Enhancement of Anti-allergic Effect of Diethylcarbamazine Citrate in Asthmatic Mouse Model: Testing of Anti-drug Antibodies and Quercetin

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ABSTRACT

Diethylcarbamazine citrate (DEC) is known as an effective treatment for bronchial asthma because of its ability to reduce eosinophil trafficking to the lung tissue. The current study aimed to potentiate the anti-allergic effect of the drug by passive immunization of the asthmatic model with anti-DEC antibody or prior treatment with quercetin (Qur).

Eight mice groups were categorized into control, the model of lung asthma, treated with DEC, passively immunized with anti(α)-bovine serum albumin Ab, anti-DEC Ab, prior exposure to 10, 20, or 40 mg Qur/Kg. b.wt. Both eosinophil peroxidase (EPO) and eotaxin2 in the lung tissues were performed. Serum levels of cytokines, bronchoalveolar lavage fluid (BALF) IgE, rabbit anti-bovine serum albumin (anti-BSA), and DEC IgG in lung tissue homogenates were assayed by ELISA.

Regarding the effect of anti-DEC Ab and Qur on DEC-induced recovery of histopathological alterations showed that the Ova group had peri-bronchial hyperplasia, mononuclear leukocyte infiltration, thickening in the wall of alveoli, and congested blood vessels. However, the reduction of inflammatory cells and thickened alveolar walls was dependent on the Qur dose. Qur40 enhanced the anti-allergic effect of DEC. Moreover, the present data revealed high levels of Th2 cytokines (IL-4 and IL-5) and IgE in the Ova group. An increased leukocyte infiltration/thickening of the alveolar wall and lung tissue EPO/eotaxin2 were also observed. Qur-40 could show an enhancement effect on DEC for the reduction of IL-4, IL-5, IgE, EPO, and eotaxin 2. Consequently, the IFN-γ/IL-4 ratio was increased.

Qur at 40 mg/Kg could be recommended to enhance the DEC effect suggesting a novel approach for treatment.

Keywords: Antibodies; Anti-allergic agents; Asthma; Diethylcarbamazine; Quercetin

INTRODUCTION

Asthma is a chronic inflammatory disease of the lung characterized by recurrent airway obstruction and increased bronchial hyperresponsiveness.[1] Most current mouse models induce T helper type 2 (Th2) immune

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responses, which is associated with allergic/eosinophilic asthma in humans.[2] These models typically use chicken egg ova, the archetypal Th2-inducing antigen. Sensitization and subsequent challenge with ova in the lung induces hallmark features of allergic asthma, including the production of antigen-specific IgE, mucous hypersecretion, airway hyperresponsiveness, and eosinophilic infiltration. The level of chemokines like eotaxin2 and eosinophil granule protein eosinophil peroxidase (EPO) are diagnostic markers for lung eosinophilia or asthma.[3] These manifestations were dependent on IL-4-mediated activation of the STAT6 pathway.[4] The resulting Th2 cells secrete amongst others IL-5 and IL-13.

Current treatments for asthma mainly consist of inhaled corticosteroids and long-acting bronchodilators which act in reducing the symptoms without curing the disease. Although these treatments are effective in most patients, they can be unsuccessful in some patients and may have side effects.[5] Thus, there is a continuous search for better-targeted treatment strategies that are based on mechanistic insights into disease development and progression. Diethylcarbamazine citrate (DEC) has been known as an effective treatment for bronchial asthma because of its ability to reduce eosinophil numbers as a result of inhibition of 5-lipoxygenase.[6] In addition, the drug was also able to induce ultrastructural changes in pneumocytes (types I and II) which were important for improving asthma symptoms.[7] Eosinophils were also reduced after DEC treatment of patients with tropical pulmonary eosinophilia.[8] The potentiation of DEC microfilaricidal action by anti-DEC Ab was previously indicated.[9] This was depended on the development of Ab to 4-methylpiperazine-1-carboxylic acid (MPCA) which bears a free carboxyl group while retaining the methylpiperazine function of DEC.[10]

Quercetin (Qur) is a flavonoid which is existed in some vegetables and fruits and was characterized as an anti-oxidant/ allergic substance. Its anti-allergic effects were referred to as inhibition of histamine, IL-4, IL-5, lipoxygenase, and IgE formation.[11-13] The current study aimed to test both anti-DEC Ab and Qur as enhancement agents for DEC anti-allergic effect in an experimental model of asthma in mice.

