectively in cells. Values are expressed as means \pm S.E (n = 6). Statistical significance expressed as *p < 0.05, **p < 0.01 compared to control, \$p < 0.05, compared to LPS group, & p < 0.01 compared to LPS group.

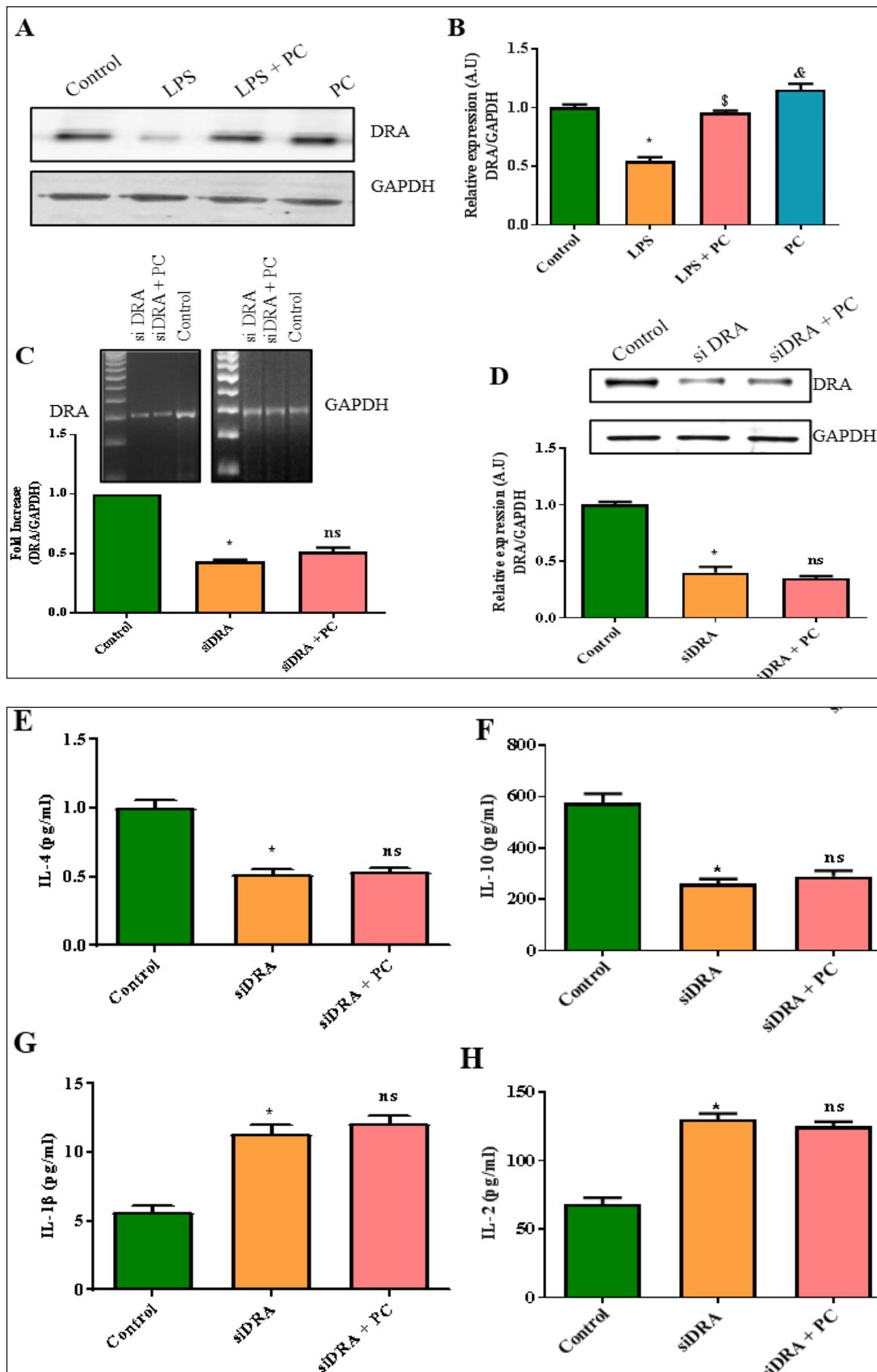
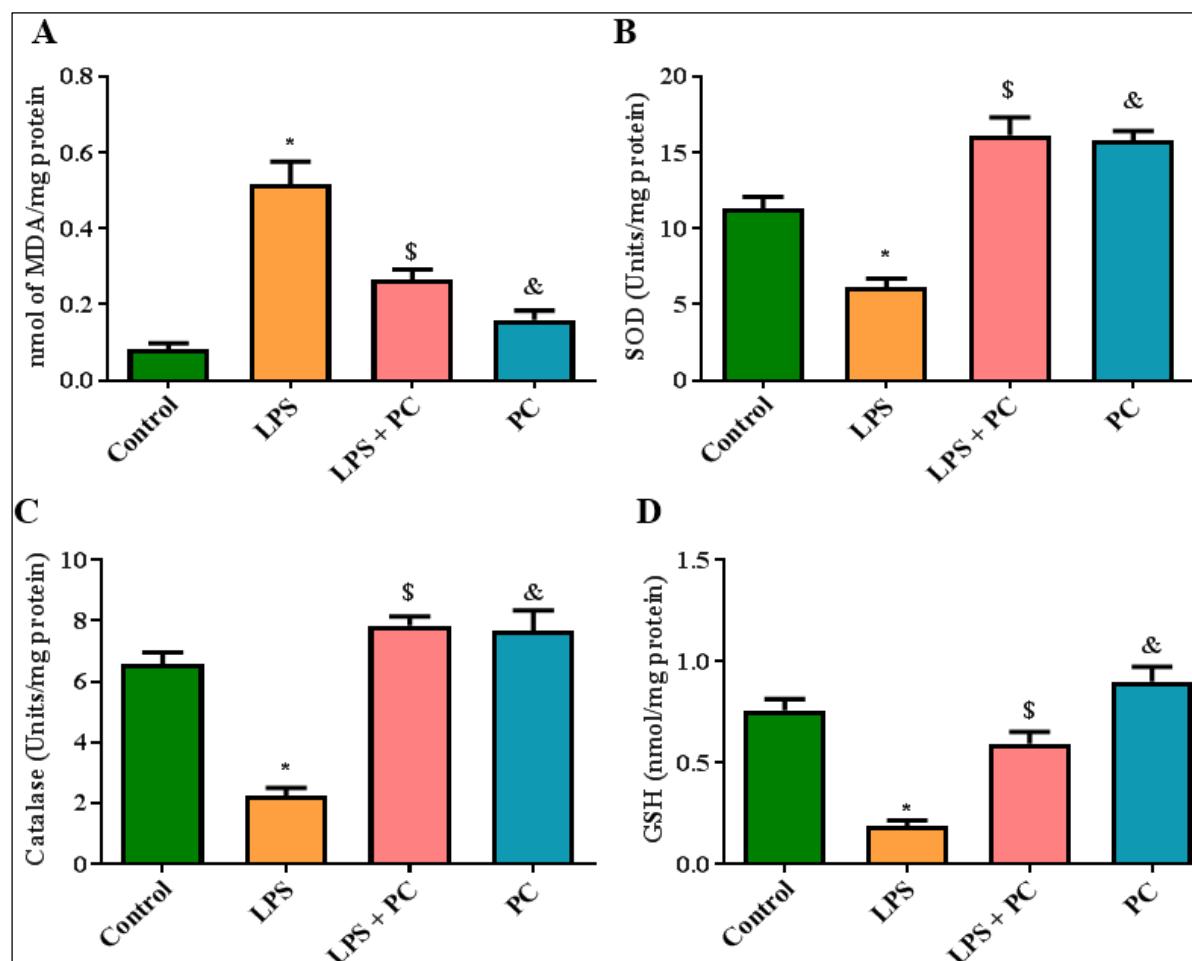
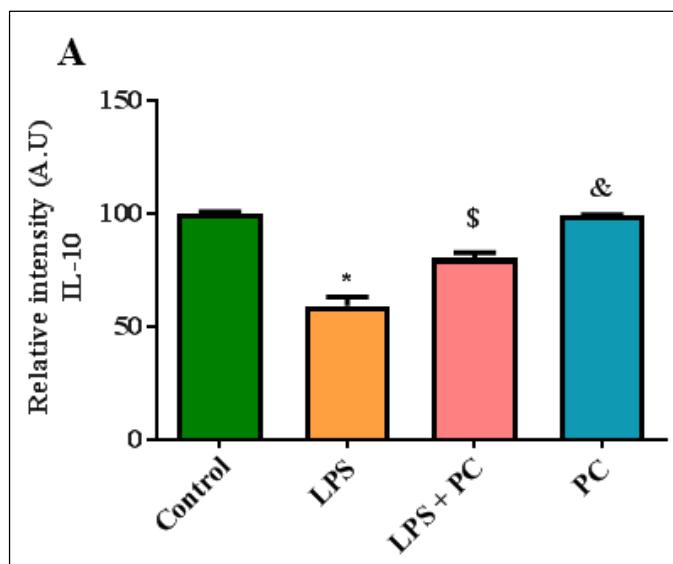


