List of Publications

1. **Panda B**, Momin A, Devabattula G, Shrilekha C, Sharma A, Godugu C. Peptidyl arginine deiminase-4 inhibitor ameliorates pulmonary fibrosis through positive regulation of developmental endothelial locus-1. International Immunopharmacology. 2024 Oct 25;140:112861.
2. **Panda B**, Momin A, Devabattula G, Doneti R, Khandwaye A, Godugu C. A review on mechanistic aspects of litchi fruit induced acute encephalopathy. Toxicon. 2024 Jul 27:108052.
3. Sakla AP, **Panda B**, Mahale A, Sharma P, Laxmikeshav K, Khan MA, Kulkarni OP, Godugu C, Shankaraiah N. Regioselective synthesis and in vitro cytotoxicity evaluation of 3-thiooxindole derivatives: Tubulin polymerization inhibition and apoptosis inducing studies. Bioorganic & Medicinal Chemistry. 2023 May 1:117297.
4. Sakla AP, **Panda B**, Laxmikeshav K, Soni JP, Bhandari S, Godugu C, Shankaraiah N. Dithiocarbamation of spiro-aziridine oxindoles: A facile access to C3-functionalised 3-thiooxindoles as apoptosis inducing agents. Organic & Biomolecular Chemistry. 2021;19(48):10622-34.
5. Kadagathur M, Shaikh AS, **Panda B**, George J, Phanindranath R, Sigalapalli DK, Bhale NA, Godugu C, Nagesh N, Shankaraiah N, Tangellamudi ND. Synthesis of indolo/pyrroloazepinone-oxindoles as potential cytotoxic, DNA-intercalating and Topo I inhibitors. Bioorganic Chemistry. 2022 May 1;122:105706.
6. Anchi P, **Panda B**, Mahajan RB, Godugu C. Co-treatment of Nimbolide augmented the anti-arthritic effects of methotrexate while protecting against organ toxicities. Life Sciences. 2022 Apr 15;295:120372.
7. Gurram S, Anchi P, **Panda B**, Tekalkar SS, Mahajan RB, Godugu C. Amelioration of experimentally induced inflammatory arthritis by intra-articular injection of visnagin. Current Research in Pharmacology and Drug Discovery. 2022 Jan 1;3:100114. Sakla AP, **Panda B**, Laxmikeshav K, Soni JP, Bhandari S, Godugu C, Shankaraiah N. Dithiocarbamation of spiro-aziridine oxindoles: A facile access to C3-functionalised 3-thiooxindoles as apoptosis inducing agents. Organic & Biomolecular Chemistry. 2021;19(48):10622-34.
8. Devabattula G, **Panda B**, Yadav R, Godugu C. The potential pharmacological effects of natural product withaferin A in cancer: opportunities and challenges for clinical translation. Planta Medica. 2024 Apr 8.
9. Evariste Leonce AA, Devi P, Richard TS, **Panda B**, Devabattula G, Godugu C, Phelix Bruno T. Anti-melanoma and antioxidant properties of the methanol extract from the leaves of Phragmenthera capitata (Spreng.) Balle and Globimetula braunii (Engl.) Van Tiegh. Journal of Complementary and Integrative Medicine. 2024 Mar 20;21(1):88-100.
10. Leonce AA, Chilvery S, Richard TS, **Panda B**, Godugu C, Bruno TP. Phragmenthera capitata (spreng.) Balle methanol leaves extract Is used safely to reduce tumor growth in c57bl/6j mice bearing melanoma b16-f10.

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Peptidyl arginine deiminase-4 inhibitor ameliorates pulmonary fibrosis through positive regulation of developmental endothelial locus-1

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ABSTRACT

Recurring lung injury, chronic inflammation, aberrant tissue repair and impaired tissue remodelling contribute to the pathogenesis of pulmonary fibrosis (PF). Neutrophil extracellular traps (NETs) are released by activated neutrophils to trap, immobilise and kill invading pathogen and is facilitated by peptidyl arginine deiminase-4 (PAD-4). Dysregulated NETs release and abnormal PAD-4 activation plays a crucial role in activating pro-fibrotic events in PF. Developmental endothelial locus-1 (Del-1), expressed by the endothelial cells of lungs and brain acts as an endogenous inhibitor of inflammation and fibrosis. We have hypothesised that PAD-4 inhibitor exerts anti-inflammatory and anti-fibrotic effects in mice model of PF. We have also hypothesised by PAD-4 regulated the transcription of Del-1 through co-repression and its inhibition potentiates anti-fibrotic effects of Del-1. In our study, the PAD-4 inhibitor chloro-amidine (CLA) demonstrated anti-NETotic and anti-inflammatory effects *in vitro* in differentiated HL-60 cells. In a bleomycin-induced PF mice model, CLA administration in two doses (3 mg/kg, I.P and 10 mg/kg, I.P) improved lung function, normalized bronchoalveolar lavage fluid parameters, and attenuated fibrotic events, including markers of extracellular matrix and epithelial-mesenchymal transition. Histological analyses confirmed the restoration of lung architecture and collagen deposition with CLA treatment. ELISA, IHC, IF, RT-PCR, and immunoblot analysis supported the anti-NETotic effects of CLA. Furthermore, BLM-induced PF reduced Del-1 and p53 expression, which was normalized by CLA treatment. These findings suggest that inhibition of PAD-4 results in amelioration of PF in animal model and may involve modulation of Del-1 and p53 pathways, warranting further investigation.

1. Introduction

Pulmonary fibrosis (PF) results from an abnormal response to tissue injury, leading to excessive connective tissue accumulation and alterations in the extracellular matrix (ECM). The repair process after lung injury involves immune cell infiltration, such as neutrophils, macrophages, leukocytes, T cells, and B cells, which release pro-inflammatory and pro-fibrotic factors. These signals stimulate fibroblasts to become myofibroblasts, initiating tissue remodelling by synthesizing collagen and other ECM components. Under normal conditions, this repair process is tightly regulated, with myofibroblasts undergoing apoptosis to conclude wound healing. However, an overabundance of initiating signals, including transforming growth factor-β1 (TGF-β1), fibroblast growth factor (FGF), and aberrant signalling pathways, can disrupt the regulation, leading to deposition of ECM proteins such as collagen, increase in transcription factors that causes epithelial mesenchymal transition (EMT) such as SNAIL [1,2] and SLUG [1] that results in decrease in epithelial cells markers and increase in mesenchymal cells markers such as α-smooth muscle actin (α-SMA) [3,4] and N-cadherin (N-cad) [3,4], and abnormal tissue growth [5–8]. Currently, FDA has approved only two drugs against PF i.e. pirfenidone (PFD) and nintedanib (NTD). The irregularity in the fibrotic matrix and complex network of ECM protein has limited the availability of anti-fibrotic medications. While, the mechanisms of the two drugs are distinct and majorly unknown, PFD is thought to inhibit fibroblast proliferation and TGF-β1 signalling and EMT [9]. On the other hand, Nintedanib is a small molecule that inhibits the tyrosine kinases of several growth factor receptors such as fibroblast growth factor (FGF), vascular endothelial growth factor receptor (VEGFR), platelet derived growth factor receptor (PDGFR) thereby it inhibits TGF-β1 signalling and also inhibits

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expression of proteins of ECM such as fibronectin (FN) and collagen 1A1 (COL1A1) [10]. However, both the drugs are associated with similar side effects including gastrointestinal complications such as anorexia, nausea, abdominal pain, dyspepsia, and gastrointestinal reflux diseases (GERD). Moreover, PFD is associated with photosensitivity reaction and hepatic dysfunction and NTD is associated with thromboembolism, hypothyroidism. To increase the spectrum of anti-fibrotic drugs, there is a great need of novel anti-fibrotic drugs that can inhibit cells of innate immune system such as neutrophils and macrophages, adaptive immune systems such as lymphocytes and fibroblast proliferation, thus targeting the inflammatory stage and wound healing or repair stage of fibrosis may provide accumulated anti-fibrotic effects.

Citrullination of histone peptidyl arginine residues by peptidyl arginine deiminase (PAD)-4, one of the PAD isozyme that plays a pivotal role in antimicrobial defence mechanisms of neutrophils triggers chromatin decondensation, resulting in the release of neutrophil extracellular traps (NETs). NETs are intricate mesh-like structures formed by neutrophils, composed of decondensed chromatin, alongside bactericidal enzymes and components such as reactive oxygen species (ROS), neutrophil elastase (NE), matrix metalloproteinase (MMP), myeloperoxidase (MPO), and cathepsin-G. Collectively, these components collaborate to eliminate invading pathogens, an attempt to mend the damaged tissue [11,12]. Dysregulated or excessive release of NETs, coupled with ineffective termination, can trigger a cascading inflammatory response thus exacerbating the wound healing process, leading to PF and organ malfunction [13,14]. Interestingly, NETosis has also been implicated in induction of epithelial-mesenchymal transition (EMT) in COVID-19 related fibrosis and chronic obstructive pulmonary disease (COPD) wherein they showed a higher concentration of NETs and a co-expression of epithelial and mesenchymal markers in the BALF [15,16]. Additionally several other studies have reportedly studied the effect of exaggerated NETs release on several pulmonary diseases [15,17–20].

The investigation into the protective role of various anti-inflammatory factors and mediators has revealed developmental endothelial locus-1 (Del-1) as one such mediator, primarily expressed by endothelial cells in lungs and neural cells in brain. This protein, homologous to milk fat globule epidermal growth factor-8 (MFG-E8) can restrict leukocyte recruitment by inhibiting the interaction of leukocyte function antigen (LFA) to its receptors, such as intercellular adhesion molecule-1 (ICAM-1) on immune cells' surfaces. Del-1 is also involved in regulating extracellular matrix (ECM) formation and composition. Due to its anti-inflammatory properties, Del-1 exerts protection against disorders like experimental encephalitis, multiple sclerosis, pulmonary inflammation, and autoimmune disorders. Del-1 inhibits TGF-β1 activation and also possess anti-fibrotic properties [22]. Furthermore, Del-1 enhances efferocytosis, phagocytosis of apoptotic cells by macrophages, by bridging the gap between apoptotic cells and macrophages. Additionally, Del-1 inhibits the nuclear factor kappa B (Nf-κB) pathway, indirectly suppressing multiple pro-inflammatory pathways [23,24].

The interaction between PAD-4 enzyme, NETosis, and Del-1 remains unestablished despite Del-1′s role as an anti-inflammatory mediator and inhibitor of leukocyte recruitment. PAD-4 has been reported to interact with and repress the p53 gene, a tumor suppressor and regulator of various signalling pathways, which in turn regulates multiple p53 target genes, including Del-1 [25]. The presence of multiple p53 response elements in the promoter region of the Del-1 gene suggests a direct upregulation of Del-1 transcription by p53, with inhibition of p53 leading to reduced Del-1 expression in murine primary endothelial cells, indicating a potential interaction between PAD-4 and Del-1 through p53 gene [26,27]. Therefore, the principal objective of this study is to investigate anti-fibrotic role of standard PAD-4 inhibitor i.e. Chloroamidine (CLA) and its effects on Del-1 expression in bleomycin (BLM) induced PF. The anti-fibrotic effects of CLA will be compared with the standard anti-fibrotic drug PFD.

2. Materials and methods

2.1. Chemicals and reagents

BLM was purchased from Celon labs (India). CLA, 5, 5-dithio-bis (2–nitrobenzoicacid) (DTNB), bovine serum albumin (BSA), phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich (USA). Most of the other chemicals used for biochemical assays including bradford reagent, sodium dodecyl sulphate (SDS), 2-thiobarbituric acid (TBA), tetra-methoxy propane (TMP), radioimmunoprecipitation assay (RIPA) lysis buffer, 4′,6-diamidino-2-phenylindole (DAPI), 2′,7′-dichlorofluorescin-diacetate (DSCFDA) were purchased from Sigma-Aldrich (USA). Chemicals necessary for staining techniques like direct red, picric acid, toluidine blue, and haematoxylin and eosin solutions were also obtained from Sigma-Aldrich (USA). Enzyme-linked immunosorbent assay (ELISA) kits were purchased from Invitrogen (USA). Antibodies including anti-NE, anti-citrulline-H3 (CitH3) were purchased from Novus Biologicals (USA). Other primary antibodies and secondary antibodies generated against rabbit, mouse and goat were purchased from Santa Cruz Biotechnology (USA) Primers for RT-PCR experiments were procured from Integrated DNA Technologies (USA) and GCC Biotech (India).

2.2. Cell culture, differentiation and treatment

Human promyeloblast (HL-60) cells were cultured and maintained in Roswell Park Memorial Institute (RPMI) media with 10 % fetal bovine serum and 1 % penicillin–streptomycin solution in 5 % CO2 incubator with temperature maintained at 37 °C. The HL-60 cells need to be differentiated in order to gain the phenotype of neutrophils and to produce NETs. HL-60 cells were plated and differentiated with 1.25 % of dimethylsulfoxide (DMSO), 70 mM dimethylformamide (DMF) and 10 μM retinoic acid (RA) and maintained as such for 5 days. These 5 days incubation is required to achieve differentiated (dHL-60) cells. These dHL-60 cells were stimulated with PMA (500 nM)for 4 h to induce NETs. The status of NETs was studied through sytox green (200 nM) staining for 15 min. The cells were then harvested and evaluated for NETs positive cell count through flow cytometry analysis (BD Biosciences, USA). Cells were treated with CLA at 100 μM in the presence of PMA (500 nM) and incubated for 4 h following which the cells were harvested and taken for evaluation of anti-NETotic and anti-inflammatory effects.

2.3. Measurement of NETs by fluorescence intensity

Following 4 h incubation with PMA and CLA, sytox green was added to the cells at 200 nM concentration and relative fluorescence intensity readings were taken in a multi-mode spectrophotometer with excitation at 485 and emission at 523 nm (Perkin-Elmer, USA). Further, the NETs were isolated and separated on agarose gel electrophoresis unit and the gel was visualised in a chemi doc system (Azure Biosystems, USA). NETs visualisation was also carried out through a DAPI DNA dye binding study. Following incubation with PMA and CLA, DAPI at 10 μM concentration was added to the cells and DAPI images were taken using fluorescent microscope (Nikon, Japan).

2.4. Immunofluorescence

Moreover, after PMA and CLA incubation, the cells were fixed with 4 % paraformaldehyde and permeabilised using 0.1 % triton-X100. This was followed by blocking with 3 % BSA for one hour and incubation with primary antibody against PAD-4 (1:500) TBS overnight. Next day, cells were washed with TBST and incubated with anti-mouse FITC for 2 h. Following incubation, cells were again washed with TBST, mounted with DAPI fluorescent mounting media and visualised under fluorescent microscope (Nikon, Japan).

2.5. Measurement of nitrite and MPO levels

Nitrite and MPO levels were measured as per the standardised laboratory protocols mentioned in our previous reports. Nitrite was measured in dHL-60 cells media following treatment with PMA and CLA. Griess reagent in equal volume was added to the cell culture media. For MPO assay, the cells were lysed in chilled 50 mM potassium phosphate buffer containing 0.5 % hexadecyl trimethyl ammonium bromide (HTAB) and centrifuged at 9168*g* for 10 min at 4 °C. The obtained pellet was resuspended in the same buffer containing O-dianisidine dihydrochloride and centrifuged at 9168*g* for 10 min at 4 °C. The absorbance was recorded at 540 nm and 460 nm for nitrite and MPO levels respectively using multi-mode spectrophotometer (Perkin Elmer, USA).

2.6. Measurement of cellular ROS levels

Cellular ROS production was measured by DCFDA assay in dHL-60 cells. Following incubation with PMA and CLA for 4 h, DCFDA (10 μM) was added in each well and further incubated for 30 min at 37 °C in the dark following standard protocol. The DCFDA fluorescence images were taken using a fluorescent microscope (Nikon, Japan).

2.7. Experimental animals and study design

Male Swiss albino mice (6–8 weeks) weighing 25–30 g were purchased from Teena biolabs pvt. Ltd., Telangana, India and were allowed free access to food and water ad libitium. Throughout the study period, animals were maintained with 12/12 h light and dark cycle. Protocol for in vivo experimentation was approved by institutional animal ethics committee (IAEC) of NIPER-Hyderabad (NIP/08/2021/PC/427). After 1 week of acclimatization, animals were randomised into five groups i.e. A) Normal control; B) BLM control (1.5 IU/Kg); C) BLM+CLA low dose (3 mg/kg); D) BLM+CLA high dose (10 mg/kg) and E) BLM+PFD (200 mg/kg)with n = 8. BLM was administered oropharyngeally as single dose on day 0 as shown in Fig. 1. CLA was administered through intraperitoneal (IP) route once daily for 28 days. Standard FDA approved anti-fibrotic drug i.e. PF was administered orally once daily for 28 days. On 28th day, all the animals were anaesthetized followed by blood and broncho-alveolar lavage fluid (BALF) collection. The BALF cells were counted, centrifuged and supernatant was stored at −80 °C. Further the lungs were collected wherein part of lungs were stored in −80 °C for molecular studies and other part was stored in 10 % non-buffered formalin (NBF) for histological evaluations.

2.8. Assessment of lung functional parameters

On the 26th day, animals were acclimatized for whole body plethysmography (WBP) instrument and the readings were taken on the 27th day. Parameters such as breathe frequency (Bf), tidal volume (Tvb), minute volume (Mvb), airway resistance (Penh), inspiration time (Ti), expiration time (Te) were recorded using WBP.

2.9. Estimation of BAL fluid

BAL fluid was collected after anaesthetising the animals by making a small incision over the neck, exposing the trachea and inserting a catheter into the trachea followed by passing 1.5 ml of ice-cold PBS through it into the lungs to aspirate the fluid and the process was repeated thrice. The collected BAL fluid was subjected to total and differential cell count. Further, it was centrifuged at 3000 rpm for 5 min at 4 °C and the supernatant was subjected to albumin, LDH and total protein estimation by using commercially available kits.

