Bull. of Egyp. Soc. Physiol. Sci.

(Official Journal of Egyptian Society for Physiological Sciences)

(pISSN: 1110-0842; eISSN: 2356-9514)

Selective Serotonin Reuptake Inhibitors Targeting Autophagy/Mitophagy Pathways in an asthma model.

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Submit Date :03 Oct. 2025
Revised Date :21Oct. 2025
Accept Date :26Oct. 2025

Keywords

- Ovalbumin
- airway hyper-responsiveness,
- mTOR
- SSRIs

Abstract

Background: Chronic airway inflammation, remodeling, and hyperresponsiveness are hallmarks of neutrophilic asthma. Both its pathogenesis and severity have been related to mitophagy and autophagy. Selective serotonin reuptake inhibitors (SSRIs), which are antidepressants, have been shown in several experiments to have anti-inflammatory properties. Consequently, the purpose of this study was to examine how two SSRIs—fluoxetine and citalopram—affect autophagy and mitophagy in neutrophilic asthma and how they differ from the glucocorticoid dexamethasone as a reference anti-inflammatory medication in terms of their effects on airway inflammation. **Methods:** The asthma model was established in mice using ovalbumin (OVA) sensitization and subsequent OVA challenges. The administration of citalopram, fluoxetine, and dexamethasone preceded each challenge. In addition to estimating total and differential cell counts in bronchoalveolar lavage (BAL) fluid, airway hyperresponsiveness was assessed before sensitization and following the last challenge. IgE, inflammatory markers, oxidant, antioxidant parameters, and p-mTOR were all measured. Additionally, the expression of autophagy and mitophagy markers was determined, and histopathological changes were evaluated. **Results:** SSRIs substantially improved airway hyperresponsiveness and reduced BAL fluid neutrophilic count and inflammatory cell infiltration, goblet cell hyperplasia, and airway fibrosis in histological sections compared to dexamethasone. SSRIs also significantly reduced IgE, inflammatory markers, and oxidative stress compared to dexamethasone. In addition, the expression of proteins linked to autophagy and mitophagy was inhibited, likely due to the elevated expression of p-mTOR.**Conclusion:** SSRIs can attenuate airway inflammation and hyperresponsiveness by suppressing autophagy and mitophagy, possibly through their modulatory effect on p-mTOR.

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Introduction

Bronchial asthma is a chronic condition defined by airway remodeling, airway hyperresponsiveness (AHR), and inflammation caused by cellular and molecular changes (1).

Airway remodeling, which involves structural changes in the airway wall such as a thickened basement membrane, smooth muscle hypertrophy, collagen deposition, and subepithelial fibrosis, is a key feature of asthma. Fibrosis and remodeling are pathogenic features that are associated with disease severity and resistance to therapy. Current asthma medications are confined to addressing inflammation and AHR. Research has established thatthat airway remodeling and phenotypic changes in neutrophilic asthma, along with dysregulation of mitophagy and autophagy, have been linked (2). Consequently, pharmacological modulation of autophagy and mitophagy may be an acceptable approach for resistant asthma. (3).

Autophagy is a homeostatic process that is observed in all eukaryotic cells. During this process, damaged proteins or organelles are confined in double-membrane autophagosomes, where they combine with a lysosome to facilitate the destruction and recycling of the cytoplasmic components to synthesize new proteins. The peptides produced serve as a source of antigens for CD4+ T lymphocytes, which are essential for initiating subsequent alterations linked to the pathogenesis of asthma (2-4). Autophagy-related changes can also impact profibrotic cytokines, like transforming growth factor (TGF)-β, which can activate smooth muscle cells and myofibroblasts. This, in turn, can stimulate the release of fibrogenicextracellular matrix (ECM) proteins, which can cause airway remodeling and fibrosis (3).

Apart from autophagy, mitophagy—selective autophagy of dysfunctional mitochondria—has also been associated with the primary phenotypic alterations related to asthma. Mitophagy is a natural defense mechanism of the body that supports cell survival. It is an essential process that maintains the quality of mitochondria and the cellular homeostasis. However, patients with asthma can experience negative consequences if this process is not appropriately regulated. It can increase oxidative stress, airway hyperresponsiveness, epithelial barrier dysfunction, fibroblast activation, inflammation, and steroid resistance (5-7). Additionally, the disruption of mitochondrial degradation by mitophagy may result in the build-up of fragmented mitochondria and the initiation of mitochondrial apoptosis. The PTEN-induced kinase1 (PINK)/Parkin pathway is triggered in the nucleus by the increased production of reactive oxygen species (ROS) associated with allergens in individuals with asthma. This enhances the process of mitophagy by promoting the transcription of BNIP33/NIX, LC3/BNIP3, and p62 (2, 7). Therefore, monitoring and regulating mitophagy is important to prevent these detrimental effects.

The regulation of autophagy by the phosphorylated mammalian target of rapamycin (p-mTOR) has been demonstrated to have a significant part in the pathogenesis of asthma. The inhibition of the mTOR pathway is linked to autophagy activation, which increases the synthesis of interleukin (IL)-4, IL-12, IL-23, IL-6, and tumor necrosis factor (TNF)-α and decreases the production of IL-10 and other anti-

inflammatory mediators (8). Conversely, silent mating type information regulation 2 homolog 1 (SIRT1) signaling negatively regulates mTOR signaling. Consequently, in asthmatics, autophagy is modulated by the interaction between the mTOR and SIRT1 signaling pathways (9). While it is difficult to address how autophagy and mitophagy activation/inhibition affect inflammatory mediators, pharmacologically targeting one or both processes may benefit severe asthma (2).

Patients suffering from depression frequently receive therapy with selective serotonin reuptake inhibitors (SSRIs), which include citalopram and fluoxetine. SSRIs are well tolerated drugs with few side effects including gastrointestinal disturbances, headache, emotional agitation, sedation and male sexual dysfunction(10, 11). Patients with asthma often experience the latter and vice versa (12). Research has shown that the central and peripheral immune systems may be activated due to a shortage of monoamine neurotransmitters (13). 5-hydroxytryptamine (5-HT) may affect the innate and adaptive immune responses by influencing immune cell activation, migration, and recruitment, blocking nuclear factor kappa B (NF-KB) stimulation, reducing the release of inflammatory mediators, and raising anti-inflammatory cytokines. Additionally, 5-HT shields cells from the damaging effects of oxidative stress. Moreover, SSRIs showed anti-inflammatory and antioxidant actions in previous asthma models (14, 15). Thus, the immune system and inflammation may be modulated and controlled by pharmacological regulation of the serotonergic system (15). Additionally, SSRIs-mediated modulation of the autophagy/mitophagy pathways in different tissues and pathologies has been recently the focus of further research (11,16). However, to our knowledge, the effect of SSRIs on autophagy and mitophagy has not yet been studied in a neutrophilic asthma model.

Thus, this study aimed to examine the impact of fluoxetine and citalopram on autophagy and mitophagy and their potential to regulate inflammation in a mouse model of acute allergic airway inflammation induced by ovalbumin (OVA).

2. MATERIALS AND METHODS

2.1. Statement of ethics

The Alexandria Faculty of Medicine's Ethics Committee approved all the experimental protocols used in this study (IRB No. 00012098-FWA No. 00018699-Protocol serial No. 0305587), and the procedures followed both the institution's policies and the guide of US for the Care and Use of Animals.