The current study aims to potentiate the effect of DEC which is a strong anti-inflammatory and anti-allergic drug on asthmatic mice. The novelty of the current study is based on the potentiation by anti- DEC antibodies and their biological effects in the case of asthma.

In addition, the present study aimed to delineate the potentiation of the DEC when combined with anti-DEC antibodies (Abs) or a natural organic compound (Qur) which is an anti-inflammatory organic drug. This proposal is a novel approach since no study discussed the co-effect of anti-drug Abs or Qur on the effect of DEC within the trials to enhance its action in case of asthma.

MATERIALS AND METHODS

Experimental Animals

A total of 120 conventionally raised 6 to 8-week-old female white albino mice (weighing 18-20 g) and two 6-month-old female New Zealand white rabbits (1.5–2 kg) were included in the study. Animals were kept in hygienic cages in air-conditioned rooms at a constant temperature of 22˚C on a 12 hour light/dark cycle. Animals were fed with a commercial diet ad libitum for the experiment. All experimental procedures complied with the requirements of the Zoology Department, Faculty of Science, Beni-Suef University Animal Care and Ethics Committee.

Study Design

Allergic airway inflammation model was performed through intradermal sensitization with 100 μL 0.3% ova in corn oil at 31 and 33 days followed by exposure to 0.3% ova aerosol using an ultrasonic nebulizer (NE-U22, Omron, Kyoto, Japan) for 43, 50, 53 and 54 days (Figure 1). DEC (Sigma, St. Louis, MO, USA) was given orally at 12 mg/Kg, while anti-bovine serum albumin (anti-BSA) (Sigma) or anti-DEC Ab were injected intraperitoneally (0.5 mL/mouse).[14] Mice were divided into 8 groups (16 mice/ group): (I) Control group exposed to vehicles only, (II) Ova group (untreated), (III) Ova-DEC group (DEC-treated), (IV) Pre-exposed ova to passive immunization with anti-BSA Ab followed by DEC-treatment (ova-αBSA-DEC group), (V) Pre-exposed ova to passive immunization with anti-DEC Ab followed by DEC-treatment (ova-αDEC-DEC group), (VI) Pre-exposed ova to Qur10 (10 mg/Kg) followed by DEC treatment (ova- Qur10-DEC group), (VII) Pre-exposed ova to Qur20 (20 mg/Kg) followed by DEC (ova- Qur20-DEC group), and (VIII) pre-exposed ova to Qur40 (40 mg/Kg) followed by DEC (ova- Qur40-DEC group).

Preparation of Anti-DEC Ab

Preparation of MPCA (4-Methylpiperazine-2-carboxylic acid; Sigma)-BSA conjugate was prepared as previously described.[10] MPCA (27.47 mg) which is an acid hydrolysis product of DEC was dissolved in 2.5 mL of methanol and combined with 144 mg 1-ethyl-3-(3-dimethylaminopropyl)-carbdiimide (EDC; Sigma) /2.5 mL of 20 mM potassium phosphate buffer (pH 5.0) at room temperature for 2 min. This reaction mixture was then added to a suspension of 30 mg BSA in 8 mL of 200 mM phosphate buffer (pH 8.0) and allowed to incubate overnight at room temperature. EDC and unreacted MPCA were removed from the mixture by dialysis against 10 mM phosphate-buffered saline (PBS; pH 7.2) at 4°C for time intervals of 2 hr, 4 hr, and finally overnight. For immunization in rabbit, 500 µL MPCA-BSA conjugate or BSA (1 mg/mL) in PBS (pH 7.2) was emulsified with the same volumes of Freund's complete adjuvant (Sigma) and inoculated intramuscularly into two rabbits at day 0. The second,

third, and fourth inoculations were performed with the same volumes on days 15, 30, and 45, respectively with Freund's incomplete adjuvant (Sigma). After the final immunization, blood samples were taken from the rabbits and the production of Ab was investigated by ELISA tests.