2.10. Estimation of primary neutrophils

The collected blood was centrifuged at 10,000 rpm for 10 min at 4 °C followed by collection of the whitish buffy coat layer and resuspending it in a mixture of 1 ml of 1X PBS and 9 ml of percoll solution. The percoll solution was prepared by using 10 ml of 10X PBS and 90 ml of percoll solution. Following resuspension and addition into an ultra-centrifuge tubes, then ultra-centrifuged at 60,650*g* for 20 min at 4 °C. The neutrophils appearing at the second opaque layer were washed, counted and subjected to NETotic effects by using sytox green staining.

2.11. Estimation of collagen in lung tissues

Hydroxyproline (HP) assay was performed in lung tissues to estimate the levels of collagen as per our established protocol [30]. Briefly, lung tissues were homogenised and hydrolysed in 6 M HCl and the total mixture was heated for 90 min at 120 °C followed by neutralising the samples with 2.5 N NaOH. Chloramine T reagent was added to the above mixture and incubated at room temperature for 20 min followed by addition of Ehrlich reagent and further incubated for 15 min at 60 °C. The absorbance was taken immediately at 550 nm. Standard curve was prepared by using serial dilutions of HP. Hydroxyproline concentration was represented as μg/mg of protein.

2.12. Estimation of nitrite, malondialdehyde (MDA) and glutathione (GSH) content in lung tissues

The levels of nitrite, MDA and GSH were quantified as per previously described protocols [29,31]. Briefly, the lung tissues were homogenised in PBS and the homogenates were used for MDA estimation. Remaining homogenates were centrifuged at 8000 rpm for 10 min at 4 °C. The

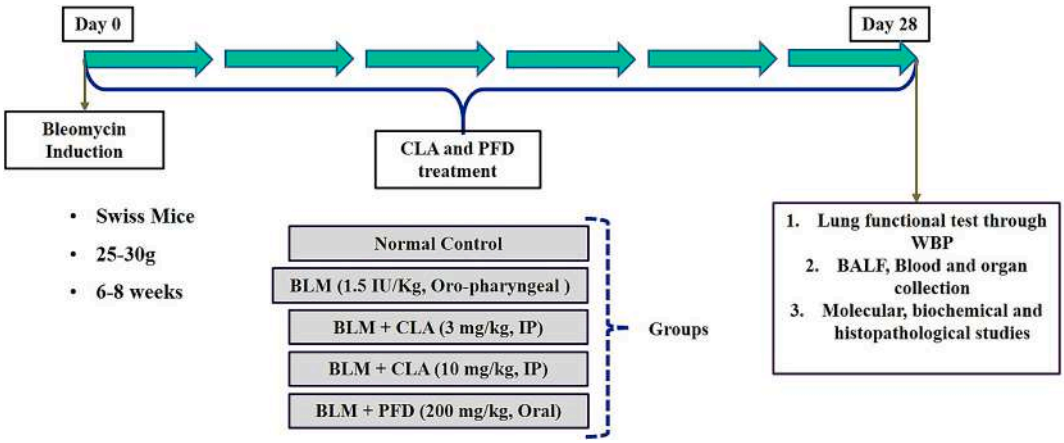

Fig. 1. Study design and intervention of CLA in BLM induced PF animal model. BLM: Bleomycin, CLA:Chloro-amidine, PFD-Pirfenidone.

obtained supernatant was used for estimating GSH and nitrite levels. Protein estimation was done by using Bradford's reagent. Nitrite and GSH levels were expressed in μM/mg of protein and MDA levels in nM/mg of protein.

2.13. Estimation of MPO levels in lung tissues

MPO in the lung tissues was estimated as per our standardised protocol [28]. Briefly, the lung tissues were homogenised in chilled potassium phosphate buffer containing 0.5 % HTAB. The homogenates were centrifuged at 9168*g* for 10 min at 4 °C. Pellet obtained was again resuspended in the same buffer containing O-dianisidine dihydrochloride and centrifuged at 9168*g* for 10 min at 4 °C. The absorbance of the supernatant was recorded at 460 nm. Protein estimation was done by using Bradford's reagent. MPO levels were expressed in IU/mg of protein.

2.14. Estimation of TNF-α, IL-6, IL- 1β and TGF-β1 levels by ELISA

ELISA for estimation of pro-inflammatory and pro-fibrotic cytokines such as TNF-α, IL-6, IL- 1β and TGF-β were performed as per manufacturer's instructions (Invitrogen, USA, catalogue no. 88-7346-88, 88-7066-22, 88-7261-22 and 88-50390-22). The results were expressed as pg of cytokine/mg of protein.

2.15. Histopathological analysis

The lung tissues fixed in 10 % NBF, processed with a series of gradient alcohols and xylene. The tissues were embedded in paraffin and 5 μm thick tissue sections were taken using microtome (Leica, Germany). Haematoxylin & Eosin (H&E) staining was performed as per previously described method [29].Additionally, Masson's Trichrome (MT) and Picrosirius red (PSR)stainings were performed to evaluate the extent of collagen deposition. The images were quantified by using ImageJ software.

2.16. Immunohistochemical analysis

The lung tissue sections were deparaffinised at 65 °C and subjected to rehydration using xylene and gradient alcohols (100, 90, 80 and 70 %). Antigen retrieval was done by using 1X citrate buffer at 95 °C for 20 min. Endogenous peroxidases were inhibited by using 3 % H_2O_2 followed by blocking with 3 % BSA for 1 h. The sections were incubated with primary antibodies against α-SMA (1:500), fibronectin (1:500) and COL1A1 (1:500) (Santa Cruz Biotechnology, USA) at 4 °C overnight. Next day, the sections were subjected to incubation with regents of poly Excel HRP/DAB Detection System (PathnSitu Biotechnologies Pvt. Ltd., India) to develop a colour reaction. Later, the sections were counterstained with haematoxylin and mounted with DPX mounting media. The expression of the protein was confirmed by DAB colour development. The Images were captured using light microscope (Olympus, Japan) and quantified by using ImageJ software.

2.17. Immunofluorescence analysis

The lung tissue sections were deparaffinised and subjected to rehydration. Non-specific binding was blocked with 3 % BSA for 1 h. The sections were incubated with Primary antibody against NE (Novus Biologicals, CA) (1: 200), Del-1 (1:500) and p53 (1: 500) (Santa Cruz Biotechnology, USA) at 4 °C overnight. Next day, the sections were washed and incubated with anti-mouse FITC (1: 2000) and anti-rabbit rhodamine (1:400) secondary antibody for 2 h. The sections were then washed with TBST, mounted with DAPI fluorescent mounting media and visualised under Leica TCS SP8 scanning spectral confocal microscope (Leica, Germany).

2.18. RT-PCR analysis

RNA was isolated from lung tissues using RNA isolation kit (Qiagen, Germany) as per the manufacturer's instructions. This was followed by cDNA synthesis with PrimeScript 1st strand cDNA synthesis kit (Takara, Japan) and real-time-PCR was performed on a Quantstudio 7 Pro (Thermo Fisher, USA) using TB Green Premix Ex Taq II qPCR kit (Takara, Japan) by using forward and reverse primers for PAD-4 (forward primer: TCTGCTCCTAAGGGCTACACA, reverse primer: GTCCAGAGGCCATTTGGAGG), Del-1 (forward primer: CCTGTGAGATAAG CGAAG, reverse primer: GAGCTCGGTGAGTAGATG) and p53 (forward primer: ACAGTCGGATATGAGCATCG, reverse primer: CCATGGAATTAGGTGACCCT) (GCC biotech, India), MMP-2 (forward primer: TTCCCCCGCAAGCCCAAGTG, reverse primer: GAGAAAAGCGCAGCGGAGTGACG), MMP-9 (forward primer: AAGGACGGCCTTCTGGCACACGCCTTT, reverse primer: GTGGTATAGTGGGACACATAGTGG), ICAM-1 (forward primer: CAATTTCTCTATGCCGTACAG, reverse primer: AGCTGGAAGATCGAAAGTCCG) (IDT, USA). GAPDH (forward primer: CATCACTGCCACCCAGAAGACTG, reverse primer: ATGCCAGTGAGCTTCCCGTTCAG) was used as an internal control to determine fold changes followed by which the graphs were plotted.

2.19. Western blot analysis

The lung tissues were homogenised in RIPA lysis buffer containing protease and phosphatase inhibitors. The homogenates were probe sonicated and centrifuged at 8000 rpm for 10 min at 4 °C and protein concentration was determined. Same amounts of protein was separated on 10 % SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose (NC) membrane. The membranes were blocked with 3 % BSA for non-specific binding followed by incubation with primary antibodies against CitH3 and NE (Novus biologicals, CA), MPO Cell Signalling Technology, USA) and IL-1β, IL-6, TGF-β1, α-SMA, SNAIL, SLUG, N-cad, PAD4, Del-1, MMP-2, MMP-9 and ICAM-1 (Santa Cruz Biotechnology, USA). The next day, membranes were washed and incubated with respective secondary antibodies and proteins were visualised with an enhanced chemiluminescent (ECL) reagent using chemidoc instrument (Azure Biosystems, USA). The obtained protein bands were quantified and analysed using ImageJ software and the fold change was calculated and plotted by taking β-actin as an internal standard.

2.20. Statistical analysis

All the results were reported as mean ± SEM of at least three independent experiments. Statistical analysis was performed by one way ANOVA and Dunnett's multiple comparison test. All the statistical analyses were performed using GraphPad Prism® Version 6 software. Probability < 0.05 levels was considered as statistically significant.

3. Results

3.1. CLA decreased NETosis and inflammatory parameters in neutrophil like HL-60 cells

We first evaluated the anti-NETotic effects of CLA in dHL-60 cells. It was observed that among the cell differentiating agents such as DMSO (1.25 %), DMF (70 mM) and RA (10 μM), DMF (70 mM) was the suitable differentiating agent that resulted in maximum NETs release upon PMA induction. It was evident from the flow cytometry experiment, the DMF differentiated group showed higher percentage of cells that were positive for sytox green dye which was illustrated by a greater rightward shift in the curve (Fig. 2A). Moreover, the relative sytox fluorescence units of sytox green affirmed this finding of DMF differentiated cells showing highest relative fluorescence compared to DMSO or RA differentiated cells. Hence, for further experiments the HL-60 cells were

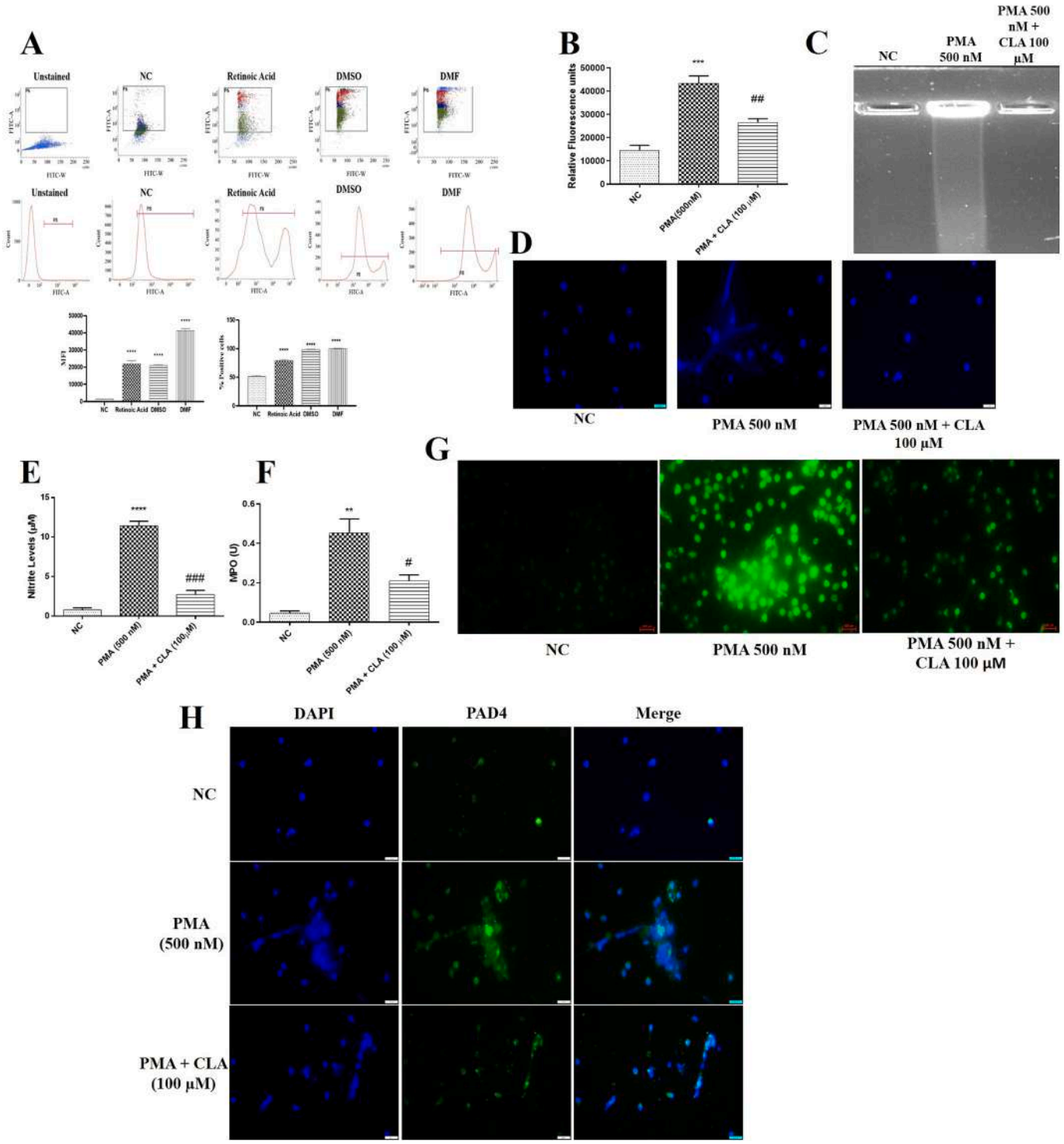

Fig. 2. Effects of CLA on NETosis and inflammation *in-vitro*. A) Flow cytometric analysis of the effect of differentiating agents on HL-60 cells, B) Quantification of relative fluorescence units of dHL-60 cells, C) Representative image depicting DNA bands of isolated NETs on agarose gel electrophoresis. D) Representative fluorescence images of DAPI staining. E and F) Quantification of nitrite and MPO levels in dHL-60 cells treated with PMA and PMA+CLA. G) Representative fluorescence images showing DCFDA stained cells. H) Representative images depicting expression of PAD-4 in the dHL-60 cells. All results are expressed as mean ± SEM of n = 3. **p < 0.01, ***p < 0.001 and ****p < 0.0001 vs NC, #p < 0.05, ##p < 0.01 and ###p < 0.001 vs PMA. (NC– normal control, PMA- phorbol 12-myristate 13-acetate control, PMA+CLA- phorbol 12-myristate 13 + chloro-amidine).

differentiated with 70 mM DMF. Then PMA (500 nM) stimulated dHL-60 cells treated with CLA (100 µM) showed decreased relative fluorescence of sytox green stain compared to PMA (500 nM) induced dHL-60 control cells depicting CLA treatment reduced total population of cells undergoing NETosis (Fig. 2B). Further, agarose gel electrophoresis of isolated NETs revealed a broadly intensified DNA bands upon PMA induction which was reduced upon CLA treatment, thus indicating the anti-NETotic effects of CLA (Fig. 2C). Further, DAPI staining performed for nuclear changes and NETs visualisation showed thread like structures resembling NETs in the PMA induced cells which were markedly decreased upon CLA treatment (Fig. 2D). Moreover, nitrite assay reflected a significant increase in the nitric oxide levels in PMA induced

cells which were significantly decreased in CLA treated cells (Fig. 2E). Similar pattern was obtained from MPO assay, where PMA led to significant increase in MPO levels which were decreased after CLA treatment (Fig. 2F). Treatment with CLA also decreased the ROS levels in cells which is evident from the DCFDA imaging data (Fig. 2G). In addition, we observed significantly higher expression of PAD-4 in PMA induced cells, further, CLA treatment markedly controlled the expression of PAD-4, thus substantiating the findings that CLA effectively ameliorated NETs release and associated events (Fig. 2H).