2.2. Animals

Forty male CD1 mice, six weeks old and weighing 20 ±2 g, were obtained from the Animal House of Medical Physiology Department at Alexandria University in Egypt. Before the commencement of the experiments, mice were acclimatized for one week and housed in settings free of pathogens and ovalbumin (OVA). These conditions included a 12-hour light-dark cycle, a 22 ± 2 °C temperature, and a 45–55 percent humidity. Five groups of mice with normal lung function—each with eight mice—were randomly assigned into normal control (NC), OVA-induced asthma, OVA+ dexamethasone, OVA+ fluoxetine, and OVA + citalopram.

2.3. Sensitization and challenge

Mice from every group—aside from the NC group—were exposed to the following stimuli and challenges: On days 0 and 14, 20 µg OVA (OVA, grade V; Sigma-Aldrich, St. Louis, MO, USA) was adsorbed in 200 µL PBS with 1 mg of aluminum hydroxide (Imject Alum; Pierce, Rockford, IL, USA) and injected intraperitoneally (IP). Then, an ultrasonic nebulizer (NE-U22, Omron, Kyoto, Japan) was utilized for 20 minutes on days 21–23. During this period, mice were placed in a 50 × 30 × 40 cm3 plastic box and exposed to OVA aerosols 1% w/v (10 mg/ml) in PBS for the initial inhalational challenge. On days 30-32, a secondary inhalational challenge was followed using 1% w/v OVA in PBS for 20 minutes. In contrast, the NC group was sensitized with 200 µL PBS and challenged by PBS aerosols (1,17). (**Figure 1**)

2.4. Treatment protocol

Half an hour before the primary and secondary OVA challenges, The following medications were dissolved in 0.9% saline and administered intragastrically to each mouse according to its treatment group: 1 mg/kg dexamethasone (18) (Deltasone tablet, The Nile Co. for pharmaceutical & Chemical Ind, Egypt), 10 mg/kg fluoxetine (16) (Philozac capsule, Amoun pharmaceutical Co.S.A.E, Egypt), and 10 mg/kg citalopram(19) (Citalo tablet, Delta pharmaceutical Ind, Egypt). On the same schedule, 0.5 ml/20 g body weight (BW) of 0.9% saline was given orally to control mice (normal and OVA). (**Figure 1**)

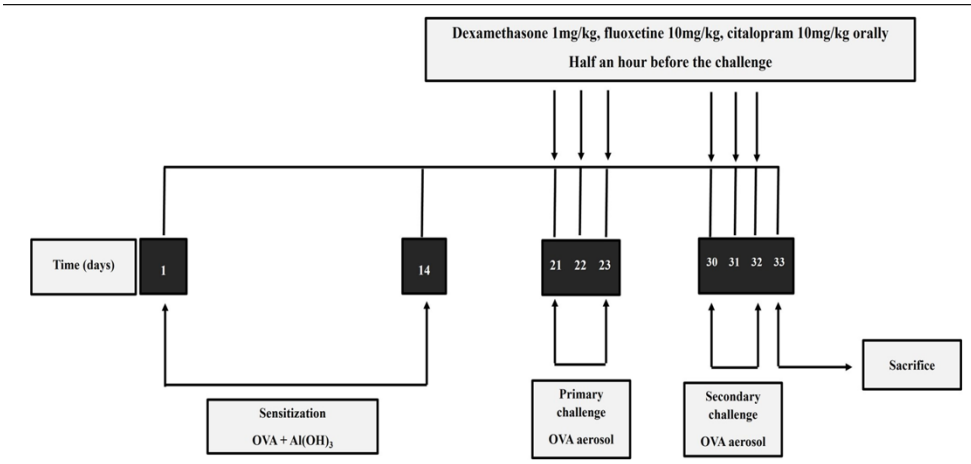

Figure 1: Administration protocol of dexamethasone, fluoxetine, and citalopram to mice sensitized and challenged with OVA.

2.5. Pulmonary function tests

Pulmonary functions were evaluated in unrestrained, non-anesthetized mice, using whole-body plethysmography. Mice were put inside transparent plastic plethysmograph chambers with an internal diameter of 10.2 cm and an external length of 5.0 cm, with a final capacity of 150 mL. They were left for 60 to 90 minutes to get used to the chamber's environment (20).The pneumotachometer MLT1L (Lab chart 8, AD Instruments, Castle Hill, NSW, Australia) is linked to two inlets. The first inlet takes in a gas mixture (N2, 21% O2, and/or oral CO2), and the second inlet is used as an exit. The chamber stayed closed, and the airflow was stopped for two minutes throughout each recording. The program Power

Lab Chart 8 was used to examine the recordings (21). During every minute ventilation volume (VE) measurement, a volume calibration was conducted by infusing the chamber with an air volume of 100 µL. Before sensitization, lung functions were assessed, and any mice with abnormal breathing patterns in the form of a respiratory rate more than 400 or less than 200 breaths/minute, minute respiratory volume less than 2.5 ml/gm/min, and forced expiratory volume 1% (FEV1%) less than 75% indicating any existing pulmonary disease were excluded from the study. Whole body plethysmography was also performed 24 hours after the last OVA challenge to detect airway hyperresponsiveness (AHR) to OVA and to be compared with the initial baseline data.

2.6. Scarification of mice, samples, and biochemical analysis

Mice were put under anesthesiawith ketamine/xylazine cocktail, and blood was drawn from the abdominal aorta into plain tubes following the pulmonary function test. Tubes were centrifuged, and serum was refrigerated at -80°C until analysis after the blood was allowed to coagulate for 30 minutes at room temperature.

2.6.1. Bronchoalveolar lavage and lung histology

Tracheal cannulation was used to obtain bronchoalveolar lavage (BAL)using 2 ml of ice-cold PBS. After centrifuging BALF at 500 × g for 10 min at 4 °C, the supernatants were stored at -80°C until cytokine analysis was performed. A hemocytometer was used to measure the total leukocytic counts in the resuspended cell pellet, and light microscopy was used to perform cell differentiation on cytospin slides stained with Wright-Giemsa.

Afterward, the right lung was removed following a mid-ventral laparotomy and the closure of the right major bronchus. The lung was then cleaned with ice-cold phosphate-buffered saline (PBS) and weighed to facilitate homogenization and biochemical analysis. Then, 1.5 ml of formalin was administered into the animal's left lung through the endotracheal tube. Once the left lung was dissected, it was fixed in 10% neutral buffered formalin, immersed in paraffin wax blocks, and sliced at a thickness of 5 µm in the coronal plane. Sections were stained with hematoxylin and eosin (H&E) to identify inflammatory cells, periodic acid Schiff (PAS) to identify cells secreting mucus, and Masson's trichrome (MT) to measure the amount of collagen deposition after deparaffinization. An Olympus CX23 light microscope was used to examine each segment.

The degree of inflammation, mucus production, and collagen deposition were evaluated using semi-quantitative scores based on a modified version of a previously published approach (22). Peri-bronchial inflammation was measured and assigned a score of 0 (absent or rare), 1 (mild: localized), 2 (moderate: < 5 layers of cells), and 3 (severe: > 5 layers of cells). PAS-positive cell percentage was graded as follows: 0 (absent); 1 (mild:1-30%); 2 (moderate:31-60%); and 3 (severe>60%). A score of 0 (absent), 1 (mild: 1-30%), 2 (moderate: 31-60%), and 3 (severe: >60%) was assigned to the proportion of peri-bronchial collagen deposition. The sum of the three scores produced a final score of 0–9. While performing the histopathological evaluation, the examining pathologist was blind to the group.

2.6.2. Homogenization of lung tissue and estimation of total protein

To prepare the right lung for assays, it was homogenized using a SCILOGEX D160 Homogenizer on ice in lysis buffer (150 mM NaCl, 10 mM Tris solution, 1% Triton X-100, pH 7.4) containing protease inhibitor (Sigma-Aldrich, St. Louis, MO, USA). The supernatant was then collected after being centrifuged for 10 minutes at 4°C at 13,000 rpm. The lung homogenate's total protein content was then ascertained using Lowry's method (23).