Purification of Rabbit Polyclonal IgG and Inhibition ELISA

Polyclonal IgG Abs were purified from rabbit serum using ammonium sulfate precipitation followed by caprylic acid purification method. [15] Briefly, 100% ammonium sulfate (Oxford Laboratory, Maharashtra, India), pH 7.2 was added to rabbit serum dropwise to reach 50% saturation with continuous stirring. The resultant suspension was centrifuged at $3500 \times g$ for 20 min at 4°C. The precipitate was dissolved in 0.01 M PBS, pH 7.2, and ammonium sulfate was removed by dialysis against 0.01 M PBS, pH 7.2 for 72 days at 4°C.
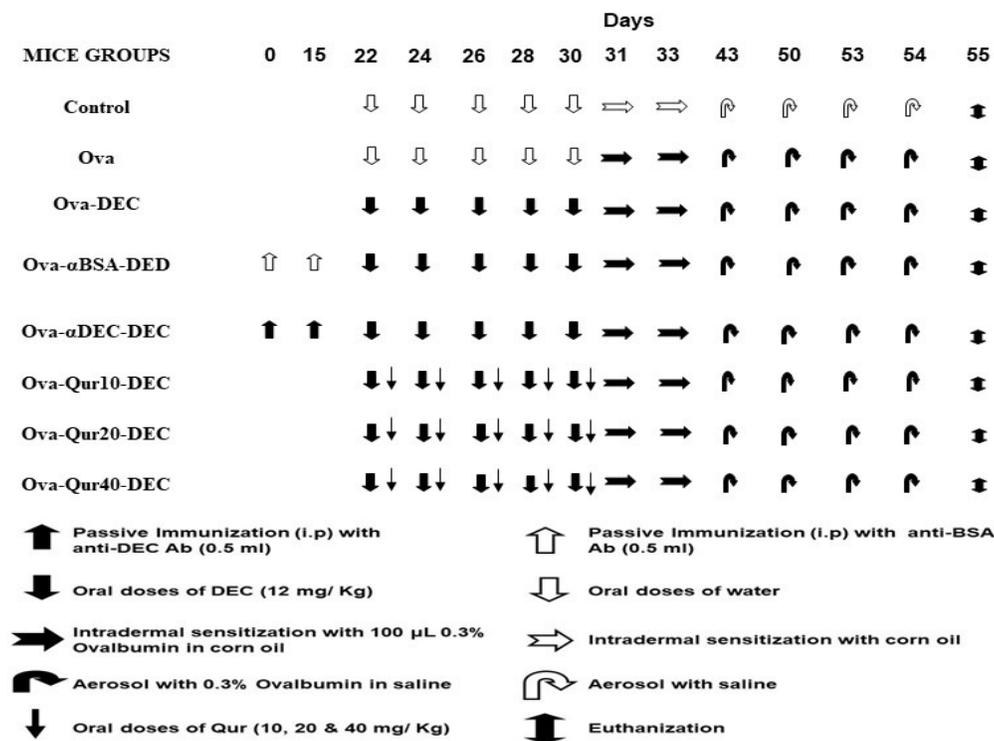
Figure 1. Schematic presentation of passive immunization of mice with rabbit anti-bovine serum albumin (anti-BSA) and diethylcarbamazine citrate (DEC) IgG Abs, sensitization, inhalation exposure, and the drug administration times in the mouse model of ova-induced allergic airway inflammation
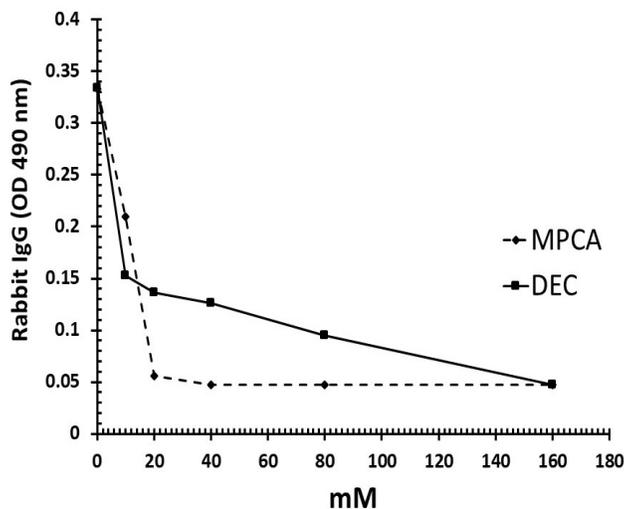
Figure 2. Effect of both methylpiperazine-1-carboxylic acid (MPCA) and diethylcarbamazine citrate (DEC) at different concentrations (0–40 mM) on the reactivity of rabbit anti-MPCA-bovine serum albumin (BSA) conjugate polyclonal IgG Abs to MPCA-BSA conjugate coated wells. MPCA and DEC were pre-incubated with rabbit Abs