3.2. CLA alleviated BLM induced abnormal lung functional parameters, body weight, lung gross appearance and lung weights in mice model of PF

Evaluation of lung functional parameters such as breathe frequency, tidal volume, minute volume, airway resistance, inspiration time and expiration time through WBP showed decrease in breathe frequency, tidal volume and minute volume and increase in airway resistance, inspiration time and expiration time in BLM treated animals due to fibrotic changes in the lungs which were significantly normalised dose dependently by CLA treatment, these effects were similar to the effects shown by standard anti-fibrotic drug PFD (Fig. 3A). Further, BLM alone instillation showed a significant decrease in body weight, which was restored dose dependently upon CLA treatment (Fig. 3B). Photographs of the lungs revealed development of fibrotic lesions, swelling and discolouration resembling inflammation in the BLM control group which led to significant increase in lung weight and lung/body weight index. However, treatment with CLA restored the lung morphological changes and reduced the lung weight and thus a significant decrease in the lung/

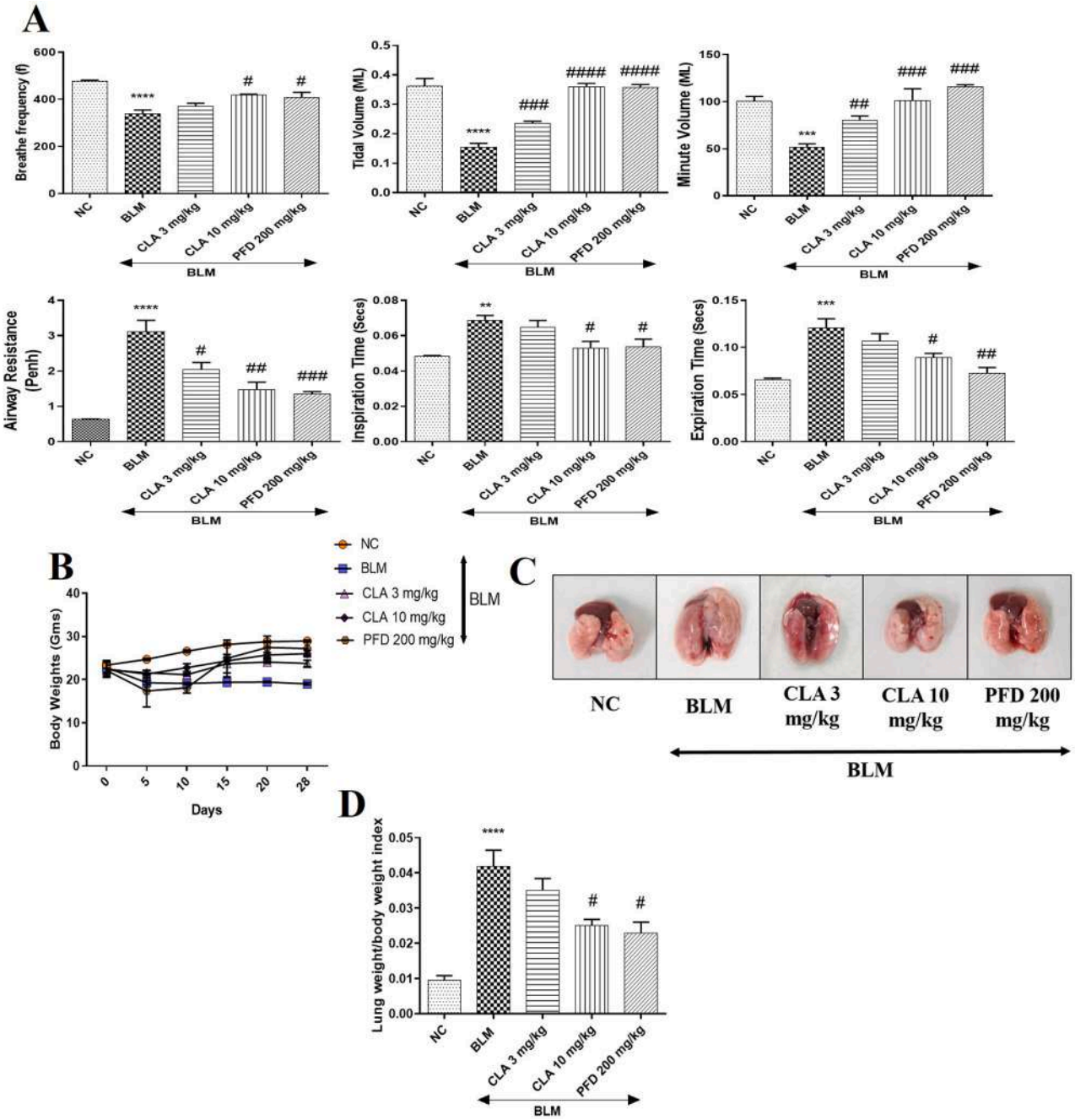

Fig. 3. Effects of CLA on lung functional parameters, body weight, and lung gross appearance and lung weights. Graphical representation of A) Lung functional parameters and B) Body weights of the animals, C) Photographs of lungs, D) Graphical representation of lung-body weight index. All results are expressed as mean ± SEM of n = 8. **p < 0.01, ***p < 0.001 and ****p < 0.0001 vs NC, #p < 0.05, ##p < 0.01, ###p < 0.001 and ####p < 0.0001 vs BLM.

body weight index was observed (Fig. 3C & D).

3.3. CLA treatment normalised BALF parameters and neutrophilic parameters in fibrotic animals

Assessment of several BALF parameters such as total cell count, differential cell count, albumin and total protein levels revealed that BLM increased the total BALF cells, neutrophils and lymphocytes percentage in BALF due to inflammatory response leading to influx of these inflammatory cells. Treatment with CLA decreased this inflammatory cells influx dose dependently, these effects are found to be similar like PFD treatment group (Fig. 4A-C). Further, it was observed that the albumin and total protein levels in BALF were increased in BLM control group due to increased inflammation and vascular permeability.

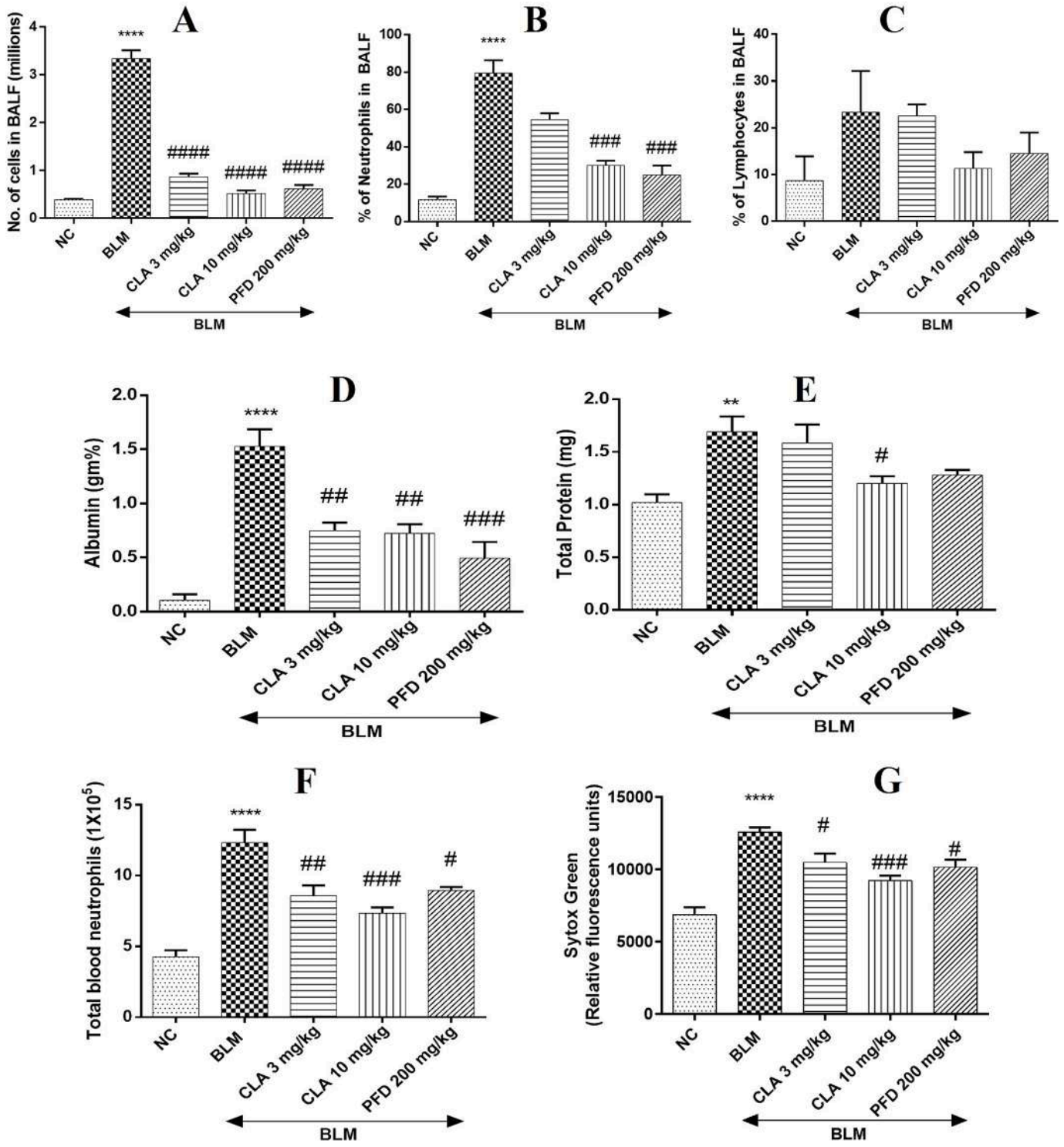

Fig. 4. Effects of CLA on BALF and neutrophilic parameters. Graphical representation of A) Total BALF cell count, B & C) Percentage of neutrophils and lymphocytes, respectively in BALF, D & E) Quantification of albumin and total protein levels in BALF, F & G) Graphical representation of total blood neutrophils and their NETotic potential. All results are expressed as mean ± SEM of n = 4. **p < 0.01 and ****p < 0.0001 vs NC, #p < 0.05, ##p < 0.01, ###p < 0.001 and ####p < 0.0001 vs BLM.

Treatment with CLA decreased these levels significantly in both the doses, effects are identical to standard drug PFD (Fig. 4D & E). Further, we isolated the neutrophils from whole blood and evaluated total neutrophil count and their NETotic ability. It was observed that BLM instillation led to a significantly higher neutrophil count with increased NETotic effects as evident from higher sytox green fluorescent values. Where, CLA dose-dependently decreased total neutrophil count and sytox green fluorescence intensities (Fig. 4F & G).

3.4. CLA restored lung histological features, inhibited collagen deposition and decreased oxidative stress in fibrotic lung tissues

We performed H & E staining to observe the effects of CLA on lung histological changes. BLM administration significantly damaged the lung architecture with features such as alveolar septa degradation, alveolar thickening and infiltration of inflammatory cells. These abnormal features were effectively restored upon CLA treatment with high dose showing maximum protection, which was similar to the effects shown by PFD as evidenced from Ashcroft scoring (Fig. 5A). Further, we evaluated the deposition of collagen, which is the major component of ECM in the lung tissues through MT and PSR staining methods. It was observed that there was a coherent deposition of collagen throughout the lung sections in BLM alone group which was dose-dependently reduced by CLA treatment (Fig. 5A). Furthermore, CLA treatment led to significant reduction in the levels of MDA and nitrite compared to BLM alone group suggesting attenuation of oxidative and nitrosative stress (Fig. 5B & C). In line with this, CLA treatment also significantly increased the levels of anti-oxidant GSH compared to BLM control group which was comparable to the standard drug PFD (Fig. 5D).

3.5. CLA effectively impedes inflammatory and fibrotic cytokines in fibrotic lung tissues

Further, tissue levels of TNF-α, IL-6, IL-1β and TGF-β1 were

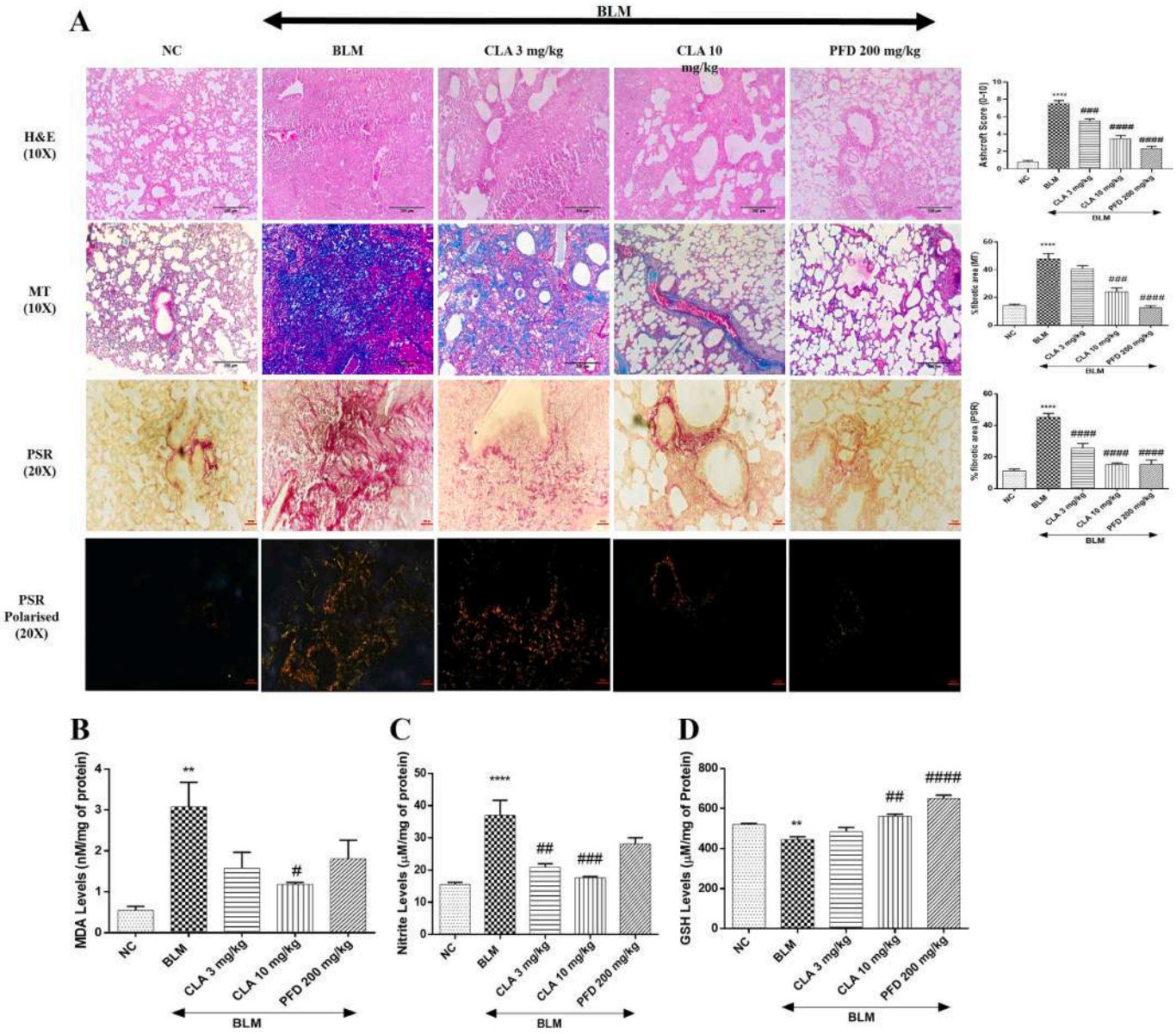

Fig. 5. Effects of CLA on lung histological features, collagen deposition and oxidative stress. A) Representative H&E, MT and PSR (normal and polarised) images, Ashcroft scoring and quantitative analysis (n = 3), B, C & D). Graphical representation of MDA, nitrite and GSH levels in lung tissues (n = 8). H&E and MT images were taken at 10X magnification. PSR images were taken at 20X magnification. All results are expressed as mean ± SEM. **p < 0.01 and ****p < 0.0001 vs NC, #p < 0.05, ##p < 0.01, ###p < 0.001 and ####p < 0.0001 vs BLM.

significantly increased to several folds in BLM treated animals, moreover these levels were significantly reduced by CLA treatment (Fig. 6A-D). To confirm these results, we also performed immunoblotting analysis for these proteins and found that significant downregulation was observed in CLA treated lung tissues compared to BLM control group (Fig. 6E-H). The lung tissue specific expression of FN, a component of ECM evaluated through IHC revealed a higher immunopositivity of this protein in BLM alone group. On the other hand, CLA treatment dampened this signal as evident from the CLA treated lung tissue images, these reductions are found to be consistent to PFD treated lung tissues (Fig. 6I).

3.6. CLA significantly alleviated fibrosis related markers in fibrotic lung tissues

Next, we evaluated the effect of CLA on the fibrosis related markers. It was observed CLA significantly decreased the immunopositivity of α-SMA and COL1A1 in the lung tissues compared to BLM alone group, which was identical to the effects produced by PFD (Fig. 7A). Hydroxyproline is an important biomarker for collagen estimation in lung tissues. It was evident from the hydroxyproline assay that CLA and PFD had a prominent decreasing effect on BLM induced increased hydroxyproline levels in the fibrotic lung tissues (Fig. 7B). Further we evaluated the tissue expression of α-SMA, SNAIL, SLUG and N-cad through immunoblotting and found that CLA markedly downregulated the expression of these fibrotic markers compared to BLM control group as evident from the western blots bands and quantitative analysis (Fig. 7C-G).

3.7. CLA normalised molecular markers for fibrogenesis and matrix remodelling

There is significant increase in several molecular markers namely MMP-2, MMP-7, MMP-9, ICAM-1, and lysyl oxidase (LOX) upon induction of fibrosis through BLM. They play a crucial role in PF development such as promoting EMT, increase activities of pro-fibrotic and pro-inflammatory mediators and promote fibroblast differentiation and myofibroblasts migration. Similarly, we evaluated the protein and mRNA expression of MMP-2, MMP-9 and ICAM-1 following fibrosis induction in fibrotic and treated tissue. BLM group led to significant higher expression of protein and mRNA expression of MMP-2, MMP-9 and ICAM-1. Although, the low dose of CLA did not show any significant effects, high dose of CLA significantly inhibited these expression which was comparable to the standard drug PFD (Fig. 8).