2.6.3. Assay for lipid peroxidation

The measurement of lipid peroxidation in lung homogenate was consistent with that previously reported by Ohkawaet al. (24). After mixing the lung homogenate with 1.5 mL of 20% acetic acid (AcOH), 1.5 mL of thiobarbituric acid (TBA), and 0.2 mL of sodium dodecyl sulfate (SDS), the mixture was heated for 60 minutes at 100°C. Following cooling, 5 mL of n-butanol-pyridine (15:1) and 1 mL of distilled water were added, and the mixture was vortexed. The organic layer was separated by centrifugation at 1200 g for 10 minutes, and an ELISA plate reader was used to detect the absorbance at 532 nm. When coupled with thiobarbituric acid, malondialdehyde (MDA), an end product of lipid peroxidation, created a reactive molecule of pink chromogen– TBA.

2.6.4. Assessment of Glutathione (GSH)

The Jollowet al. (25) technique was used to assay glutathione (GSH). To summarize, 100 µL of the sample, distilled water, or GSH were combined with 100 µL of 4% sulphosalicylic acid (SSA) and stored at 4°C for at least one hour. The mixture was centrifuged at 1200 g for ten minutes at 4°C. Subsequently, 2.7 mL of 0.1 M pH 7.4 phosphate buffer and 0.2 mL 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) were added to the supernatant and left for 5 minutes. The resultant yellow color was seen at 412 nanometers. Using standard GSH, a standard curve was created. Ultimately, the GSH content per milligram of protein was determined.

2.6.5. Superoxide Dismutase measurement

The technique described by Rai et al. (26) was used to evaluate the activity of superoxide dismutase (SOD). 100 µL of supernatant, 1.2 mL of sodium pyrophosphate (SPP) buffer (pH 8.3; 0.052 M), 0.3 mL of nitro blue tetrazolium (NBT) (300 m), 0.1 mL of phenazine methosulphate (PMS) (186 m), and 0.2 mL of NADH (750 m) were all added in the test solution. NADH triggered a reaction, followed by adding 0.1 mL of glacial acetic acid to stop the response after it had been incubated for 90 seconds at 30 °C. Then, n-butanol (4.0 mL) was added to the reacted mixture and vigorously agitated. SOD concentration was expressed in units per milligram of protein, and the color intensity of the chromogen in butanol was determined using spectrophotometry at a wavelength of 560 nm.

2.6.6. Immunoblot

The Bradford method (27) (Coomassie Plus Protein Assay Reagent-Thermo Scientific, Rockford, IL, USA) was used to estimate the concentration of protein in supernatants. Fifty micrograms of protein lysates from each lung sample were mixed with 2X loading buffer (130 mM Tris-HCl, pH 8.0, 30% (v/v) Glycerol, 4.6% (w/v) SDS, 0.02% Bromophenol blue, and 2% DTT), which was boiled for five minutes before cooling at 4°C. Samples were separated and processed at 120 V on SDS-PAGE mini-gel. Then, using the Mini-Protean Tetra Cell and Mini Trans-Blot systems from Bio-Rad in Hercules, California, the proteins were transferred to polyvinylidene difluoride membranes (Roche,

Mannheim, Germany). Afterward, the membrane was blocked with a blocking buffer (TBST containing 5% nonfat dry milk) for an hour at room temperature. It was then washed three times with TBST (50 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween-20), and primary antibodies diluted in Tris-buffered saline containing Tween 20 (TBST) were incubated overnight. The membrane was incubated with the secondary antibody for an hour at room temperature following three TBST washes. Following three TBST washes, blots were detected utilizing the ChemiDoc MP Imaging System (Bio-Rad) and a chemiluminescence western blotting kit (Luminate Forte Western HRP Substrate, Millipore). Lastly, Image Lab 6.1 software (Bio-Rad) was used to quantify the bands. All utilized compounds were sourced from Sigma-Aldrich Co. (USA). The following antibodies were bought from Cell Signaling Technology, USA: LC3B (3868), p62 (39786), PINK1 (6946), PRKN (32833), phospho-mTOR (Ser2448) (5536), β-actin (4970), and peroxidase-conjugated anti-rabbit antibody (7074).

2.6.7. Enzyme-Linked Immunosorbent Assay

Following the manufacturer's instructions, a sandwich enzyme-linked immunosorbent assay (ELISA) was used to measure Interleukin (IL)-4 and tumor necrosis factor (TNF)-α in the BALF supernatant, p-mTOR (NOVA, Beijing, China) levels in the right lung homogenates, and total IgE in the serum (Fine test, Wuhan, China) of mice in each group. For IL-4, the sensitivities were 0.5 pg/ml, TNF-α, 2.2 pg/ml, p-mTOR, 0.5 ng/L, and total IgE, 1.875 ng/ml. Briefly, standards and samples were pipetted into wells and allowed to incubate. Detective antibodies were added and allowed to incubate following a wash. HRP conjugate and subsequent substrate solution incubation were used to visualize the immunological activity. Once the reaction was stopped, a microplate reader was used to measure the values at 450 nm.

2.7. Data analysis

Statistical analysis was finished with SPSS 25 (SPSS Inc., USA). The results are displayed as mean ± standard deviation (SD). The intergroup and intragroup comparisons were performed using a one-way analysis of variance (ANOVA) and Tukey's multiple comparisons. The differences were deemed statistically significant if the P-value was less than 0.05 (28).

3. Results

3.1. Pulmonary function

Animals with normal lung function were randomly assigned to five groups before sensitization, whereas abnormal animals were kept out. After the OVA challenge, the mice's regular breathing pattern significantly changed to a prominent, characteristic pattern of increased respiratory rate ($p<0.05$; Figure 2A). At the same time, the tidal volume and minute ventilation were reduced considerably ($p<0.05$; Figure 2B&C). The forced vital capacity (FVC), FEV1% (forced expiratory volume 1%), and PEF (peak expiratory flow) were all reduced by about 50% ($p<0.05$; Figure 2D-F), while peak inspiratory flow (PIF) demonstrated no significant difference ($p>0.05$; Figure 2G). Meanwhile, there was a significant decrease in the mean expiratory flow (MEF) ($p<0.05$; Figure 2H), an indicator of bronchoconstriction. Moreover, the time of expiration (Te) was nearly doubled ($p<0.05$; Fig 2I). However, the time of inspiration (Ti) did not change from normal ($p>0.05$; Figure 2J). Dexamethasone, fluoxetine, and citalopram all significantly attenuated the airway obstructive effects of OVA as evidenced by increased PEF and FVC to be insignificantly different from normal control group as well as increased FEV1%, which is an indicator of relief of the obstructive pattern ($p<0.05$; Figure 2).