Fig 4: A). The representative Western blot analysis for the DRA expression performed in the chicken intestinal epithelial cell lysate. B). Quantitative data expressing the protein levels assessed densitometrically. C and D). siRNA silencing of DRA shown in PCR gel for the mRNA expression and Western blotting protein expression with densitometer analysis respectively. E-H). Represents the levels IL-4, IL-10, IL-1 β and IL-2 cytokine in cells respectively. Details of the experiments were given in "Materials and Methods" section. Values are expressed as means \pm S.E (n = 6). Statistical significance expressed as *p < 0.05 compared to control, ns denotes non-significant compared to siDRA group.



Supplementary Fig 1: A-D). Effect of LPS and PC on the MDA levels, Superoxide dismutase (SOD), Catalase and GSH of control and experimental cells respectively. Values are expressed as means \pm S.E (n = 6). Statistical significance expressed as * $p<0.05$, compared to control, \$ $p<0.05$ compared to LPS group, & $p<0.05$ compared to LPS group.



Supplementary Fig 2: The relative fluorescence measurements of the cells. Values are expressed as means \pm S.E (n = 6). Statistical significance expressed as * $p<0.05$ compared to control, \$ $p<0.05$ compared to LPS group, & $p<0.05$ compared to LPS group.

Acknowledgement

The authors thank Dr. N. Devipriya, SASTRA University, Thanjavur, Tamil Nadu, India for providing excellent technical help in performing experiments.

Disclosures

No conflicts of interest, no ethical issues in performing the experiment, no financial conflicts or otherwise, are declared by the author(s).

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