3.8. CLA attenuated NETotic markers in BLM induced PF lung tissues and caused elevation in Del-1 and p53 levels in fibrotic lungs

We evaluated the expression of NETosis specific markers such as PAD-4, CitH3, MPO and NE in lung tissues. Treatment with CLA lead to significant reduction in immunopositivity and downregulation in the protein expression of NE in comparison to BLM induced fibrotic animals as noticeable from IF and immunoblotting (Fig. 9A, C & G). Furthermore, we estimated the levels and expression of MPO in the lung tissues and noticed that BLM had significantly increased the levels and expression of MPO in the lung tissues as reflected from MPO assay and immunoblotting. CLA treatment attenuated the increased levels and

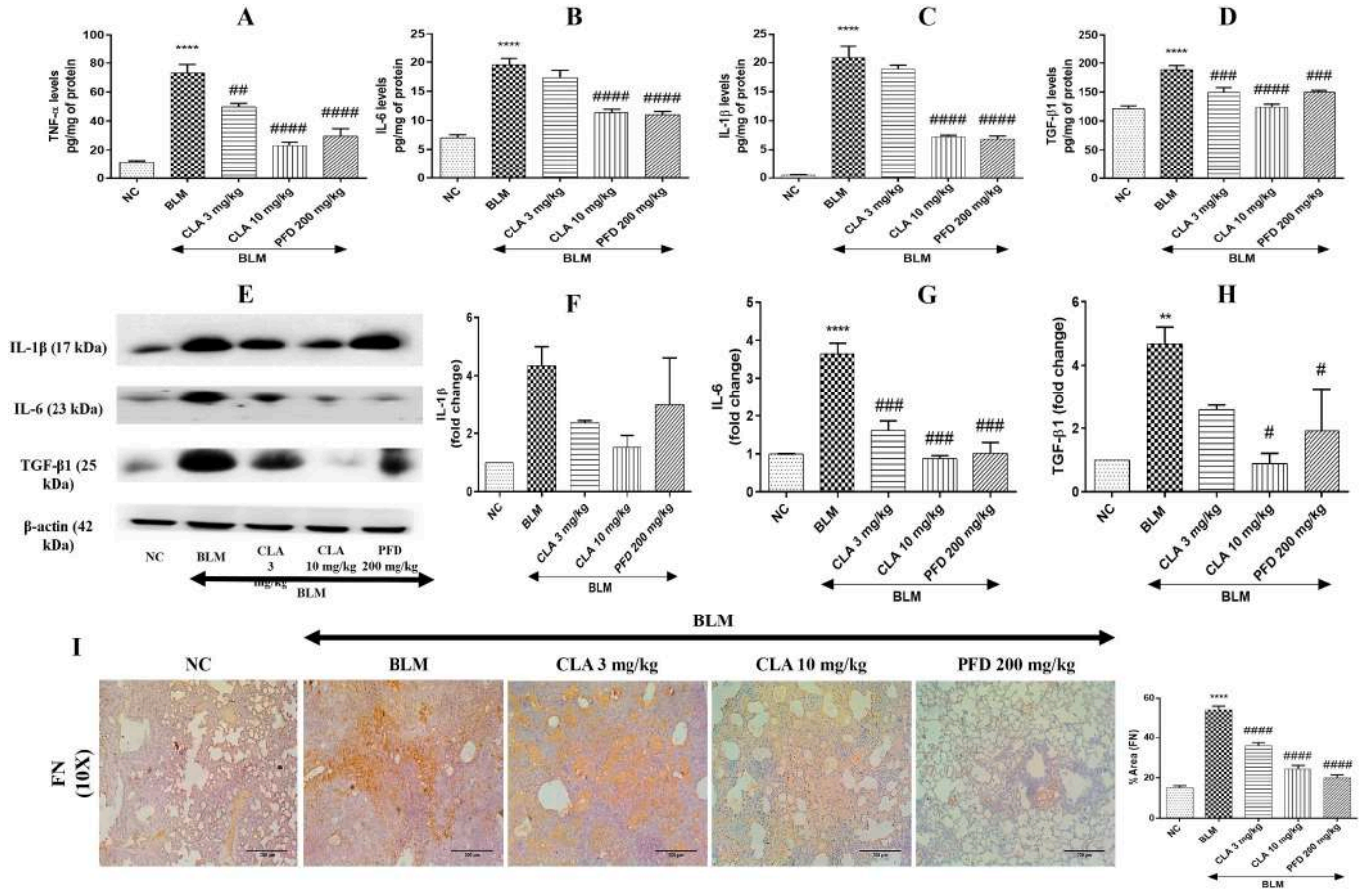

Fig. 6. Effects of CLA on inflammatory and fibrotic cytokines. A-D) Graphical representation of levels of inflammatory and fibrotic cytokines in the lungs namely, TNF-α, IL-6, IL-1β, and TGF-β1 quantified by ELISA (n = 8), E-H) Immunoblotting expression and quantitative analysis of the immunoblots (n = 3), I) Representative images showing immunohistochemical staining of FN (n = 3). The images were taken at 10X magnification. All results are expressed as mean ± SEM. **p < 0.01 and ****p < 0.0001 vs NC, #p < 0.05, ##p < 0.01, ###p < 0.001 and ####p < 0.0001 vs BLM.

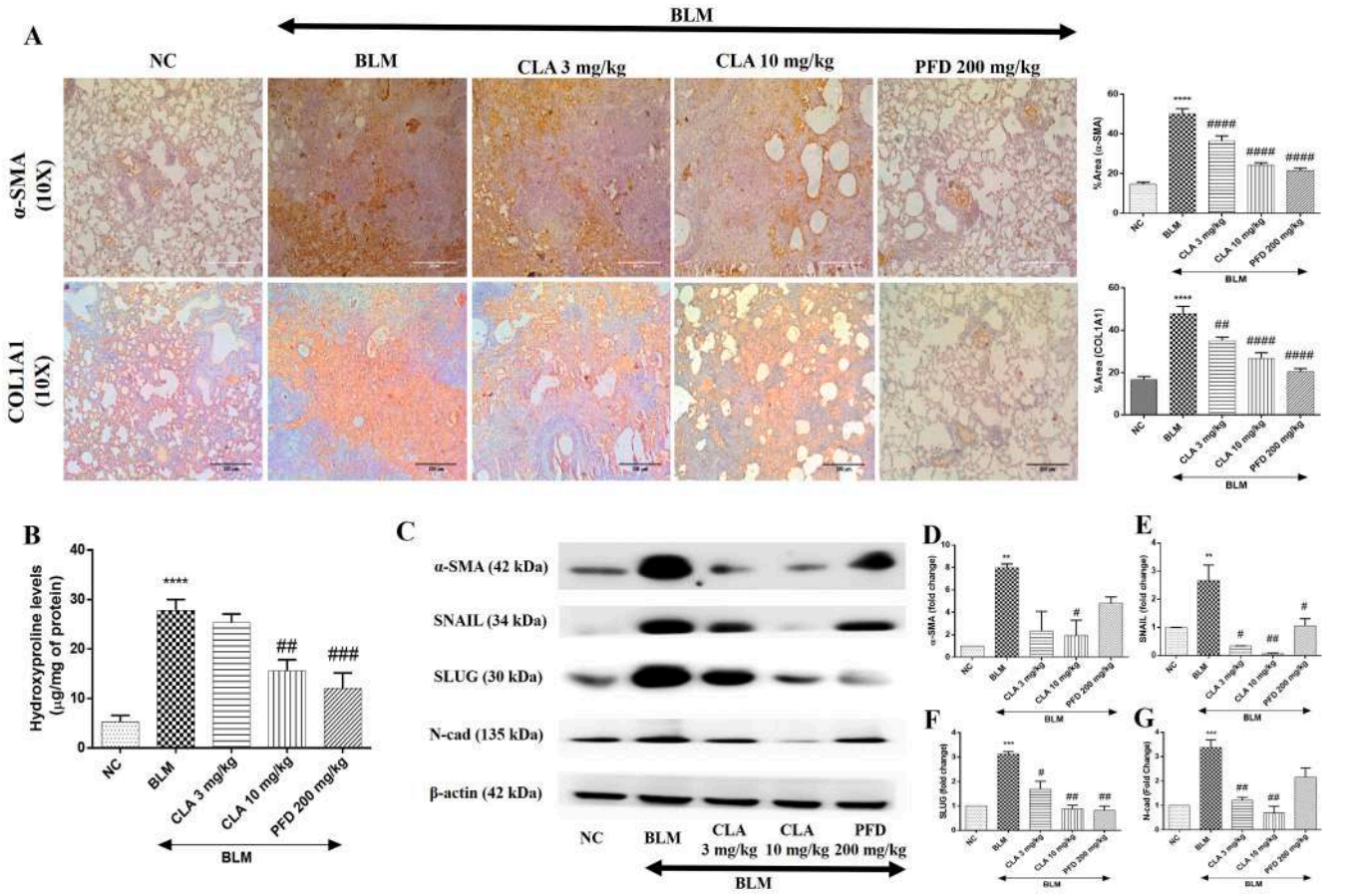

Fig. 7. Effects of CLA on fibrosis related markers. A) Representative images showing immunohistochemical staining of α-SMA and COL1A1 in the lung tissue and its area percentage quantification (n = 3), B) Graphical representation of the hydroxyproline levels in the lung tissues (n = 8), C-G) Representative immunoblots and densitometric analysis (n = 3). The images were taken at 10X magnification. All results are expressed as mean ± SEM. **p < 0.01, ***p < 0.001 and ****p < 0.0001 vs NC, #p < 0.05, ##p < 0.01, ###p < 0.001 and ####p < 0.0001 vs BLM.

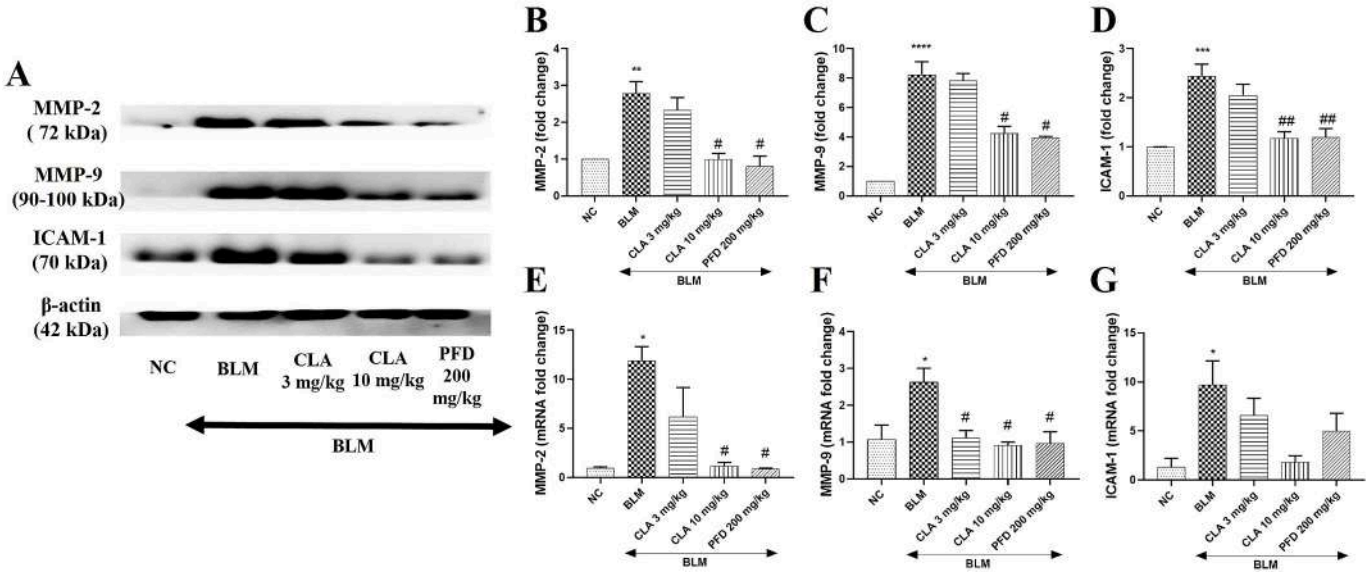

Fig. 8. Effects of CLA on the molecular markers for fibroproliferation and matrix remodelling. A-D) Immunoblots and densitometric analysis of MMP-2, MMP-9 and ICAM-1 (n = 3), E-G) Graphical representation of fold change at mRNA expression of MMP-2, MMP-9 and ICAM-1 (n = 4). All results are expressed as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001 vs NC, #p < 0.05 and ##p < 0.01 vs BLM.

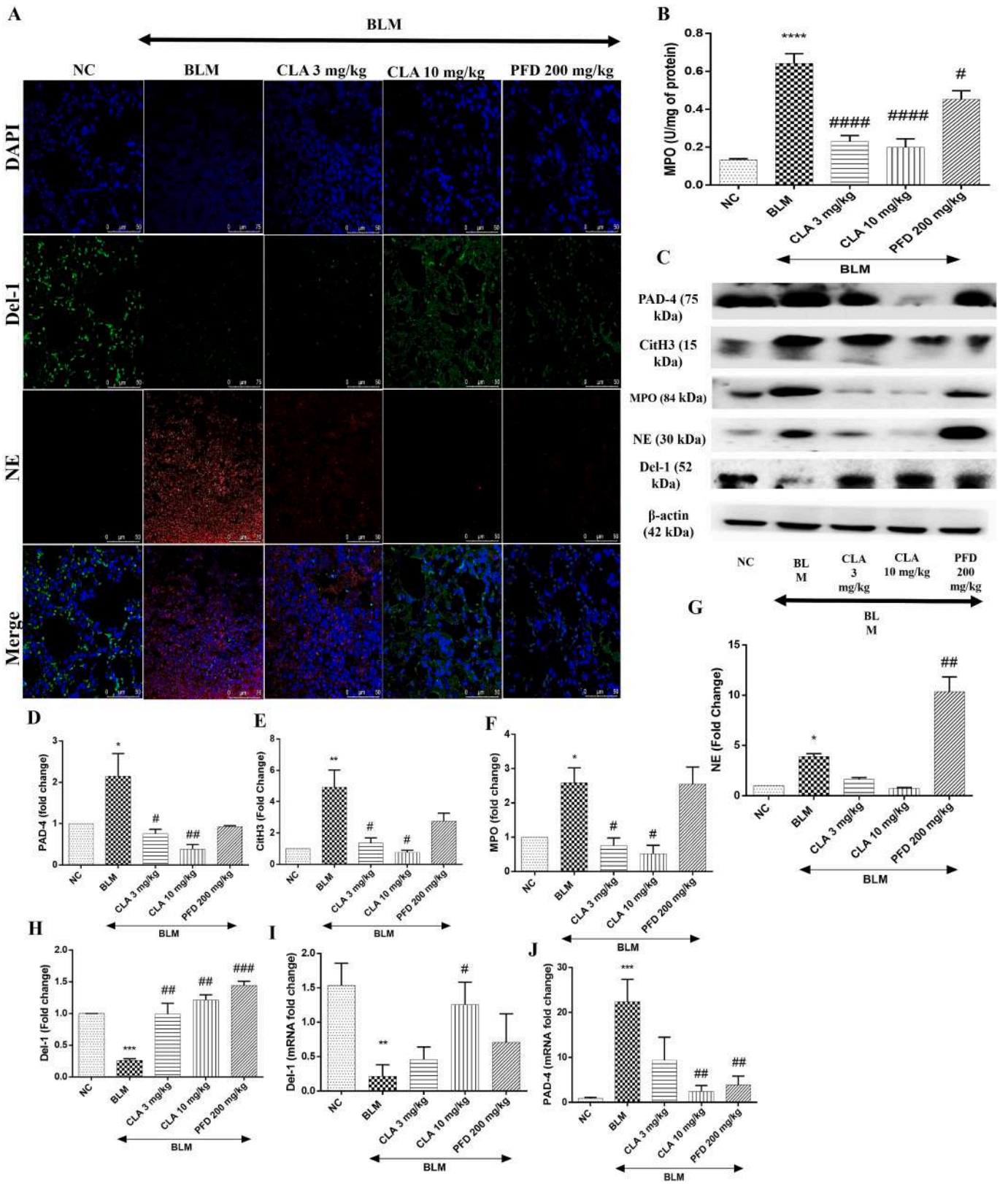

Fig. 9. Effects of CLA on NETotic markers and Del-1 expression. A) Representative confocal images of immunofluorescence staining showing expression of Del-1 and NE on lung tissues, B) Graphical representation of MPO levels in lung tissues (n = 8), C–H) Immunoblots and quantification of the tissue expression of NETotic markers and Del-1 (n = 3), I & J) Graphical representation of fold changes of mRNA expression of Del-1 and PAD-4 in lung tissues (n = 3 to 5). The images were taken at 40X magnification. All results are expressed as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001 vs NC, #p < 0.05, ##p < 0.01, ###p < 0.001 and ####p < 0.0001 vs BLM.

downregulated the expression dose dependently (Fig. 9B, C & F). Evaluation of PAD-4 revealed significant upregulation in the protein and mRNA expression in BLM alone group, which was significantly ameliorated by CLA treatment dose dependently (Fig. 9C, D & J). Additionally, CLA treatment also led to downregulation in the expression of CitH3 as compared to BLM alone group (Fig. 9C & E). We estimated the expression of anti-inflammatory mediator Del-1 in lung tissues through IF, immunoblotting and RT-PCR analysis. It was evident that BLM notably decreased the immunopositivity and significantly downregulated the protein and mRNA expression of Del-1 in fibrotic lung tissues as compared to the normal control lung tissues. Inhibition of PAD-4 with CLA dose dependently upregulated the mRNA and protein expression of Del-1 in lung tissues (Fig. 9A, C, H & I). Thus, it is evident that CLA increases the expression of Del-1 in lung tissues. We further investigated the effects of CLA on the expression of p53. It was observed that fibrosis induction with BLM lead to significant decrease in the