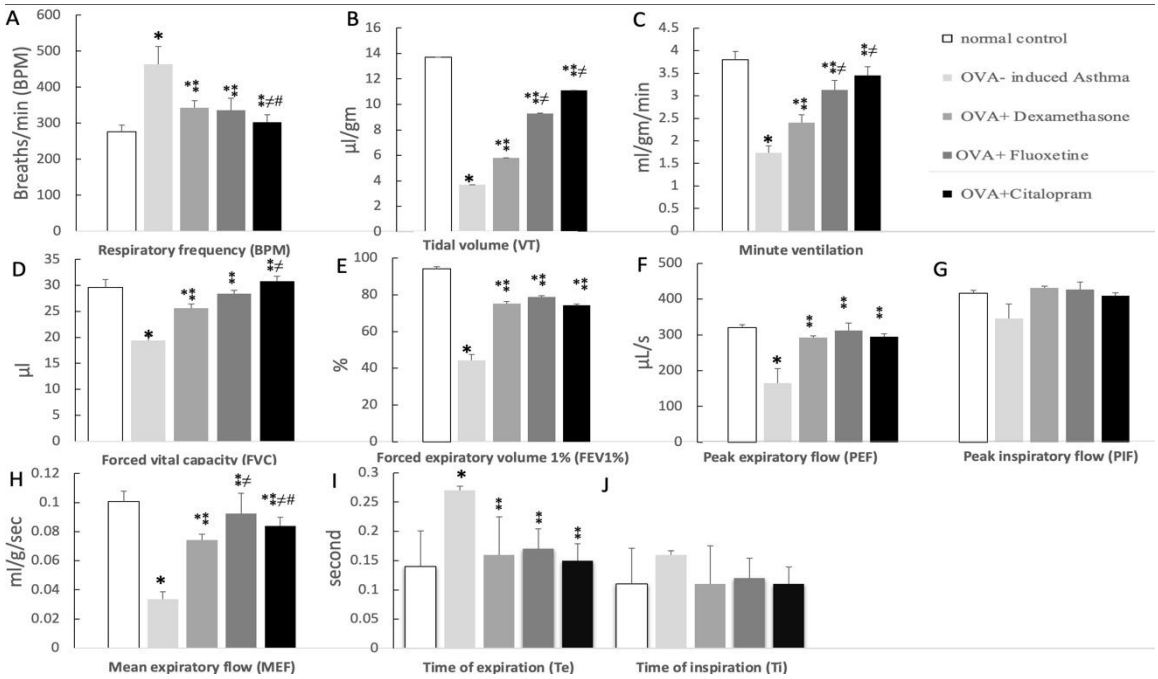

Figure 2: **Change in pulmonary function in mice sensitized and challenged by OVA.** (A): Respiratory frequency (BPM), (B): Tidal volume (VT) (µl/gm), (C): Minute ventilation (ml/gm/min), (D): Forced vital capacity (µl), (E): Forced expiratory volume 1% (FEV1%) (%). (F): Peak expiratory flow (PEF) (µl/sec), (G): peak inspiratory flow (PIF) (µl/sec), (H): Mean expiratory flow (ml/gm/sec), (I): time of expiration (Te) (second) and (J): time of inspiration (Ti) (second). The data of eight animals per group is shown as the mean ± standard deviation. (*) significantly varies from the normal control group ($P < 0.05$); (‡) significantly varies from the OVA-induced asthma group ($P < 0.05$); (≠) significantly varies from the OVA+ Dexamethasone group ($P < 0.05$); (#) significantly varies from the OVA+ fluoxetine group ($P < 0.05$).

3.2. Lung histopathological changes and total semi-quantitative score

The normal control group showed neither peri-bronchial inflammation, goblet cell hyperplasia, nor fibrosis (Figure 3A, F& K, respectively). The OVA-induced asthma group showed severe peri-bronchial inflammation with an average of six layers of chronic inflammatory cells, notably neutrophils, lymphocytes, and plasma cells, numerous hyperplastic goblet cells occupying 50% of the bronchial epithelium, and moderate fibrosis (Figure 3B, G& L respectively). The OVA + dexamethasone group showed moderate peri-bronchial inflammation with an average of three layers of chronic inflammatory cells and a few scattered hyperplastic goblet cells occupying about 20% of the bronchial epithelium but no fibrosis (Figure 3C, H& M respectively). The OVA + Fluoxetine group showed mild focal peri-bronchial lymphocytes and plasma cells but neither goblet cell hyperplasia nor fibrosis (Figure 3D, I& N, respectively). The OVA + Citalopram group showed moderate peri-bronchial lymphocytes and plasma cells arranged in two layers, no goblet cell hyperplasia, and mild fibrosis (Figure 3E, J& O, respectively).

The total semi-quantitative scores, representing infiltration by inflammatory cells, goblet cell hyperplasia, and collagen deposition in lung tissues of asthmatic mice, were markedly higher than those from normal mice ($p<0.05$; Figure 3). On the contrary, the scores of lung tissues of treated mice with dexamethasone, fluoxetine, and citalopram were lower than

asthmatic non-treated mice ($p<0.05$). The scores of the citalopram-treated group were less than those of the dexamethasone but without statistically significant differences ($p>0.05$). While fluoxetine treatment was more effective in reducing lung sores than dexamethasone and citalopram ($p<0.05$).