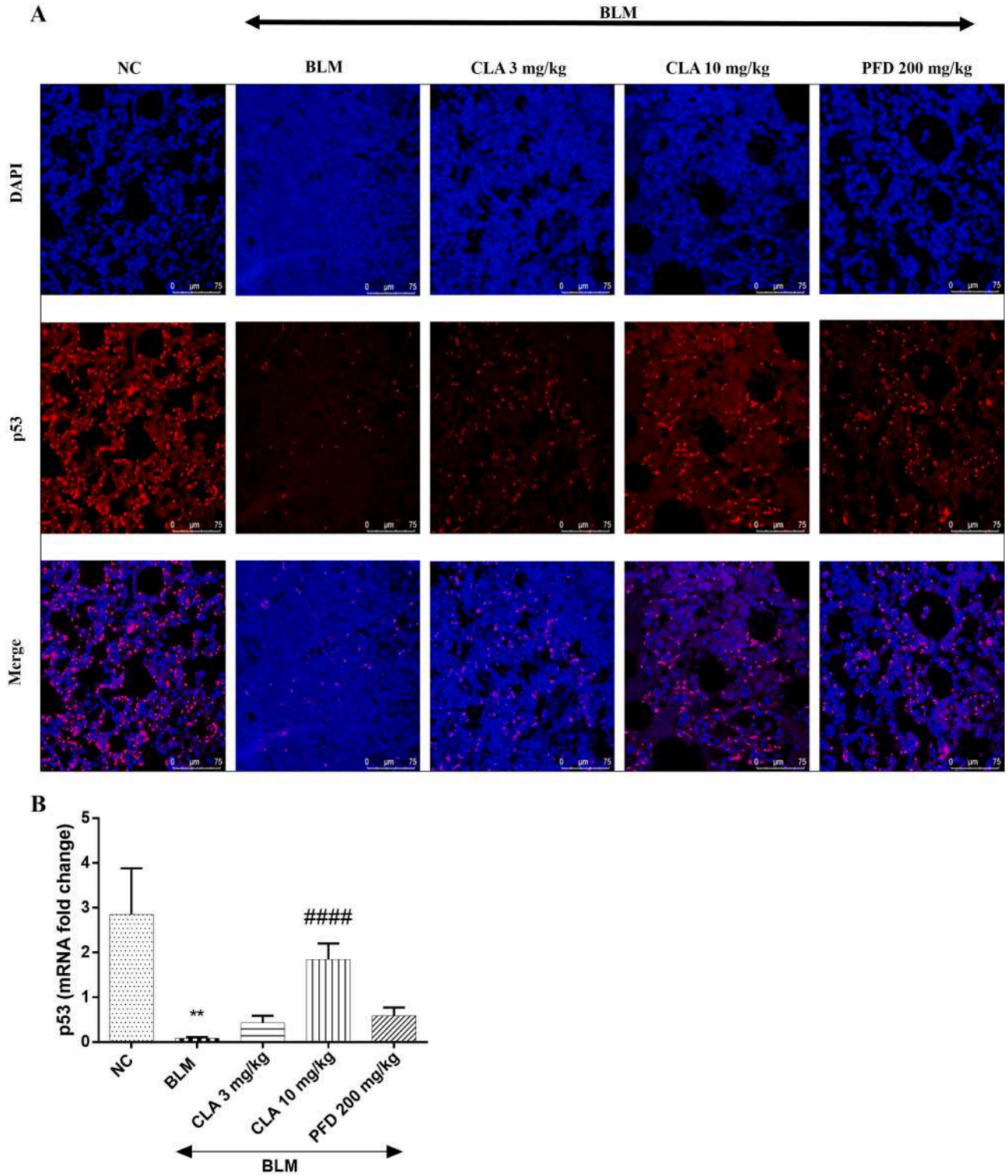

Fig. 10. Effects of CLA on p53 expression. A) Representative confocal images of immunofluorescence staining of p53 in lung tissues, B) Graphical representation of p53 mRNA fold change (n = 3 to 5). The images were taken at 40X magnification. All results are expressed as mean ± SEM. **p < 0.01 and ####p < 0.0001 vs BLM.

protein and mRNA expression of p53 as reflected from IF, and RT-PCR data. However, PAD-4 inhibition with CLA dose dependently increased the protein immunopositivity and mRNA expression of p53 (Fig. 10A-B).

4. Discussion

Pulmonary fibrosis is one of the progressive lung disorder characterised by scarring of the lung tissue developed from an extended wound healing process. When the lungs sustain an injury, the local environment initiates a repair process involving the infiltration of immune cells such as neutrophils, macrophages, leukocytes, T cells, and B cells into the alveoli. These immune cells release a multitude of pro-inflammatory and pro-fibrotic cytokines, chemokines, and inflammatory mediators in an attempt to mend the damaged tissue. They stimulate fibroblasts to differentiate into myofibroblasts, which play a crucial role in the synthesis and secretion of collagen and other ECM components, thus kick-starting tissue remodelling. However, bizarre initiation and dysregulated termination of myofibroblast tend to cause a relentless accumulation of ECM, thus causing alveolar epithelium disintegration leading to organ dysfunction [32]. Exaggerated immune response by cells of the innate immune system such as neutrophils and macrophages spearhead this extensive differentiation and deposition of ECM. Neutrophils are one of the first cells to appear at the injury site releasing inflammatory mediators and forming NETs through a process called NETosis to fight infection. NETosis is an extensively conserved process of anti-microbial defense by neutrophils, wherein they release their DNA in the form of decondensed chromatin due to the action of the enzyme PAD-4. PAD-4 is activated by several stimuli such as increased ROS, lipopolysaccharide (LPS), PMA, protein kinase C (PKC) activators and Fc components of antibodies, which in turn cause post-translational modification in terms of citrullination to the histones of neutrophils [12]. These modifications cause the histones to be less positive, thereby weakening their contact with DNA leading to the loss of compacted chromatin structure and subsequent decondensation and release. This DNA along-with several anti-microbial components such as MPO, NE, cathepsin-G, ROS, PAD-4 and CitH3 form NETs which are released from the cytoplasmic granules through disruption of plasma membrane. The role of PAD-4 in chromatin decondensation and NETs release has been supported through several studies. For example, mice deficient of PAD-4 neither show citrullinated histone moieties and decondensed chromatin nor do they undergo NETosis [33–36]. More so, PAD-4 has been implicated for development of NETosis independent of pathogenic load in several disorders [37,38]. Nevertheless, NETs aim at either immobilising or terminating the microbial load. However, neutrophils often act as a double edged sword. While, they carry out this anti-microbial activity, a dysregulated termination can cause a surge of inflammatory response. This excessive and exaggerated NETosis can form a vicious cycle, thereby causing continuous release of NETs attracting the myriad of immunogenic cells, cytokines and chemokines and activating both cellular and humoral mediated immune response [13].This persistent inflammation contributes to the deposition of excess extracellular matrix proteins, leading to tissue scarring and fibrosis.

For instance, It has been reported that the airway microenvironment in the cystic fibrosis (CF) patients lungs tend to promote neutrophils survival and influence their NETotic capacity and thus give rise to exaggerated NETs release [20]. Further, presence of neutrophils, extracellular DNA and neutrophil elastase in BALF of IPF patients has confirmed the role of NETosis in progression of PF [39]. Pulmozyme®, the brand name for Dornase alfa, is nothing but DNase enzyme that has been approved by FDA for use in CF patients [40].Interestingly, presence of serum anti-PAD-4 auto antibodies and anti-double stranded DNA auto antibodies in CF patients at as young as one year of age is very much compelling. There are multiple reports suggesting elevated inflammation following aftermath of COVID19 pathology such as acute respiratory distress syndrome (ARDS) involving NETs. Researchers have majorly emphasised on hyper inflammation, cytokine storm and exaggerated NETosis in these reports. A 2-center patient cohort study conducted in Israel in the year 2023 reflected that NETosis induction correlates strongly with COVID-19 severity long term pathologies of COVID-19. They have also significantly reported that with long term COVID, there is higher induction of NETosis. NETosis has also been implicated in induction and development of EMT in COVID-19 related fibrosis, wherein they showed a higher concentration of NETs and co-expression of epithelial and mesenchymal markers in BALF [15]. It has also been brought to the light that NETosis is the way to deliver PAD-4 outside the cells leading to auto-antibody generation which can be a novel mechanism for contributing to systemic diseases such as multiple sclerosis and rheumatoid arthritis (RA) [41]. Thus, targeting NETosis may offer new therapeutic avenues for the treatment of pulmonary fibrosis by mitigating inflammation and fibrosis progression.

Del-1, also known as epidermal growth factor (EGF)-like repeats and discoidin I-like domains 3 (EDIL3), is a target gene of p53 due to the presence of p53 response element in Del-1 gene [26]. Del-1 itself is an anti-inflammatory and anti-fibrotic mediator due to the fact that it causes inhibition of binding of integrins to its receptors thereby reducing leukocyte adhesion and migration. It also inhibits neutrophil recruitment and potentiates inflammatory clearance. Further, it also enhances macrophages mediated killing of diseased neutrophils, induce resolvin-D1 and also inhibits TGF-β1 [21]. Additionally, Del-1 possess anti-fibrotic and anti-aging effects [22,42,43].Furthermore, it has also been reported to be an important biomarker in COPD patients [44]. Del-1 overexpression has protected mice from cardiovascular hypertrophy, improved endothelial function and reduced fibrosis of aorta [45]. It also inhibited inflammation, reduced endoplasmic stress and protected mice spinal cord astrocytes from hypoxic injury [46].

BLM is a potent anti-cancer medication with pulmonary fibrosis being one of its major adverse effect. BLM cause increase in inflammatory cells infiltration and oedema of the interstitial cells leading to inflammation [47]. It is involved in increase in ROS production and initiation of lipid peroxidation [47,48]. The increase in capillary permeability of the alveoli attracts several immunogenic cells including neutrophils thereby causing NETosis. Moreover, anti-fibrotic effects of therapeutic anti-citrullinated protein antibody (tACPA) in BLM induced PF animal model has shown reduced tissue damage and NETs clearance by macrophages [49]. Trafficking of neutrophils towards the lung tissues and BALF has also been reported in BLM induced PF mouse model [39]. Thus, we have hypothesised that PAD-4 inhibition can ameliorate pulmonary fibrosis effectively and this protection is also exerted partly by Del-1 owing to its increased expression in the CLA treatment groups.

Our sytox green and DAPI staining studies confirmed CLA as a direct PAD-4 inhibitor, reducing NETs release and MPO levels. Immunocytochemistry revealed decreased PAD-4 expression with CLA treatment, reinforcing its anti-NETotic effects. CLA also controlled inflammatory markers like reactive oxygen and nitrogen species.

BLM instillation led to shrinkage of the alveolar space and thickening of the alveolar wall in addition to degradation of the alveolar septa. This in turn results in decreased breathing frequency, tidal volume and minute volume and increased airway resistance, inspiration time and expiration time. Treatment with CLA exerted protective effects which was attributed to normalising these lung function parameters. In accordance with this, there was development of fibrotic lesions inflammatory spots in the lungs which led to increase in lung weights. CLA further alleviated these features and the lungs were significantly normalised following CLA treatment which was reflected in the lung weights as well. Inflammatory cells migrate to the site of inflammation by disrupting the endothelial barrier. In the BLM induced PF, there was increase in total cell count, percentage of neutrophils and lymphocytes in the BALF that corroborated our pulmonary fibrosis research conducted in our lab [4,7,50]. Supporting these findings are increase in total protein and albumin content in the BALF in BLM control group. These parameters were significantly reduced upon CLA treatment.

Inflammation in the lungs triggered increased production and circulation of neutrophils in the whole blood which had greater than normal NETotic capacity. Out study testified the same where there was significant increase in total neutrophils in the whole blood with increased NETotic potential as depicted from sytox green dye binding assay. CLA treatment normalised these parameters as well. PFD, being a standard FDA approved drug against PF also had significant reduction of these fibrotic and inflammatory parameters. The treatment effects of CLA and PFD went simultaneously implicating anti-fibrotic effects of CLA.

Enhanced infiltration of inflammatory cells and oedema disrupt the normal architecture of the lungs upon BLM induction. It was significantly improved upon CLA treatment. Micro-injury to the lungs with BLM leads to persistent tissue injury initiating a cycle of repair and remodelling leads to increased deposition and reduced degradation of ECM, increase in adhesive proteins and markers for EMT which are inherent characteristics of fibrotic development [5]. The effect of CLA and PFD treatment on deposition of the collagen which is the primary component of the ECM was evaluated. Hydroxyproline is the major component of collagen which plays a role in collagen stability and buildup. Hydroxylation of proline by the enzyme prolyl hydroxylase is an important step in the deposition of collagen in fibrotic disorders. The levels of hydroxyproline was evaluated in the lung tissues where induction of fibrosis by BLM reflected higher hydroxyproline content which was reduced upon CLA and PFD treatment. Corroborating this is the deposition of collagen on the lung tissues which were evaluated by collagen specific staining techniques such as MT and PSR. They revealed a significant increase in collagen deposition in the BLM alone group whereas reduced collagen deposition was observed in the CLA and PFD treated lung sections indicating lesser degree of collagen deposition. Moreover, the expression of the collagen subtype COL1A1 was also evaluated through immunohistochemistry experiment. BLM treated mice resulted in higher COL1A1 expression which was significantly lowered upon CLA and PFD treatment. Furthermore, the expression of fibronectin which is an adhesive glycoprotein and has an important role to play in tissue repair and α-SMA which is a mesenchymal marker were greatly increased in the diseased tissue sections. Anti-fibrotic effects of CLA and PFD were also confirmed upon decreased expression of these proteins. These markers play a significant role in development of hallmark characteristics of fibrosis including myofibroblasts differentiation, instigation of EMT and deposition of ECM proteins. The fact that PFD had significant reduction in these levels had further strengthened its anti-fibrotic effects. However, treatment with CLA also resulted in significant inhibition of these fibrotic mediators. Additionally, sustained inflammation in the lung epithelium as a result of continuous and dysregulated stimulation by several factors contribute to the progress of PF. BLM induced lung tissue showed several fold increase in pro-inflammatory and pro-fibrotic cytokines such as TNF-α, IL-6, IL-1β and TGF-β as evidenced from ELISA studies. Treatment with CLA and PFD dampened these expressions thus validating anti-inflammatory and anti-fibrotic effects of PFD and CLA.

As mentioned above, migration of inflammatory cells into the alveolar spaces and BALF is an important feature of PF. Neutrophil migration is one of the critical step in inflammatory infiltration having paramount importance. Upon migration into the alveolar spaces, they carry out phagocytosis and NETosis and form the vicious cycle of inflammation. NE, MPO, CitH3 and PAD-4 are the decisive markers for NETosis. Although, it is pretty straight forward that the Citrullination activity of PAD-4 in the nucleus is fundamental for NETs release. These NETs are coated with proteases and peroxidases like NE, cathepsin-G and MPO, however their role in initiation of NETs through chromatin degradation is unclear [51–53]. It has been reported that inhibition of NE led to significant reduction in NETs release and mice having deficiency of serine protease inhibitor that degrades NE showed higher NETs release upon PMA stimulation [54,55]. Contradicting to another study reported that NE activity is not significant in NETs release upon ionophore or PMA stimulation [56,57]. Similarly, inhibition or deficiency of MPO has resulted decreased NETs release and MPO also enhance NE mediated chromatin degradation [55,57,58]. Moreover, citH3 is yet another marker for NETosis implying histone citrullination. Pharmacological inhibition of PAD-4 leads to decreased chromatin decondensation and thereby the expression of citH3 [34,59]. Evaluation of these markers have become important molecular events to evaluate the PAD-4 mediated anti-NETotic and anti-fibrotic effects of CLA. In line with the literature, the levels of MPO and expression of NE, and PAD-4 had increased to several folds in the fibrotic lung implicating NETs release, inflammation and fibrosis. Additionally, the expression of citH3 had increased several folds as well due to higher PAD-4 activity. On the other hand, treatment with CLA predominantly decreased the levels of MPO and expressions of NE, CitH3 and PAD-4 in a dose-dependent manner in the treated mice tissues, where the effects shown by CLA is more pronounced than that of PFD implicating noteworthiness of these markers in NETs inhibition mediated anti-fibrotic study. Moreover, our study has also complimented another study conducted by Masaki et al. on effects of PAD-4 knock out and CLA on BLM induced PF. They have reported decreased NETs levels in the BALF and isolated primary bone marrow neutrophils with reduction in NETotic markers such as CitH3 and NE. Additionally, they have also showed decreased fibrotic development with reduction in collagen deposition and decrease in levels of inflammatory and fibrotic markers [60].

We also evaluated the role of PAD-4 inhibitors on p53 and Del-1 expression in lung tissues. Owing to the fact that Del-1 is prominently expressed in the lungs endothelium, the normal lung showed a significant expression of Del-1 as evident from immunofluorescence, immunoblotting and RT-PCR experiments. Moreover, p53 was also expressed significantly in the normal lung tissue. However, the expression and positivity of Del-1 and p53 declined significantly to several fold in the diseased lung tissues. Interestingly, PAD-4 inhibitor CLA effectively normalised the expression of both p53 and Del-1 in both the dose groups with higher dose showing significant normalisation. The array of results obtained affirmed the fibrogenesis effects of PAD-4 and PAD-4 inhibitor can significantly result in anti-fibrotic effects. It also corroborates our hypothesis that expression of anti-inflammatory mediator Del-1 is decreased in a significant way in the diseases fibrotic lung tissues and treatment with PAD-4 inhibitor resulted in significantly normalised expression. Moreover, due to the fact that p53 expression also followed a similar pattern, it can be deduced that PAD-4 can have a significant role to play in co-repressing the expressions of Del-1 and p53. Hence, it was confirmed that PAD-4 inhibition leads to increase in Del-1 expression, thereby Del-1 can exert its anti-fibrotic effects.

The protective effects observed with PAD-4 inhibitor CLA are very much comparable with the standard PFD treated groups where anti-fibrotic effects of both CLA and PFD were pronounced, it was observed that CLA had achieved higher ant-NETotic effects indicating specific importance of both fibrotic and NETotic markers. Owing to the fact that CLA achieved a) significant inhibition of neutrophils and released NETs, b) inhibited the release and expression of pro-inflammatory cytokines and pro-fibrotic cytokines, c) ameliorated fibrogenesis through inhibition of ECM and mesenchymal proteins and also inhibited molecular markers of fibrogenesis such as MMP-2, MMP-9 and ICAM-1, the development of specific inhibitors against PAD-4 can prove to be another alternative of anti-fibrotic medications apart from the available inhibitors. In addition to PAD-4, PAD-2 also plays an important role in various auto-immune disorders including rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus and ulcerative colitis is also documented [61]. Additionally, CLA is also known to inhibit PAD-2 and the development of novel chemical entities against PAD-2 similar to CLA are being developed [61]. Moreover, the effect of CLA on PAD-2 in cancer cell lines also reported CLA to have lesser degree of inhibition on PAD-2 compared to specific PAD-2 inhibitor [62]. CLA had negligible effects on development of extracellular vesicle and cell viability of cancer cells [62]. However, our data indicate that PAD-4 inhibition through CLA is viable protective approach for PF. However,

there is a need to understand in detail various safety issues associated with PAD-4 inhibition and also there is a potential for scope for development of novel druggable therapeutic agents by targeting PAD-4 and evaluate them in suitable models.

5. Conclusion

We demonstrated that PAD-4 inhibitor CLA exerted anti-fibrotic effects in BLM induced PF mice model. It normalised lung function parameters, BALF parameters, oxidative stress markers, collagen deposition in the lung tissues and lung histology. It profoundly decreased the elevated levels of pro-inflammatory and pro-fibrotic cytokines, components of ECM and mesenchymal cells markers proving that it has a combined anti-inflammatory and anti-fibrotic effects. Further, it also reduced NETosis and controlled its markers. It also confirmed the role of PAD-4 in co-repressing anti-inflammatory mediator Del-1 through p53. Upon inhibition of PAD-4, the Del-1 repression by PAD-4 was removed resulting in increased transcription of Del-1 which in turn led to Del-1 exerting its anti-inflammatory and anti-fibrotic effects. However, in future, evaluating the anti-fibrotic effects of CLA by knocking down PAD-4, Del-1 and p53 simultaneously will be intriguing and will establish a direct cross-talk between PAD-4 and Del-1.

CRediT authorship contribution statement

Biswajit Panda: Writing – review & editing, Writing – original draft, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Alfiya Momin:** Writing – review & editing, Methodology, Formal analysis. **Geetanjali Devabattula:** Writing – review & editing, Methodology, Formal analysis. **Chilvery Shrilekha:** Methodology, Writing – review & editing. **Anamika Sharma:** Methodology, Writing – review & editing. **Chandraiah Godugu:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2024.112861.

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A review on mechanistic aspects of litchi fruit induced acute encephalopathy

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ABSTRACT

Litchi (*Litchi sinensis*), a fruit with a sweet and white aril, cultivated mainly in Southeast Asia and possesses anticancer, antibacterial, antioxidant, and other therapeutic properties. It is a delicacy among children. However, an outbreak of acute encephalopathy syndrome (AES) in litchi growing regions during the seasons of litchi ripening and harvesting (May–June) resulted in symptoms of lethargy, weakness, fever, vomiting, seizures, and coma that was most common among malnourished children below 15 years. Upon successful epidemiological studies, it was confirmed that the non-protein amino acids such as hypoglycine A (HGA) and methylenecyclopropylglycine (MCPG) are responsible for the AES outbreak. Most of the underprivileged and malnourished kids with an empty stomach venture into the litchi orchards to savor the fruit during the litchi harvesting season. Their fasting condition results in decreased glucose levels in the blood. The decreased glucose levels trigger glycogenolysis. However, gluconeogenesis takes over glycogenolysis to replenish the glucose levels due to fewer glycogen stores in malnourished children. The toxins are involved in fatty acid oxidation and gluconeogenesis pathways, by blocking several steps in the former process. Depleted glycogen stores and suppression of gluconeogenesis synergistically cause hypoglycemia and accumulation of toxic intermediates from the metabolic pathway leading to metabolic failure. The incidence of AES can be prevented by creating proper awareness among the farmers, vendors and consumers on the importance of adverse effects of litchi fruit when consumed on empty stomach or fasting state. Further, elucidating detailed biochemical pathway of HGA and MCPG toxicity, improving agricultural and public health practices, keeping glucose stores and glucose banks in the areas which are highly prone to litchi induced toxicity are some of the therapeutic measures. This review highlights and discusses the AES incidences, mechanistic pathways involved in litchi fruit toxicity, and corresponding risk factors involved and possible treatment and preventive approaches.

1. Introduction

The aetiology, implications, and treatment methods of sudden outbreaks of severe and inexplicable clinical illnesses in specific geographical locations are typically not well understood. For example, the Indian state of Bihar has had repeated outbreaks of acute encephalopathy syndrome (AES) since 1995. The World Health Organisation (WHO) states that AES is a sickness that can affect anyone at any age and at any time of year. It is distinguished by a sudden onset of fever, new seizures (apart from mild febrile seizures), and altered mental status, such as coma, difficulty speaking, confusion, or disorientation. Children are primarily affected by this disease around May and June (Shrivastava et al., 2015). During the outbreaks the significant mortality with 54 children out of 147 affected, and 178 children out of 469 affected in the years 2011 and 2012, respectively (Narain et al., 2017). While, recently according to the printed media (The Hindu on June 23, 2019), around 400 children were being admitted to hospitals in the year 2019 reporting the symptoms of AES out of which more than 100 have lost their lives. Similarly, another outbreak was reported in West Bengal causing fatalities to 34 children among 72 affected in the year 2014 (Bandyopadhyay et al., 2015). These recurring outbreaks highlight the urgent need for comprehensive investigation and effective intervention strategies to mitigate their impact. Initial investigations suggested that an encephalitis virus, such as “Japanese encephalitis virus” (JEV), transferred from

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birds or animals, and the usage of "pesticides" had been responsible for these epidemics. A research discovered that a definitive etiological diagnosis was obtained in 56.4% of children with acute encephalitis syndrome (AES), which is greater than most previous Indian studies, where more than half of children with AES had no detectable aetiology. The most prevalent aetiologies discovered were viral (30.4%) and bacterial (22%) infection related, with scrub typhus (11.2%) and dengue (9%) being the leading culprits. This is crucial since these aetiologies are rather prevalent and may be efficiently treated with proper therapy (Rebecca et al., 2024). The correlation of AES epidemics with the litchi harvesting season in some locations has sparked concern that litchi intake is associated to AES, however clear confirmatory studies are yet to be done. Upon further investigation, it was discovered that the litchi fruit (*Litchi sinensis*) of the Sapindaceae family was the primary cause of these AES symptoms, as the summer season is also ideal for the ripening and harvesting of these fruits (Nath et al., 2016). Litchi, known for its delicious fruit taste, had high commercial, nutritional and ethno pharmacological values. In addition to being directly eaten, litchi was used in the production of wine, juice, vinegar, jellies, and jams constituting its commercial values (Fig. 1) (Ibrahim and Mohamed, 2015). By virtue of its chemical constituents such as flavonoids, phenolic acids, sterols, triterpenes and anthocyanins, litchi fruit possessed anticancer, antibiotic, anti-hyperlipidaemic, hepatoprotective, antioxidant, antipyretic and anti-inflammatory properties. Each and every part of the plant had a few ethno pharmacological values. For example, the roots, bark and flowers are applied for amelioration of throat disorders, seeds are used for alleviation of neuralgic disorders, hernia and intestinal disorders including ulcers (Ibrahim and Mohamed, 2015). Furthermore, a peel drink of litchi had therapeutic activity towards smallpox and diarrhoea while the leaves are used for reliving symptoms of flatulence and heat stroke (Ibrahim and Mohamed, 2015). The fruits had anti-diarrhoeal, cough suppression, anti-ulcer, anti-diabetic and anti-obesity properties (Ibrahim and Mohamed, 2015). Litchi fruit, whose fleshy aril is a delicacy among children, is primarily cultivated throughout Southeast Asia including countries like India, Bangladesh, Vietnam, Taiwan, Japan, Thailand, and Indonesia (Kumar et al., 2020). Litchi fruit contain a chemical called methylenecyclopropylglycine (MCPG), which is a lower counterpart of hypoglycin A. This Hypoglycin A (HCA), a toxin present in litchi fruit seeds, especially unripe or semi-ripe fruits, can induce hypoglycemia encephalopathy in children. Both HCA & MCPG have been linked to hypoglycemia and disruption of fatty acid β-oxidation in liver cell mitochondria in experimental mice. The toxicity mechanism of MCPG is connected to the synthesis of MCP-formyl-CoA, which inhibits numerous dehydrogenases involved in gluconeogenesis, causing the depletion of glucose stores in the body leads to AES symptoms like mild to moderate fever, sweating, lethargy, vomiting, seizures, which results from hypoglycaemic condition in the brain (Kumar et al., 2020; Shrivastava et al., 2017; Paireau et al., 2012; Gray and Fowden, 1962; Melde et al., 1989, 1991).

Gluconeogenesis is the metabolic pathway primarily found in the liver and kidneys that allows glucose to be produced from non-carbohydrate substrates. This process is crucial for maintaining blood glucose levels, particularly during times of fasting or vigorous exercise. Interference with gluconeogenesis is a major factor in the development of hypoglycemia and subsequent neurological symptoms in litchi fruit-induced AES. Unripe litchi fruit contains high levels of HGA and MCPG, toxins that disrupt gluconeogenesis. The identification of urinary metabolites, HGA in stomach juices, high acylglycines in urine, and elevated acylcarnitines in blood products all have been used to confirm HGA and MCPG exposure in AES outbreaks. HGA in blood has been detected in investigations of Seasonal Pasture Myopathy (SPM) cases in horses, with serum levels measured as high as 8.5×10^3 ng/mL. Similar techniques have been used to detect HGA in human serum following the consumption of litchi (5 g/kg body weight) and ackee (1 g/kg body weight). Serum HGA levels were found to be 2.09 ng/mL (14.8 nmol/L) and 79.1 ng/mL (560 nmol/L) even 10 h after consuming ackee and litchi, respectively (Melde et al., 1991). Hypoglycin A and methylenecyclopropylglycine (MCPG) are toxic chemicals found in specific fruits and plants, mainly in the Sapindaceae family, which includes ackee, litchi, and several maple species. Both the seeds and the arils (edible component) of ackee fruit contain hypoglycin A, with unripe fruit having larger quantities. As the ackee fruit ripens, the hypoglycin A in the arils converts to hypoglycin B in the seeds, which aids in the detoxification process. Ackee fruit is a commodity exported by Jamaica, Haiti, and Belize, however the FDA controls its importation into the United States, allowing only up to 100 ppm of hypoglycin A (Isenberg et al., 2015). MCPG has been found in litchi fruit, as well as in the seeds, leaves, and seedlings of some maple species. A research discovered that MCPG and hypoglycin A are found in the arils of three different Chinese litchi cultivars, with varied quantities among them. Overall the levels of hypoglycin A and MCPG can vary significantly across different litchi and ackee cultivars, as well as between unripe and ripe fruit. Careful monitoring and regulation are required to ensure food safety, especially for ackee fruit exports (Spencer and Palmer, 2017). A pilot study offers the first indication that ingested HGA from sycamore maple (SM) might pass into dairy cow milk, emphasising the need for more investigation into the food safety implications. HGA was only discovered in the milk of cows who were exposed to SM trees on their pasture, indicating that the ingested SM toxin was transferred into the milk. The quantities of HGA in cow milk samples varied greatly, ranging from 120 to 489 nmol/L (Bochnia et al., 2021). In another research, cows were seen eating sycamore saplings while grazing on a grassland containing SM trees. Individual milk samples were analysed for HGA and MCPG metabolites as early as after the end of the first day of grazing. Cow urine samples contained more conjugated HGA and MCPG metabolites than milk. (Engel et al., 2023).

The present review focuses on the biological mechanisms behind HGA and MCPG-related AES and investigates outbreaks in India,

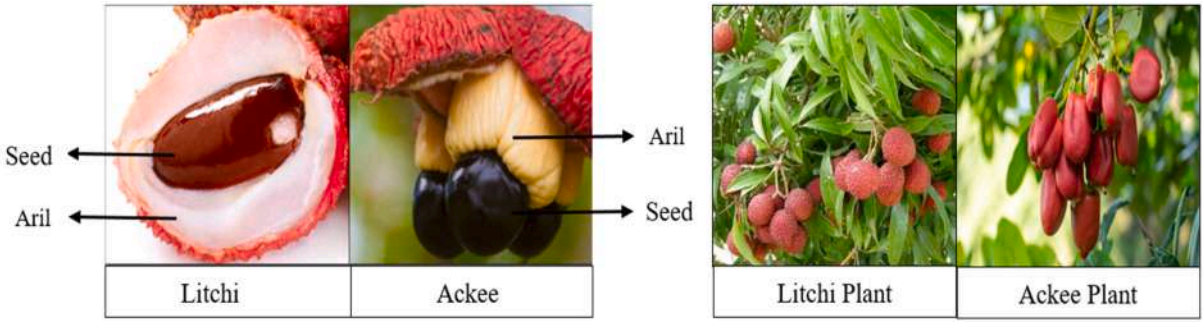

Fig. 1. The litchi and ackee fruit showing their edible aril and seed. Litchi is a fruit of delicacy in India, while ackee is the national fruit of Jamaica.
Source:https://www.britannica.com/plant/litchi-fruit. https://edition.cnn.com/2017/04/10/health/fruits-poison-litchee-ackee-nerve-disease/index.html. https://wildroots.in/shop/outdoor-plants/fruit-plants/litchi-plant/https://www.freepik.com/photos/ackee.

Vietnam, Jamaica, and Bangladesh. Furthermore, this study examines therapeutic strategies and preventative measures that can be used to control abrupt outbreaks of such medical conditions.

2. Insights into litchi related AES symptoms

Mostly economically backward and malnourished children of litchi growing regions mentioned above tend to ingest a relatively higher quantity of the fruit in the afternoon and return home with an objection to have dinner followed by waking up in the next morning with AES symptoms associated with high mortality and morbidity. This gave a hint to researchers that litchi fruit had a greater role in the development of symptoms and illness. Despite extensive investigations, the cause and risk factors for the illness remain unconfirmed among the affected individuals. To support this, the National Centre for Disease Control (NCDC) and USA Centre for Disease Control (CDC) investigated the AES based on the 2013 and 2014 outbreaks, respectively. The present review desires to figure out the risk variables associated with this syndrome using an assortment including novel laboratory testing technologies, hospital-based clinical surveillance, and an epidemiological case-control analysis on individual's biological and environmental specimens. It evaluates the potential causality of naturally existing toxins like HGA and MCPG, but leaves out the potential contribution of new infectious diseases, certain pesticides, and hazardous substances. Their reports outlined that there is no involvement of any common pathogen responsible for encephalopathy, rather there is presence of a possible toxin causing the non-inflammatory encephalopathy. Also, the laboratory findings demonstrated that the blood glucose levels had dropped down to less than 70 mg/dl of patients even before the start of therapy which, further strengthen the possible involvement of a toxin causing hypoglycaemia leading to seizures and coma (Shrivastava et al., 2015). Further, studies established the fact that the toxins involved could be the non-protein amino acids Hypoglycine A (HGA) and its lower homologue i.e. methylenecyclopropylglycine (MCPG) which are present in the seeds and arils of litchi fruit (Das et al., 2015). *Gray and Fowden* first isolated HGA and MCPG from the litchi fruit in the year 1962 (Gray and Fowden, 1962). Moreover, a report by Srivastava et al. in the year 2017 based on the 2013 and 2014 outbreaks confirmed that this unusual non-protein amino acid of the litchi plant are the primary cause of AES outbreak due to litchi fruit ingestion. Further, it was identified that skipping the evening meal worsens the symptoms leading to coma and death (Shrivastava et al., 2017). Furthermore, Ingestion of fruits belonging to the Sapindaceae family such as the Jamaican ackee fruit (*Bilghia sapida*), rambutan fruit (*Nephelium lappaceum*), longan (*Dimocarpus longan*has), have also shown the symptoms of AES indicating presence of the non-protein amino acid such as HGA and MCPG. In addition, hypoglycin B (HGB), the γ-glutamyl dipeptide analogue of HGA is also present in fruits and seeds of *Billia hippocastanum* belonging to the family Hippocastanaceae and *Acer pseudoplatanus* belonging to the family Araceae. The similar AES syndrome has been witnessed in countries such as Vietnam, Bangladesh and Jamaica (Paireau et al., 2012; Joskow et al., 2006; Gaillard et al., 2011; Biswas, 2012).