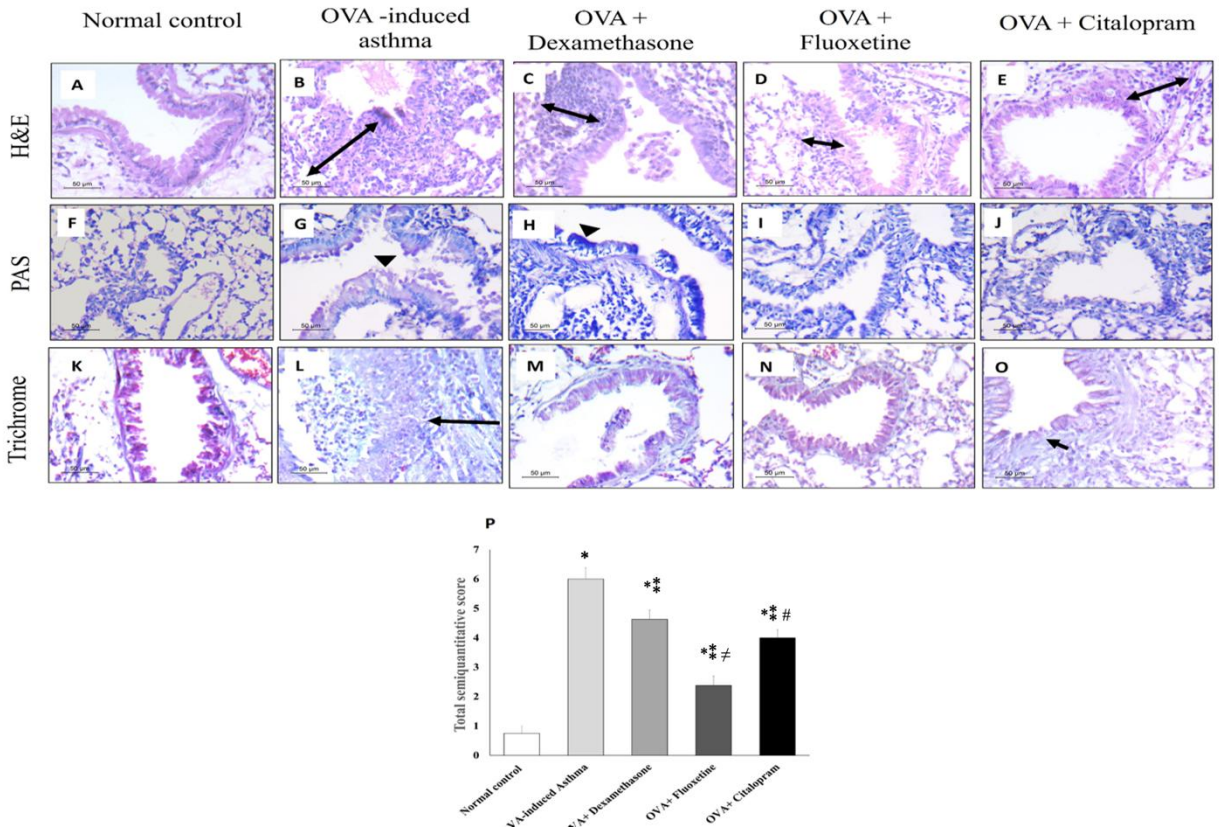

Figure 3: Histopathological changes and the total semi-quantitative score of mice sensitized and challenged by OVA.A-E: Inflammation score (A): Normal control group: showed no peribronchial inflammation: score 0. (B) OVA-induced asthma group shows severe peribronchial inflammation: score 3. (C): OVA + dexamethasone group shows moderate peribronchial inflammation: score 2. (D): OVA + Fluoxetine group shows mild peribronchial inflammation: score 1.(E): OVA + Citalopram group shows moderate peribronchial inflammation: score 2 (Peribronchial inflammation: double-headed arrow, H&E x400). **F-J: Mucous score** (F): Normal control group shows no hyperplastic goblet cells: score 0. (G) OVA-induced asthma group shows moderate goblet cell hyperplasia: score 2 (H): OVA + dexamethasone group shows mild goblet cell hyperplasia: score 1 (I): OVA + Fluoxetine group: score 0. (J) OVA + Citalopram group: score 0 (Hyperplastic goblet cells: arrowheads, PAS x400). **K-O: Fibrosis score** (K) Normal control group: score 0. (L) OVA-induced asthma group shows moderate fibrosis: score 2. (M) OVA + dexamethasone group: score 0. (N): OVA + Fluoxetine group: score 0 (O) OVA + Citalopram group shows mild fibrosis: score 1 (Fibrosis: arrows, Trichrome x400). **(P) Total semi-quantitative score**. The data of eight animals per group is shown as the mean ± standard deviation. (*) significantly varies from the normal control group ($P < 0.05$); (⁑) significantly varies from the OVA-induced asthma group ($P < 0.05$); (≠) significantly varies from the OVA+ Dexamethasone group ($P < 0.05$); (#) significantly varies from the OVA+ fluoxetine group ($P < 0.05$).

3.3. Total and Differential Leukocyte Count in BALF

The total and differential leukocyte counts, including neutrophils, eosinophils, lymphocytes, and macrophages, were markedly increased in BALF of the asthmatic group compared with the normal group ($p<0.05$; Figure 4). However, these changes were significantly reduced in asthmatic mice treated with dexamethasone, fluoxetine, and citalopram compared with the non-treated

asthmatic group (p<0.05). Moreover, fluoxetine significantly decreased the infiltration of neutrophils and lymphocytes than dexamethasone and citalopram, but all treatments were indifferent in their effect on eosinophils and macrophages (p>0.05).

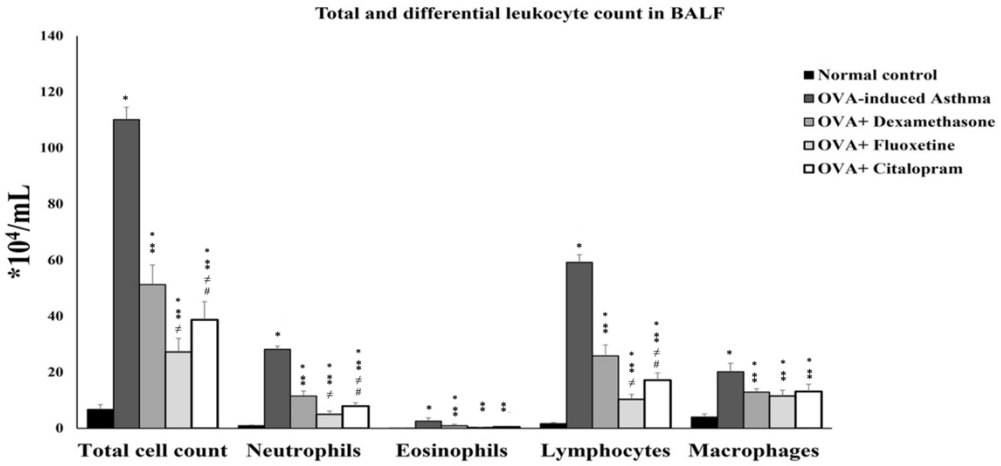

Figure 4: Total and Differential Leukocyte Count in Bronchoalveolar Lavage Fluid (BALF) of mice sensitized and challenged by OVA. The data of eight animals per group is shown as the mean ± standard deviation. (*) significantly varies from the normal control group (P < 0.05); (⁑) significantly varies from the OVA-induced asthma group (P < 0.05); (≠) significantly varies from the OVA+ Dexamethasone group (P < 0.05); (#) significantly varies from the OVA+ fluoxetine group (P < 0.05).

3.4. Alterations in serum total IgE and BALF inflammatory cytokines

Levels of total IgE in serum (Figure 5A) and cytokine markers of inflammation, including IL-4 and TNF-α in BALF (Figure 5B), were notably increased (p<0.05) in the OVA-induced asthma group relative to the normal control group. However, treatment of asthmatic mice with dexamethasone, fluoxetine, and citalopram produced a significant reversal of the effects of OVA by reducing the levels of IgE and inflammatory cytokines (p<0.05). In addition, treating asthmatic mice with fluoxetine had a greater suppressive effect than dexamethasone and citalopram. (p<0.05).

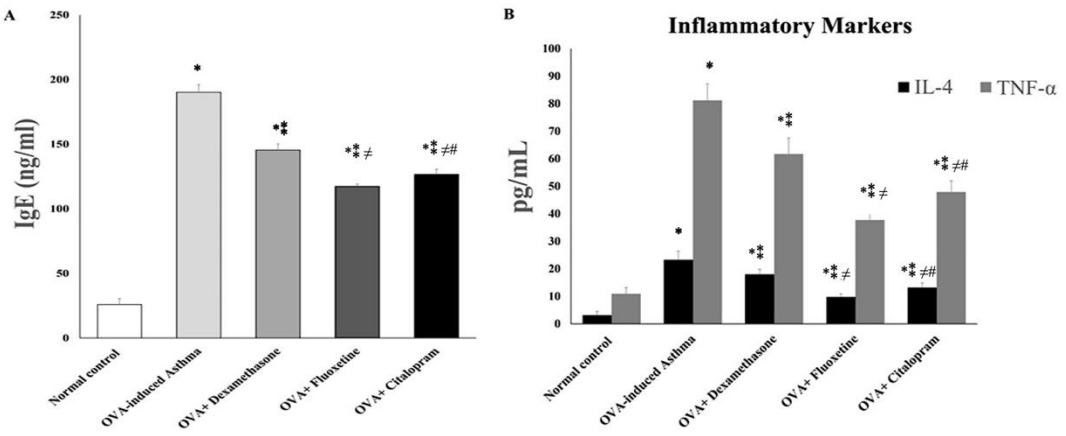

Figure 5: Alterations in serum total IgE and BALF inflammatory markers in mice sensitized and challenged by OVA. (A): Total serum Immunoglobulin E (IgE), (B): BALF inflammatory markers (Interleukin (IL)-4, and Tumor necrosis factor (TNF)-α). The data of eight animals per group is shown as the mean ± standard deviation. (*) significantly varies from the normal control group (P < 0.05); (⁑) significantly varies from the

OVA-induced asthma group (P < 0.05); (≠) significantly varies from the OVA+ Dexamethasone group (P < 0.05); (#) significantly varies from the OVA+ fluoxetine group (P < 0.05).

3.5. Variations in oxidative stress markers in lung tissue

Compared with the normal control group, the lung homogenates of the asthma group showed a substantial increase (p<0.05) in MDA generation (Figure 6A) and a reduction (Figure 6B & C) in GSH content and SOD activity. Mice treated with dexamethasone, fluoxetine, and citalopram showed a substantial suppression of oxidative stress and increased antioxidant enzymes (p<0.05) compared with the asthma group. Concurrently, the group treated with fluoxetine exhibited a significantly greater antioxidant capacity than those treated with dexamethasone and citalopram (p<0.05).