2.1. Symptoms and pathophysiology of AES

The neurons in the brain are the extensive user of blood glucose for energy requirement throughout the day. Human brain constitutes around 2% of the body weight, whereas it consumes around 20% energy derived from blood glucose (Erbsloh et al., 1958). Glucose availability and subsequent metabolism maintains brain physiology and drives neuronal and non-neuronal functions. The carbohydrate from ingested food is broken down into its simplest form i.e. glucose which is involved in the metabolic process to derive energy in the form of ATP. While, excess glucose is stored in the form of glycogen which is used for converting it back to glucose by a process called glycogenolysis under fasting conditions. However, when all the glycogen stores are drained and the glycogen reserves dwindle, the liver produces glucose from non-carbohydrate stores such as fats and amino acids in a process called gluconeogenesis to supply glucose to organs with high demand such as brain and red blood cells (RBCs). Gluconeogenesis is a group of metabolic reactions to maintain adequate levels of glucose in the blood and organs such as brain, liver and kidney and they generate glucose from non-carbohydrate sources such as fats. As mentioned earlier, brain utilises glucose during normal and fasting conditions as well. It uses 70% of the total glucose produced in fasting conditions making it one of the high end glucose user (SCHEINBERG, 1965). While in the initial several hours of fasting glycogenolysis and gluconeogenesis contribute equally to maintain the glucose levels, the contribution of gluconeogenesis rises from 54% during the first 14 h of fasting to 64% after 22 h of fasting and to 84% after 42 h of fasting (Chandramouli et al., 1997). Upon dysregulated supply of glucose to brain, it gives rise to brain hypoglycaemic conditions that progress to brain dysfunction with imbalance posture, poor attention, vomiting, seizures, memory loss and coma.

Similar symptoms were visible in the children of Bihar, India. The combination of these symptoms were then named as non-inflammatory AES or hypoglycaemic encephalopathy. The symptoms developed during litchi harvesting season and hence, litchi fruit was thought to be attributed to be contributing for the AES development. Researchers speculated the cause to be pesticide or to be any viral pathogen transmitted from an animal or a bird. It was until a report by Srivastava et al. in the year 2017 that confirmed the presence of specific toxins in the arils and seeds of the litchi fruit (Shrivastava et al., 2017). The toxin, namely HGA is known to irreversibly inhibit β-oxidation of fatty acids and gluconeogenesis resulting in impaired energy production and glucose formation (Melde et al., 1989, 1991). Ingestion of a comparatively higher quantity of litchi fruit in the afternoon and going to bed without having a proper meal results in absence of glucose availability from the diet and fasting like condition. This initiates glycogenolysis to synthesise glucose from the glycogen reserve in the liver. However, unavailability of sufficient glycogen stores in malnourished children lead to poor maintaining of an optimum glucose level in the blood resulting in initiation of gluconeogenesis as well as other energy producing pathways such as β-oxidation of fatty acids (Melde et al., 1989, 1991). While the gluconeogenesis and β-oxidation pathways initiate as directed in normal individuals, toxins from litchi fruit hinders the normal functioning of these pathways resulting in decreased glucose and energy availability to strategic organs such as brain, kidney and RBCs.

2.2. Mechanism of HGA and MCPG induced toxicity

β -oxidation of fatty acids is known to take part in generation of energy in the form of ATP along with protein and carbohydrate metabolism through generation of acetyl coenzyme A (CoA). Acetyl CoA is the initiation molecule for Krebs cycle, which in turn release energy yielding molecules. The crucial enzymes catalysing fatty acid oxidation process includes acyl-CoA dehydrogenase, enoyl-CoA hydratase, hydroxylacyl CoA dehydrogenase and thiolase. The acyl-CoA dehydrogenase catalyses the first oxidation or dehydrogenation step and uses flavin adenine dinucleotide (FAD) as its cofactor to proceed the reaction (Blake et al., 2006). The type of acyl CoA dehydrogenase to be used for catalysing the reaction depends on the size of the fatty acid metabolised. Acyl CoA dehydrogenase involved in fatty acid oxidation comprises of medium chain acyl CoA dehydrogenase (MCAD), short chain acyl CoA dehydrogenase (SCAD), long chain acyl CoA dehydrogenase (LCAD), very long chain acyl CoA dehydrogenase 1 and 2 (VLCAD) (Fagan and Palfey, 2010). Inherited genetic deficiency of MCAD in certain individuals inhibit β-oxidation involving medium chain fatty acids. If these individuals fail to eat for a longer period of time, generate symptoms of acute encephalopathy such as weakness, vomiting and seizures occur owing to unavailability of glucose. Further, due to indirect effect of fatty acid oxidation on gluconeogenesis, the latter is also suppressed and hypoglycaemia develops leading to metabolic coma (Kumar et al.,

2020). Due to similar effects and development of alike symptoms, it was thought that the toxins inhibit the acyl CoA dehydrogenase enzyme. In addition, the inhibitory activities of HGA and its lower homologue MCPG are as a result of their conversion to active metabolites mainly methylenecyclopropanylacetic acid- CoA (MCPA-CoA) and (methylenecyclopropyl)formyl-CoA (MCPF-CoA), respectively (Fig. 2) (Joskow et al., 2006). Understanding the mechanism of toxicity of these toxins has been the prime objective of a number of studies (Shrivastava et al., 2017; Melde et al., 1989; Blake et al., 2006; Feng and Patrick, 1958; Qiu et al., 2018; Li et al., 1999). The active metabolites of these toxins inhibited cofactor FAD of acyl CoA dehydrogenase irreversibly (Lai et al., 1991, 1992, 1993). Additionally, as a result of decreased fatty acid oxidation, there is increase in oxidation of residual glucose in the blood causing decline in glucose levels. Moreover, increase in glucose utilisation during glucose crisis period lead to decrease in cofactors required for gluconeogenesis. Hence, inhibition of fatty acid oxidation indirectly leads to lowering of residual glucose concentration and decline in gluconeogenesis. By virtue of the fact that fatty acid oxidation produces one molecule of acetyl CoA, this inhibition also cause decrease in acetyl CoA production and decrease in energy homeostasis. Experimental results by Qiu et al. showed decreased hepatic glucose production, depleted acetyl CoA, decreased pyruvate carboxylase and decreased ATP:ADP:AMP ratio by the toxins in experimental mice models (Qiu et al., 2018). Also, indirect inhibition of gluconeogenesis is mediated by inhibition of pyruvate carboxylase by increased accumulation of medium chain acyl-thioesters and also by reduced acetyl CoA as it is a pivotal cofactor for pyruvate carboxylase enzyme (Melde et al., 1989; Qiu et al., 2018). Moreover, HGA also has inhibitory actions on the carnitine transferase enzyme, which prevents fatty acyl CoA from entering the mitochondrial matrix further exaggerating the inhibition of β-oxidation (Li et al., 1999). Interestingly, in contrast to inhibition of enzymes reflected in most of the studies, Li et al. reported that MCPG has a reversible inhibitory activity towards enoyl-CoA hydratase (ECH) that catalyse a hydration reaction in the fatty acid oxidation process (Li et al., 1999). The impact on this pathway can also be evidenced from lower levels of its product i.e. acetoacetate (ketogenesis precursor) and 3-hydroxybutyrate (ketone body) (Melde et al., 1991; Qiu et al., 2018). It also lead to increase in concentration of fatty acids and their thio-esters conjugate leading to metabolic acidosis. Despite the fact that both of these toxins inhibit a common pathway, MCPG cause hypoketoanaemic condition while HGA cause unchanged levels of ketone bodies (Kumar et al., 2020; Qiu et al., 2018). While, the inhibitory effects of the toxins on β-oxidation of fatty acid is primarily mediated by inhibition of carnitine transferase and acyl CoA dehydrogenase the inhibition of gluconeogenesis is mediated by decreased acetyl CoA, decreased pyruvate carboxylase and decreased energy homeostasis (Fig. 3).

3. Outbreak of encephalopathy in several countries

3.1. AES in Bihar, India

Litchi growing belts of Southeast Asian countries namely India, Bangladesh and Vietnam have experienced death of a number of children during the litchi-harvesting season. The children showed symptoms of AES that coincides with encephalopathy observed in MCAD deficient individuals. As mentioned above Srivastava et al. deduced the confirmed cause of AES to be the toxins in the seeds and arils of litchi plant (Shrivastava et al., 2017). They reported that around 94% of the patients showed seizure status while 95% of the patients had mental status degradation. All the patients reported were hypoglycaemic with blood glucose level dropped to as much as 1.67 mmol/L reflecting critical hypoglycaemia. Urine analysis of more than half of the cases (64%) showed presence of metabolites of HGA and 45% showed presence of MCPG metabolites while 44% showed metabolites of both. Comparatively all the cases showed an abnormal plasma acylcarnitine values (Shrivastava et al., 2017). Interestingly the unripe fruit had significantly higher concentration of both the toxins than the ripe fruit, a characteristic similar to ackee fruit related encephalopathy in Jamaica (Blake et al., 2006). Significant factors contributing to development of illness in cases included an absence of evening meal followed by eating unripe fruit, poor nutritional and socio-economic status. Perhaps, genetic setting may also be an underlying factor. Similar outbreak was also observed in another litchi producing belt of Southeast Asia i.e. Malda district of West Bengal in June 2014. A report of epidemiological and laboratory outcome by Bandopadhaya et al. reflected that the symptoms arose during early dawn and the patients had sudden episodes of seizures followed by loss of consciousness with prominent hypoglycaemic condition. The involvement of a pathological organism caused was ruled out as the results were negative. Although specific aetiology of the development of the symptoms could not be found then and the researchers attributed JE virus to be the causative agent despite negative results for pathogenic presence (Bandyopadhyay et al., 2015). The AES has been a long-standing public health issue in several regions of Bihar, India, with seasonal epidemics over the last two decades. Agrochemicals, litchi toxins, heat stroke, and infectious agents such as Japanese encephalitis virus, Chandipura virus, and enteroviruses have all been identified as potential causes of AES. However, no definite causality has been discovered yet, and the aetiology remains unknown. Bihar's health performance on several measures has been not great, with the state ranked 20th among India's 21 largest states in terms of health indices. Therefore, strengthening primary health care, particularly in rural regions, is critical for improving outcomes and making the health system more robust to epidemics. Early access to primary care can lead to much better results. Empowering communities, increasing disease surveillance, and integrating multiple health services into the primary care system are all critical methods for preventing and responding to AES outbreaks (Kumar, 2021).

3.2. AES in Bangladesh

Dinajpur and Thakurgaon district in Bangladesh witnesses the unexplored outbreak of illness every year during May–July, which gets suppressed after onset of monsoon rains. Like Muzaffarpur in Bihar, Dinajpur and Thakurgaon districts are also major litchi producing regions that transports litchi to every part of the country. The characteristics of AES were moreover similar to the Indian outbreak that encompassed vomiting, unconsciousness, convulsions and frothy discharge from the mouth. The illness started suddenly during late night to early morning as reported by the family members of the patients. Islam et al. conducted a conventional epidemiological study of the 2012 AES outbreak taking a group of 14 affected children with a mean age of 4.7 years (Islam et al., 2017). They also confirmed that the outbreak is because of a probable toxin rather than a pathogen, although laboratory tests of serum biochemical parameters were not conducted. There was a significant correlation between onset of illness and visiting a litchi orchard or blood-relation with a litchi orchard worker. Increased ingestion of litchi, which have fallen on the ground, or litchi from diseased trees also increased the risk of illness. Apparently, workers were unaware of the quality and quantity of the pesticides used, hence the influence of pesticides in causing the illness could not be completely ruled out. In addition, a confirmed case of pesticide poisoning occurred in Bangladesh in the year 2006, showed similar symptoms such as excess sweating, frothing in mouth, seizures and coma (Martin et al., 2011).

In Bangladesh, attempts to prevent AES include vector management measures, bolstering healthcare institutions, and launching immunization campaigns against JEV. Campaigns for public health seek to increase knowledge of preventive actions including utilizing insecticide-treated nets and getting rid of mosquito breeding grounds. The World Health Organization (WHO) reported in 2019 that these measures were successful in lowering the number of AES cases in specific high-risk areas. Several socioeconomic and environmental factors contribute to

the AES burden in Bangladesh. Poor living conditions, limited access to healthcare, and inadequate vector control measures exacerbate the situation.

3.3. Ac Mong Encephalitis (AME) in Vietnam

Northern Vietnam has also experienced unidentified AES since 1999 termed as Ac Mong Encephalopathy (AME). The AME primarily affected children below the age of 15 and it occurred seasonally during May–July months. They share the same characteristics of clinical features with the reported outbreak in Bihar, India such as headache, vomiting fever and seizure onset during late night to early morning. Most patients were unconsciousness during the time of admission. The reports suggest a spatial and temporal pattern in the outbreak of AME and cultivation of litchi. Although serum biochemical levels of metabolites of MCPG and HGA, blood glucose levels were not investigated or were not available. Serum levels of inflammatory mediators such as interferon-α_2, interleukin-6 (IL-6), IL-8 and leukocytes were elevated in cerebrospinal fluid (CSF) of live patients as well as in deceased patients than controls (Paireau et al., 2012). While the viral pathological studies yielded negative results, strong correlation between litchi cultivation and number of children affected were found but due to lack of sufficient evidence against litchi fruit, it was concluded that in addition to litchi fruit, pathogenic viral aetiology could also be a probable cause for the outbreak of AME (Paireau et al., 2012).

3.4. Jamaican vomiting sickness (JVS) in Jamaica

Similar to the AES, Jamaican vomiting sickness (JVS) represent another type of encephalopathy affecting the children below the age group 10 with the first case of JVS being reported as early as 1875 (Hill, 1952; Tanaka et al., 1976). The symptoms of JVS were more or less similar to AES such as severe hypoglycaemia, vomiting, convulsions, coma and death. The autopsies of deceased patient's reports decreased glycogen reserves of liver followed by findings of lipid droplets in liver and kidney (Hill et al., 1955). In contrast to AES, the cause of JVS is not litchi but ackee fruit belonging to the litchi fruit family i.e. Sapindaceae. The ackee fruit has been a favourite in the diet of Jamaican people with the arils being the major part of the fruit eaten (Fig. 1). With the onset of JVS during the ackee harvesting months i.e. December to March, the correlation of onset of JVS and ackee became clarified (Joskow et al., 2006). But following the isolation of HGA and HGB from seeds and arils of ackee by Hassal and Reyle in 1955 and symptoms of JVS being similar to hypoglycin A toxicity, it was well interpreted that HGA and HGB can be the significant toxic substances causing the development of JVS (Tanaka et al., 1976; Hassall and Reyle, 1955). It was further reported through investigation of the ackee fruit that there is significant difference in the concentration of HGA and HGB present in unripe and ripe fruit with unripe fruit containing 100 timer higher HGA and HGB than ripe fruit (Golden et al., 2002). Ripening reduces the toxicity from 1000 parts per million (ppm) to 0.1 ppm (BROWN et al., 1991). Ingestion of cooked ripe fruit in significantly nontoxic than ingestion of unripe and cooked unripe fruit. The HGA and HGB acts in a similar manner i.e. they inhibit β-oxidation fatty acid by acting against acyl dehydrogenase and carnitine transferase. In addition, they also block malate transportation from mitochondrial matrix to outer mitochondria and thereby inhibits gluconeogenesis directly as well (Joskow et al., 2006). Biochemical investigation by researchers have reflected finding of short chain fatty acids such as propanoic acid, carnitine, urinary dicarboxylic acids and MCPA, the toxic metabolite of HGA in significant quantities in the urine (Joskow et al., 2006; Tanaka et al., 1976). However, they also reported that the metabolites are short-lived after action, due to which they are rapidly excreted from the body (Tanaka et al., 1976). In addition to these findings, any other association such as ingestion of other food items, polluted water intoxication or association of any other illness were not found (Joskow et al., 2006). Hence, the relation between unripe ackee fruit ingestion and onset of JVS became clear.

4. Inter-relationship between MCAD deficiency, AES, AME and JVS

The fatty acid oxidation or the β-oxidation of fatty acids is the primary metabolism pathway for fats, thereby producing energy. Acyl dehydrogenase enzyme is one of the mainstay metabolising enzyme catalysing a dehydrogenation reaction to the medium chain, long chain or short chain fatty acid; thereby promoting it for further metabolism. Inhibition of acyl dehydrogenase enzyme, irrespective of the type i.e. MCAD, SCAD, LCAD or VLCAD has long lasting consequences not only on fat metabolism, but also on amino acid and carbohydrate metabolism as well. This include increased serum concentration of fatty acids leading to metabolic acidosis, lowering of ketone bodies production, decreased glucose concentration, decreased concentration of gluconeogenesis cofactors, increased carnitine in the serum and urine (Melde et al., 1989, 1991; Qiu et al., 2018; Li et al., 1999). The resultant of these

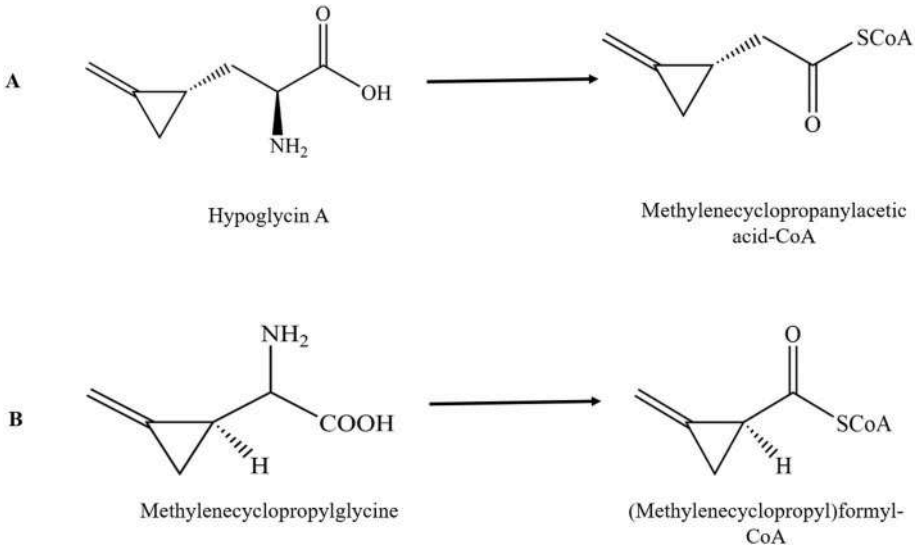

Fig. 2. HGA and MCPG are first converted to their respective metabolites such as MCPA-CoA and MCPF-CoA which act on their target enzymes to inhibit fatty acid oxidation.

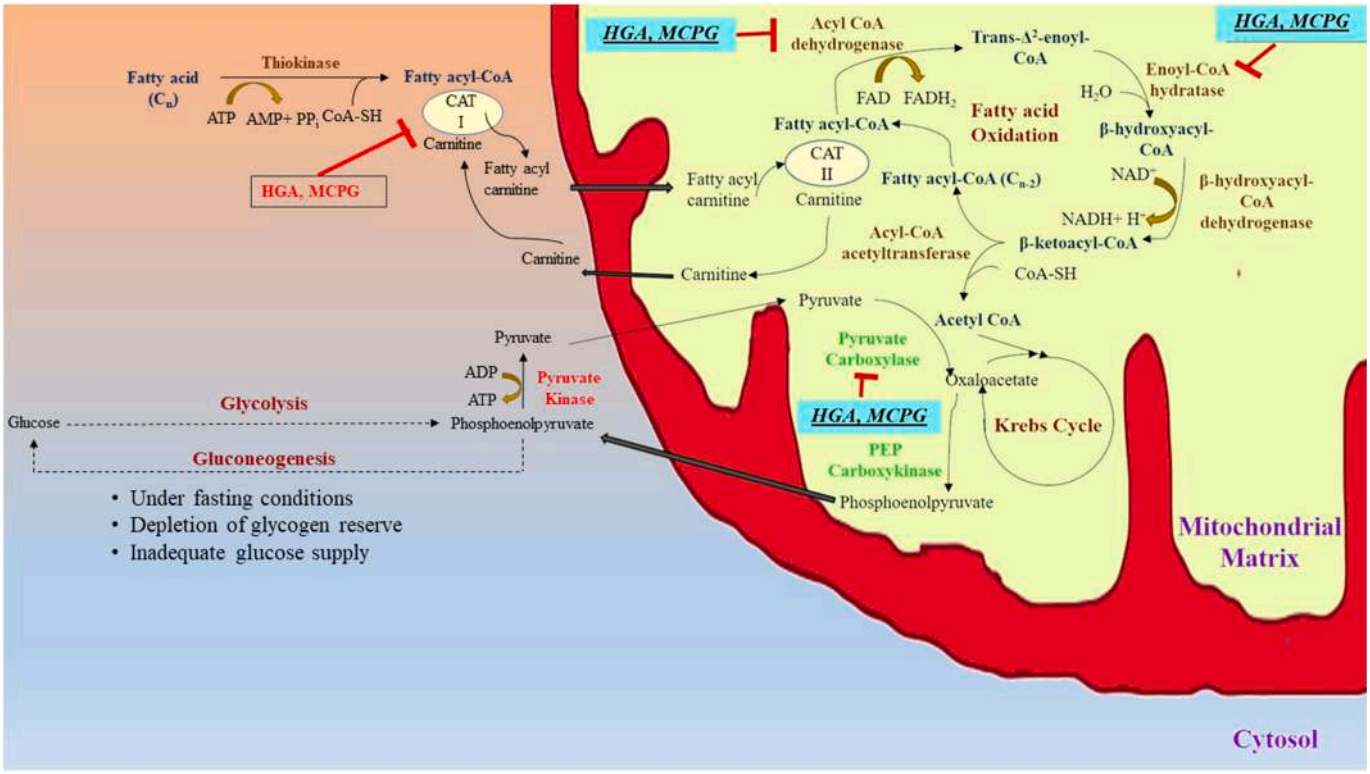

Fig. 3. Inhibitory effects of HGA and MCPG on Fatty acid oxidation and its impact on gluconeogenesis. The process of fatty acid oxidation starts in the cytosol. 1) Thiokinase converts a molecule of fatty acid (C_n) to fatty acyl-CoA by the expenditure of ATP and CoA-SH as a cofactor. 2) CAT I catalyses formation of fatty acyl-carnitine conjugate facilitating mitochondrial membrane transport of fatty acyl-CoA. 3) Once inside the matrix, fatty acyl CoA is regenerated by CAT II. 4) Acyl-CoA dehydrogenase oxidises fatty acyl CoA to trans-Δ^2-enoyl-CoA where FAD acts as an oxidising agent. 5) Trans-Δ^2-enoyl-CoA undergoes hydration by ECH with the help of H_2O forming β-hydroxyacyl-CoA. 6) It is followed by β-hydroxyacyl-CoA dehydrogenase oxidising β-hydroxyacyl-CoA to β-ketoacyl-CoA where NAD^+ is reduced to $NADH+H^+$. 7) The enzyme thiolase acts on β-ketoacyl-CoA to form a molecule of acetyl CoA with CoA-SH as a cofactor. The remaining fatty acid molecule (C_{n-2}) repeatedly undergoes same process until carbon atoms are exhausted. The generated acetyl CoA enter the Krebs cycle by conjugation with oxaloacetate further synthesising energy yielding molecules. Under fasting conditions, gluconeogenesis is the primary glucose production pathway that deals with synthesis of glucose from pyruvate through oxaloacetate. A) Pyruvate carboxylase converts pyruvate to oxaloacetate with acetyl CoA as a cofactor and B) oxaloacetate is converted to phosphoenolpyruvate by pyruvate carboxykinase. Glucose is regenerated from phosphoenolpyruvate through a similar reaction as that of glycolysis. The marked enzymes such as CAT, acyl CoA dehydrogenase, ECH and pyruvate carboxylase are the direct and indirect inhibitory targets of the toxins.

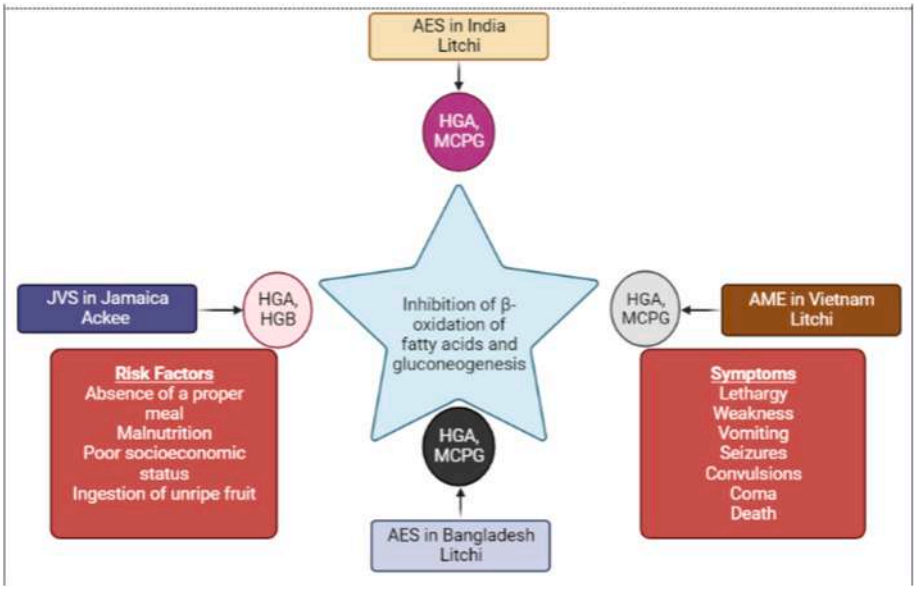

Fig. 4. The inter-relationship between world-wide incidence of AES, AME and JVS.

consequences lead to development of symptoms of AES, AME and JVS such as lethargy, vomiting, weakness, fever, seizures, convulsions, coma and death (Fig. 4). The development of these symptoms is further exaggerated by absence of an evening meal. The onset of symptoms occurs at late midnight and continues till morning or until any therapeutic measure is taken. Deficiency of MCAD enzyme greatly withheld the oxidation of medium chain fatty acids, its role in development of AES cannot be neglected as the medium chain fatty acids, in addition to short chain and long chain fatty acids, constitute energy precursors in fasting conditions. However, MCAD deficiency does not account for ingestion of litchi fruit or ackee fruit, whereas AES, AME and JVS are resultant of litchi and ackee fruit toxicity respectively. While, development of AES and AME are a resultant of MCPG toxicity than HGA, development of JVS is vice-versa. Researchers have investigated several mechanism of action of these toxicities, inhibition of carnitine transferase, acyl CoA dehydrogenase, malate transporter are identified as most probable sites of actions (Joskow et al., 2006).

5. Therapeutic and preventive measures for AES

Management of AES is principally done by infusion of 10% dextrose to meet the energy demands and stabilise mental and physical functions (Bandyopadhyay et al., 2015; Shrivastava et al., 2017; Das et al., 2015; Joskow et al., 2006; Biswas, 2012). In addition to dextrose, anti-convulsants, mannitol for oedema-like conditions are the secondary therapeutic measures administered (Bandyopadhyay et al., 2015). Furthermore, animal studies have also suggested use of L-carnitine, glycine, clofibrate have suppressed hypoglycaemia induced by HGA (Joskow et al., 2006; Marley and Sherratt, 1973; Sherratt and Al-Bassam, 1976). In spite of therapeutic measures, prevention of litchi fruit toxicity can lower of incidence of AES. Genetic intervention has a greater role to play in plant breeding techniques. Hence, manipulating the genetic structure to selectively breed comparatively non-toxic litchi plant is a primary preventive measure. Concentration of the non-protein amino acids in litchi fruit arils and seeds is determined by their specific genes (Velisek et al., 2006). Differential concentration in seeds and arils arises due to differential gene content in the arils and seeds. Manipulating with the genes responsible for producing these amino acids is the primary focus for transgenic development of non-toxic litchi plants. Gene sequencing has been carried out which will help in developing transgenic variety of litchi fruit having negligible content of the toxins (Das et al., 2016). The development of transgenic plants having lesser quantity of the toxins without compromising the yield and deliciousness of the fruit is one of the primary preventive approach to prevent these outbreaks. Application of viral and non-viral vectors for gene delivery has a significant role in gene or protein level inhibition of toxins. Secondly, all the trees in the litchi orchard should be analysed and the trees having minimal concentration of toxins as reported by Srivastava et al., should be tagged (Shrivastava et al., 2017; Isenberg et al., 2016). Fruits of the untagged plants should not be consumed by children owing to its harmful effects. Thirdly, the Parents and caretakers, who work in litchi orchard must restrict the children from overeating the litchi fruit. They also must ensure that the children should go to sleep after having a proper dinner as its effects were discussed in earlier sections (Shrivastava et al., 2017). In addition, the children must be advised not to eat unripe fruits, fruits dropped on the ground and the fruits having bite marks of any animals or birds. Creating awareness among communities living near litchi orchards and educating them on probable toxicity of litchi fruit would also help to alleviate the incidence of AES. Skipping the evening meal has been identified as a significant risk factor for the development of toxic encephalopathy caused by litchi consumption. Ensuring that children, particularly those from remote areas, have an evening meal can assist to prevent these events. Fast glucose correction via intravenous glucose infusion is required in suspected cases of litchi toxin illness. This can help manage the hypoglycemia produced by toxin exposure. In summary, the major preventative and therapeutic interventions for AES associated with litchi consumption are limiting litchi intake, providing appropriate nutrition, adopting quick glycemic correction, enhancing the monitoring, improving primary health care access, and empowering communities.

6. What we know so far

Being a delicious fruit, litchi (*Litchi sinensis*) belonging to family Sapindaceae is the prime culprit behind development of encephalitis syndrome in many parts of Southeast Asia namely India, Bangladesh and Vietnam. A fruit similar to litchi i.e. ackee is reported to develop similar encephalitis syndrome in Jamaica as well. AES occurs due to unavailability of glucose to the brain to produce energy for the neuronal cells to act. Brain can only metabolise glucose for meeting its energy needs. Under fasting conditions, when supply of glucose ceases to the brain, then body uses up the stored glycogen by the process of glycogenolysis to supply glucose to organs having higher demand such as brain and RBCs. Further, following glycogen reserve depletion, glucose is synthesised from non-carbohydrate sources such as amino acids and fats through a process called glyconeogenesis. However, inhibition of gluconeogenesis completely ceases glycose supply to the brain that leads to development of encephalitis like symptoms called AES. The symptoms include vomiting, weakness, lethargy, fever, seizures, frothing discharge and coma. Litchi fruit has a significant role in development of AES owing to the presence of toxins such as HGA and MCPG. Comparatively higher amount of litchi fruit ingestion and not having a dinner are the primary risk factors involved. Children in the litchi harvesting areas ingest a higher amount of litchi in the afternoon and go to bed at night without having an urge to have dinner. The symptoms generally start at late night and continue until medical supervision. The toxins ingested starts to get metabolised to MCPA and MCPF respectively, thereby they inhibit several stages of β-oxidation of fatty acids which then indirectly inhibit gluconeogenesis and energy production. They inhibit acyl dehydrogenase, carnitine transferase, malate transferase and enoyl CoA hydratase. Inhibition of fatty acid oxidation has an indirect inhibitory effect on gluconeogenesis leading to symptoms of AES. Increase concentration of medium chain and short chain fatty acids also lead to metabolic acidosis which probably lead to metabolic coma. Constitutively they develop the AES. AES has also been observed and reported in Vietnam, Bangladesh and Jamaica where it was known as AME, AES and JVS, respectively. Different researchers have attributed to various causes, however established common link is found to be excessive litchi and ackee fruit ingestion. Srivastav et al. in the year 2017 confirmed the role of these non-protein toxins through epidemiological study based on 2014 outbreak in Bihar, India. Development of transgenic plant with suppression of gene responsible for the toxins expression, selectively tagging dangerous litchi plant having comparatively higher toxins values, ensuring the children do not ingest a lot of litchi and they should have proper dinner before going to sleep should collectively reduce the incidence of AES. While, important phytoconstituents, either isolated molecules or extracted compounds from several medicinal and herbal plants have a great pharmacological value with few of them being researched in our own research group, care should be taken to study its toxicological profile and aspects (Thatikonda et al., 2020; Gurram et al., 2022; Sayed et al., 2019).

7. Future directions

Future objectives deals with strong and repetitive attempt to deal eradicate litchi fruit induced AES. It includes elucidation of specific biochemical pathways affected by HGA and MCPG through metabolomics and proteomics. Prevention of litchi fruit AES also involves carrying out nutritional interventions with special dietary supplements and carrying out community-based nutrition programs to prevent hypoglycaemia and optimise treatment protocols including rapid glucose administration and supportive care. Development of HGA and

MCPG antagonist, which can neutralise the actions of the toxins inside the human body. Further development of genetic intervention technique such as siRNA, shRNA, novel genes in the plants to inhibit the expression of the toxins should be another future prospective. Genetic intervention can play a major part in reducing the incidence of AES as it will inhibit the HGA and MCPG *de novo*. Designated litchi plantation area should be marked of the presence of the toxins such that researchers can gain insight to the climatic and environmental conditions controlling the synthesis of the toxins. Toxicological investigations should detect the maximum tolerated dose (MTD) such that the threshold amount of litchi ingestion should be preserved thereby avoiding excessive ingestion. Moreover, proper consumption guidelines for this delicious fruit for both children and adults may also be considered with emphasis on not having meal at nights.

8. Conclusion

Consumption of litchi by malnourished children followed by skipping the evening dinner has led to the development of AES which is caused by HGA and MCPG present in litchi plant. They inhibit several energy generating processes such as fatty acid oxidation and gluconeogenesis. The symptoms generally included vomiting, lethargy, fever, weakness, convulsions and coma in untreated conditions that starts at late night and continue until medical assistance is provided. The AES outbreaks are observed in India, Bangladesh, Vietnam and Jamaica. Advising the children not to ingest a lot of litchi fruit and going to bed only after a dinner in addition to genetic intervention described above can prove beneficial in avoiding the occurrence of AES.

Ethical statement

1) This material is the authors' own original work, which has not been previously published elsewhere.
2) The paper is not currently being considered for publication elsewhere.
3) The paper reflects the authors' own research and analysis in a truthful and complete manner.
4) The paper properly credits the meaningful contributions of co-authors and co-researchers.
5) The results are appropriately placed in the context of prior and existing research.
6) All sources used are properly disclosed.
7) All authors have been personally and actively involved in substantial work leading to the paper, and will take public responsibility for its content.

List of Abbreviations

AES	Acute Encephalopathy Syndrome
HGA	Hypoglycine A
MCPG	Methylenecyclopropylglycine
JEV	Japanese Encephalitis Virus
NCDC	National Centre For Disease Control
DCD	USA Centre For Disease Control
HGB	Hypoglycin B
FAD	Flavin Adenine Dinucleotide
MCAD	Medium Chain Acyl Coa Dehydrogenase
SCAD	Short Chain Acyl Coa Dehydrogenase
LCAD	Long Chain Acyl Coa Dehydrogenase
VLCAD	Very Long Chain Acyl Coa Dehydrogenase
MCPF-CoA	(Methylenecyclopropyl)Formyl-Coa
ECH	Enoyl-Coa Hydratase
AME	Ac Mong Encephalitis
JVS	Jamaican Vomiting Sickness
MTD	Maximum Tolerated Dose

CRediT authorship contribution statement

Biswajit Panda: Writing – review & editing, Writing – original draft, Validation, Formal analysis, Data curation, Conceptualization. **Alfiya Momin:** Writing – review & editing, Validation, Formal analysis. **Geetanjali Devabattula:** Writing – review & editing, Validation, Formal analysis. **Ravinder Doneti:** Writing – review & editing, Investigation. **Aarti Khandwaye:** Writing – review & editing, Investigation. **Chandraiah Godugu:** Writing – review & editing, Writing – original draft, Validation, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

Data availability

No data was used for the research described in the article.

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