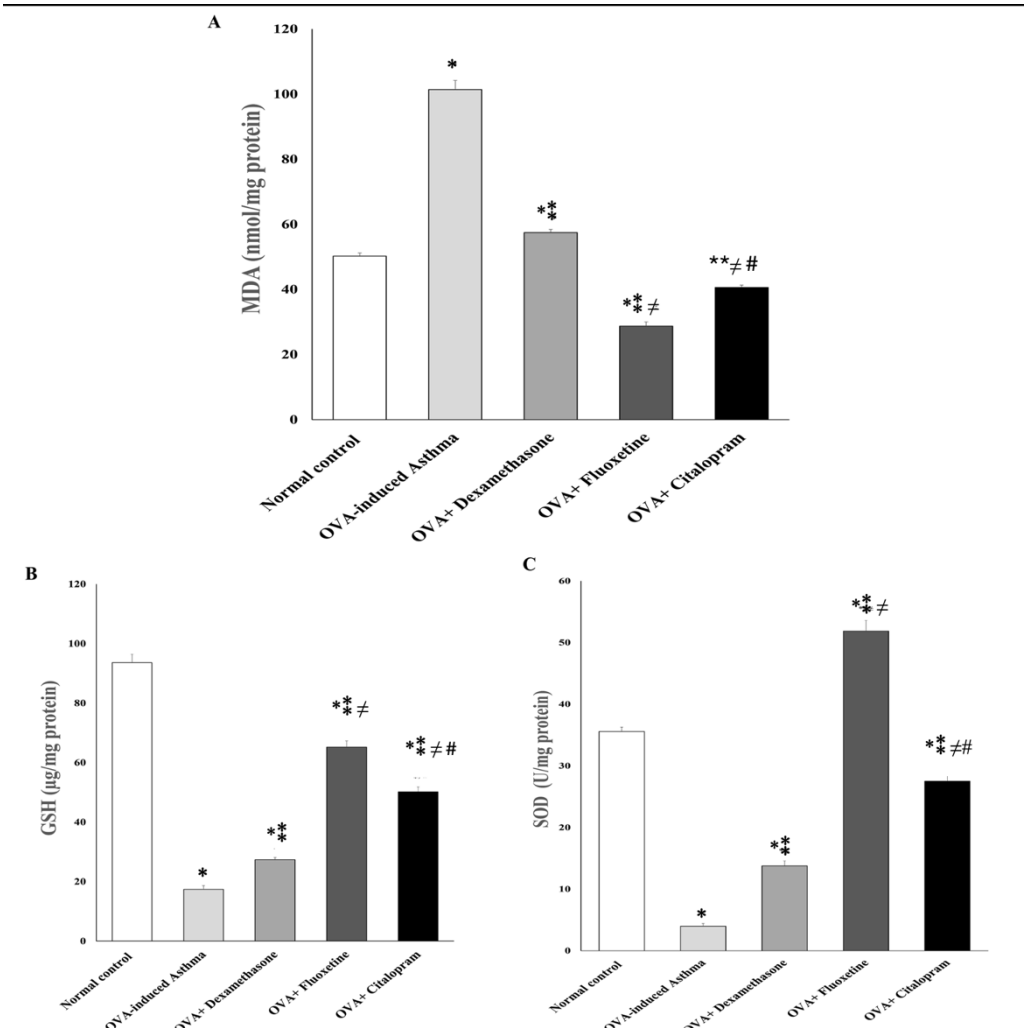

Figure 6: Variations in oxidative stress markers in lung homogenate of mice sensitized and challenged by OVA. (A): Malondialdehyde (MDA), (B): Glutathione (GSH), and (C): Superoxide dismutase (SOD) in lung tissues. The data of eight animals per group is shown as the mean ± standard deviation. (*) significantly varies from the normal control group (P < 0.05); (⁑) significantly varies from the OVA-induced asthma group (P < 0.05); (≠) significantly varies from the OVA+ Dexamethasone group (P < 0.05); (#) significantly varies from the OVA+ fluoxetine group (P < 0.05).

3.6. Modulation of p-mTOR expression

Analysis of p-mTOR by ELISA and western blot revealed a remarkable reduction in its expression in the lungs of the OVA model group (p<0.05; Figure 7A&B) compared with the normal control group. This inhibitory effect of OVA was

significantly attenuated by dexamethasone, fluoxetine, and citalopram ($p<0.05$). In contrast, fluoxetine induced more expression of p-mTOR than dexamethasone and citalopram ($p<0.05$).

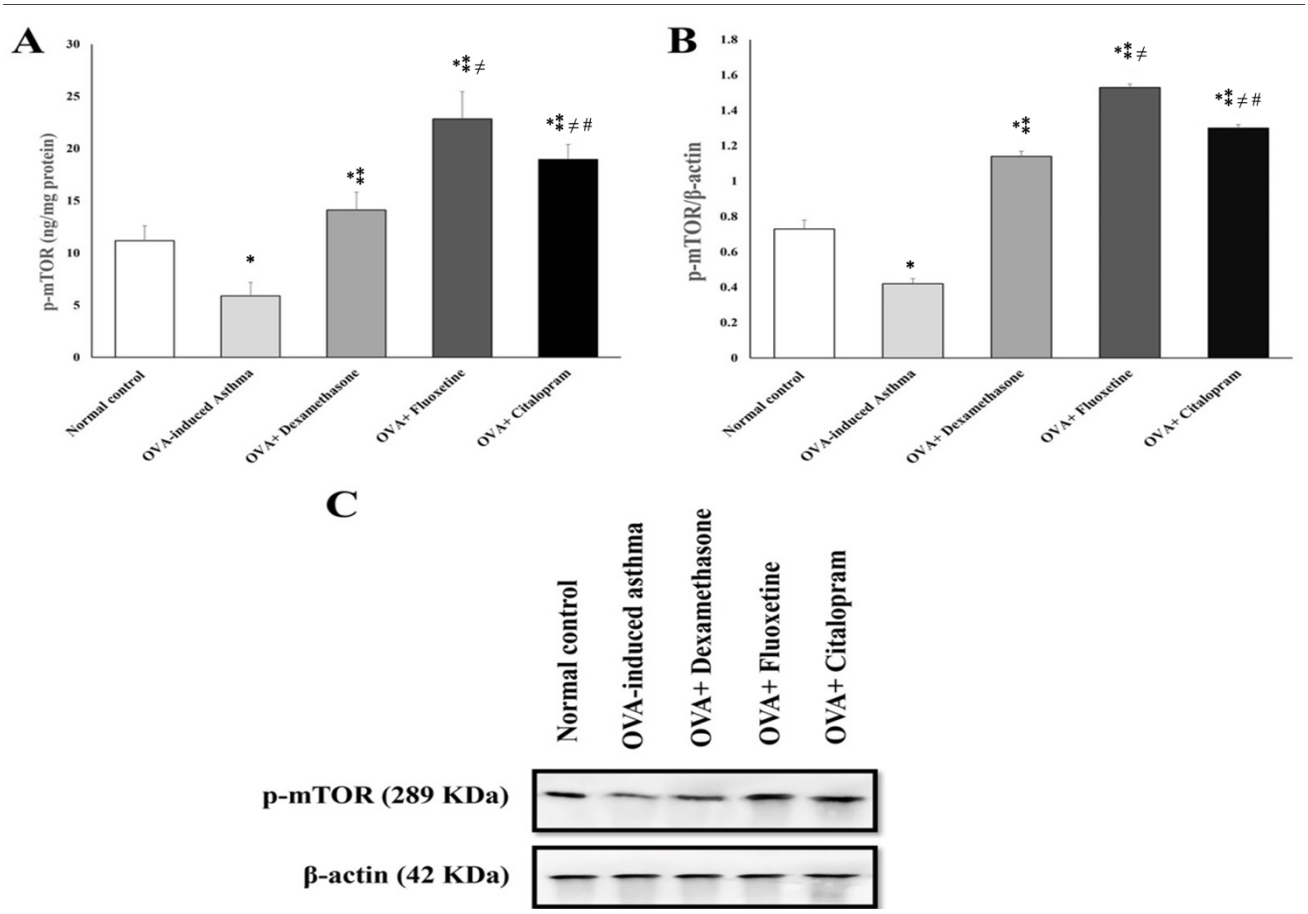

Figure 7: Modulation of p-mTOR expression in lung homogenate of mice sensitized and challenged by OVA. (A) expression of p-mTOR by ELISA. (B): the relative band intensities of p-mTOR modified to the expression of β-actin. (C) Western blot relative expression of protein levels of p-mTOR. The data of eight animals per group is shown as the mean ± standard deviation. (*) significantly varies from the normal control group ($P < 0.05$); (⁑) significantly varies from the OVA-induced asthma group ($P < 0.05$); (≠) significantly varies from the OVA+ Dexamethasone group ($P < 0.05$); (#) significantly varies from the OVA+ fluoxetine group ($P < 0.05$).

3.7. Change in expression of autophagy and mitophagy protein markers

Western blot analysis of protein expression of autophagy markers, including LC3B and P62 and mitophagy markers, involving PINK1 and PARKIN in lung tissues, showed that administration of OVA significantly increased LC3B, PINK1, and PARKIN. Additionally, it significantly decreased the expression of p62 as compared with the normal control group ($p<0.05$; Figure 8). The administration of dexamethasone, fluoxetine, and citalopram before the OVA challenge produced a marked decrease in LC3B and mitophagy markers and an increase in p62 compared with the non-treated asthmatic group ($p<0.05$). However, the asthmatic mice treated with fluoxetine showed a more prominent inhibition of LC3B expression and an increase in p62 than the rest of the treated groups that were statistically indifferent from the normal mice ($p>0.05$). Concurrently, fluoxetine and citalopram

showed insignificant differences in attenuation of PINK1 (p>0.05).

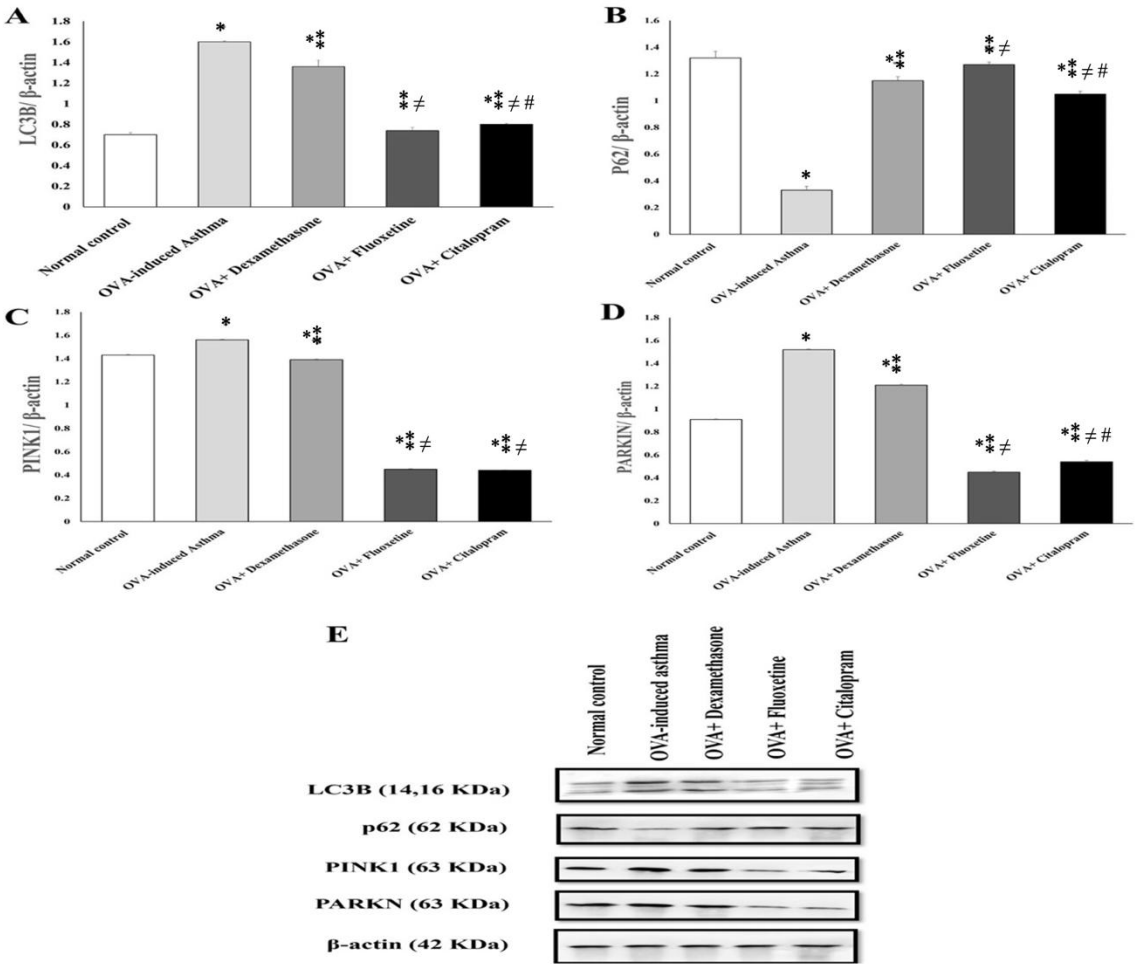

Figure 8: Change in expression of autophagy and mitophagy protein markers in lung homogenate of mice sensitized and challenged by OVA. (A-D): the relative band intensities of LC3B, P62, PINK1 and PARKN modified to the expression of β-actin. (E) Western blot analysis of LC3B, P62, PINK1, and PARKN protein levels. The data is shown as the mean ± standard deviation. (*) significantly varies from the normal control group ($P < 0.05$); (⁑) significantly varies from the OVA-induced asthma group ($P < 0.05$); (≠) significantly varies from the OVA+ Dexamethasone group ($P < 0.05$); (#) significantly varies from the OVA+ fluoxetine group ($P < 0.05$).

Discussion:

In Low- and Middle-Income Countries (LMICs), asthma patients face significant financial difficulties because of their illness. Using recently approved biologics to treat severe asthma is not widely accessible, with a reported frequency of 6.7% of asthmatics in Egypt. Additionally, oral corticosteroids (OCS) have a wide range of adverse effects and may not be helpful for neutrophilic asthma. Therefore, looking for alternative, reasonably safe, and affordable treatments is necessary (29).

Since SSRIs have been demonstrated to regulate the functions of various immune cells that express 5-hydroxytryptamine (HT) receptors, including mast cells, eosinophils, neutrophils, lymphocytes,

and macrophages, as well as modify the transcription of pro-inflammatory genes and the production of proinflammatory cytokines, they have been investigated for their potential anti-inflammatory effects (14, 15). Nevertheless, there is doubt regarding their therapeutic benefits for asthmatic patients who do not have any concurrent mental conditions, and research remains in progress to identify novel molecular pathways that may be responsible for their effects.

Using Ovalbumin (OVA) for sensitization and challenge is the most classic and widely used experimental model for studying allergic asthma. Its advantages are rooted in its reliability and its ability to mimic key features of the human disease. OVA is a potent allergen that effectively primes the immune system to mount a strong T-helper 2 (Th2) response. This is the central immunological pathway in allergic asthma, leading to the production of key cytokines (like IL-4, IL-5, IL-13) that drive the disease(17).OVA sensitization of CD1 mice resulted in a neutrophilic inflammation of the airways mimicking acute bronchial asthma, as demonstrated by the increased total and differential cell counts in the BALF, serum IgE and inflammatory markers of the untreated asthmatic group relative to normal mice, along with a marked decline in lung functions and changes in the histological characteristics of lung sections exhibiting neutrophilic infiltration of lung tissues, similar changes were reported by Elsakkar et al.,(30) and Magalhães et al.,(31). Although the changes in the acute asthma model are temporary and reverse within several weeks following the last challenge, the acute asthma model is valuable for investigating the mechanisms involved in inflammatory processes and identifying new therapeutic targets. In contrast, the long-term challenge in the chronic model has been linked to tolerance to OVA and downregulation of inflammation (32-34).

The increased levels of Th1 and Th2 inflammatory cytokines, TNF-α and IL-4, respectively, following the OVA challenge of sensitized mice, were considerably attenuated after administration of SSRIs compared to dexamethasone. The elevated levels of inflammatory cytokines suggest a mix of Th1 and Th2 immune responses, which can account for the different features of asthma observed in this study. The raised levels of TNF-α contributed to the increased recruitment of neutrophils to the lung tissue, as demonstrated by the neutrophil count in the BALF and the histological examination of lung tissues. The elevated TNF-α also accounts for the ineffectiveness of dexamethasone when compared to SSRIs in this particular model, which is consistent with earlier research showing that patients with neutrophilic asthma exhibit an inadequate response to corticosteroids (35-37).

While the raised IgE, a marker of allergic inflammation, may be attributed to the activation of B cells to release IgE by the increased production of IL-4. Prior research has also indicated that IL-4 is responsible for the hyperplasia of the lung's goblet cells, the thickening of airways, and increased airway responsiveness. (38-41). Moreover, the increased inflammation observed in the group with asthma can be attributed to the elevated levels of the oxidative stress marker, MDA, and the notable decrease in antioxidant markers, GSH and SOD. Previous studies have indicated that oxidative stress contributes to inflammation and vice versa

by increasing the expression of Th2 cytokines, particularly IL-4. Following SSRI treatment for asthmatic mice, the lung's antioxidant capacity was restored, leading to an anti-inflammatory state that supports the therapeutic potential of SSRIs in the treatment of asthma (15, 30, 42).

Antidepressants, notably SSRIs, have been shown to regulate autophagy and mitophagy processes in a variety of pathologies, such as depression, neurodegenerative illnesses, and malignancies. This is in line with the idea that they could be used as an adjuvant therapeutic agent in non-psychiatric morbidities. Although mitophagy and autophagy are crucial in airway inflammation and asthma pathophysiology, their precise roles in the disease pathophysiology remain unclear. Uncertainty regarding the overregulation;whether it is a risk element to be avoided or a positive aspect to invest in (43, 44). As a result, SSRIs may be able to rebalance this process experimentally (16).

Three autophagy-related markers were examined in this study: p-mTOR, LC3b, and p62. LC3 is a protein marker denoting the formation of autophagosomes, while p62 accumulation demonstrates the inhibition of autophagy as it is subject to proteolysis during active autophagy (45). Increased detection of p-mTOR signifies the stimulation of the PI3K/Akt/mTOR signaling pathway that downregulates autophagy in the asthmatic lungs (46). Since dexamethasone inhibits dysregulated autophagy marker LC3b, which has been shown to exacerbate the inflammatory process in asthma, a substantial rise in p-mTOR and p62 after therapy was anticipated (44). Additionally, following treatment with citalopram and fluoxetine, the expression of LC3b decreased, but p62 and p-mTOR dramatically increased. These findings suggest that SSRIs significantly affect autophagy and clarify their anti-inflammatory and antioxidant properties via enhancing the activity of p-mTOR (8, 9, 46, 47).

This study also investigated the mitophagy pathway, which is a specific type of autophagy that is responsible for removing dysfunctional mitochondria (42). In the asthmatic group, the expressions of mitophagy indicators, specifically PINK1 and PARKN, were significantly increased. This increase may be attributed to the increased generation of ROS in the nucleus, which promotes mitophagy by enhancing the transcription of LC3 and p62 (2). However, after treatment, especially with SSRIs, there was a noticeable decrease in the expression of these indicators, which has been shown in earlier research to help reduce airway inflammation and reverse airway remodeling (5, 42, 48). Prior research has also indicated that increased antioxidant activity and the expression of p-mTOR could explain the reduction of mitophagy (49, 50). In contrast to our results, citalopram and fluoxetine demonstrated pro-autophagic effects in several neurons by promoting autophagy and mitophagy (10, 11). Though the role of various autophagy inhibitors, such as chloroquine and a SIRT1 inhibitor (51), supports the idea of a positive effect of autophagy/mitophagy suppression in experimental asthma models; the modulation of both autophagy and mitophagy in allergic asthma following SSRI treatment has not yet been clearly defined.

Consequently, the noticeable improvement in PFTs and the histological results, as confirmed by the semiquantitative score, could be attributed to the inhibition of autophagy and mitophagy, as

evidenced by the different protein markers investigated.Given the limitations of our study design and scope, further experiments specifically targeting autophagy and mitophagy pathways would be valuable for confirming their involvement in the inhibitory effects of SSRIs on airway inflammation. Future investigations utilizing pharmacological inhibitors or mechanistic studies could provide deeper insights into the underlying mechanisms and validate our initial observations. Additional studies are also recommended to clarify the impact of SSRIs on the different types of lymphocytes and immune cells to establish a correlation between their anti-inflammatory properties and their effects on acute and chronic asthma models.

The suggested repurposing of SSRIs in corticosteroids-resistant neutrophilic asthma seems a logical long-term objective. But for the time being, more investigations defining the exact role of autophagy and mitophagy in asthma pathogenesis and the benefit/risk of pharmacotherapy with SSRIs in steroid-resistant asthma are critical as SSRIs have a favorable safety profile and are less financially burdensome in economically challenged countries.

Author contributions

S.E.: Conceptualization, Methodology, Investigation, Resources, Data Curation, Writing the Original Draft, Review, and editing.

S.Z.N.: Methodology, Investigation, Resources, Data Curation, Writing Original Draft.

M.M.A.: Methodology, Investigation, Resources, Data Curation, Writing Original Draft.

D.A. G.: Investigation, Resources, Data Curation, Writing Original Draft.

S.A. A.: Investigation, Resources, Data Curation, Writing Original Draft.

E.S.H.: Methodology, Software, Validation, Formal Analysis, Investigations, Resources, Data Curation, Writing Original Draft, Review & Editing.

The authors declare that all data were generated in-house and that no paper mill was used.

Ethics approval

The Alexandria Faculty of Medicine's Ethics Committee approved all the experimental protocols used in this study (IRB No. 00012098-FWA No. 00018699-Protocol serial No. 0305587). The procedures followed both the institution's policies and the US Guide for the Care and Use of Animals.

Funding

The authors received no funding for this study.

Declaration of interests

The authors declare that no financial interests or personal relationships could influence the work reported.

Data availability

Data can be provided upon request.

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