The precipitate was further dialyzed against 3 mM sodium acetate buffer, pH 4.8. Caprylic acid (Merck, MA, USA) was added (25 µL/mL) dropwise with stirring at 4°C for 30 min and the mixture was centrifuged at $1000 \times g$ for 30 min. The supernatant was taken, filtered through a 0.8 µM filter to remove fines. ELISA was performed as previously described. [16] Briefly, 100 µL of rabbit serum (1:100 diluted in PBS-T) were mixed with 100 µL of different concentrations of MPCA or DEC (0–40 mM) in PBS containing 0.1% Tween-20 and incubated for 60 min at 37°C. The mixture was then added in quadruplicates to the antigen-coated plates and titrated. The background absorbance value obtained against BSA was subtracted from MPCA-BSA values for each of the samples. With inhibition ELISA, the reactivity of IgG Abs with DEC appeared, however, reactivity with MPCA was higher (Figure 2).

Collection of Serum and Bronchial-alveolar Lavage Fluid (BALF)

At day 55, blood was collected by retro-orbital puncture immediately before mice were euthanized. The blood was left overnight at 4°C to clot before centrifugation for 15 minutes at $1000 \times g$. The serum was separated and aliquoted. After the mice were euthanized by a lethal dose of pentobarbital sodium salt (CAS Number: 57-33-0; Sigma-Aldrich), 3 mL of PBS saline (pH 7.4) was instilled into the lungs via a tracheal cannula, recovered immediately and set into a siliconized glass tube on ice. After the lavage, the tubes were centrifuged at $120 \times g$ for 10 min and the supernatant was collected. Serum and BALF were collected and stored at -80°C. [17]

Lung Tissue Histology

Equal pieces from both sides of the lung tissue from each animal were washed in phosphate buffer salt saline (PBSS, pH 7.4), then fixed in 10% buffered formalin solution for 24 h. Later, tissues were washed and immersed in ascending serial dilutions of ethyl alcohol for dehydration. The specimens were cleared in xylene and embedded in paraffin at 56°C in an oven for 24 h. Paraffin tissue blocks were prepared for sectioning at 4 µm thickness by the microtome. The obtained tissue sections were collected on glass slides, deparaffinized, and stained with hematoxylin & eosin for examination using the light microscope. [18]

Mouse IL-4, IL-5, and IFN-γ Assay

The procedure for the quantitative measurement of mouse IL-4, IL-5, and IFN-γ in serum using ELISA was performed according to the instructions provided by RayBiotech (Georgia, USA) with codes: ELM-IL4-1, ELM-IL5-1, and ELM-IFN-γ-1, respectively.

Detection of Ova-specific and Total IgE in BALF

Anti-ova specific IgE was measured by ELISA, as previously described.[19] Briefly, microtiter plates (Nunc, Roskilde, Denmark) were coated overnight with 5 µg/mL of ova in a 50 mM of carbonate buffer (pH 9.6) at 4°C. Nonspecific binding was blocked with 2% bovine serum albumin for 1 hr at room temperature. After incubation of the test sera for 2 hr, the plates were incubated with horseradish peroxidase-labeled goat anti-mouse IgE (Pharmingen, San Diego, U.S.A.) for 1 hr at room temperature. The reaction was developed with an *O*-phenylenediamine (Sigma, St. Louis, U.S.A.) and stopped by adding 2N H_2SO_4. Total mouse IgE in BALF was determined using a commercial kit (CUSABIO, Houston, USA). The procedure was performed according to the guidelines provided with the kit.

EPO Assay in Lung Tissue

The right and left caudal lobes and each of one-half of the trachea plus main bronchi were suspended (200 mg/mL) in 0.5% hexadecyl trimethyl ammonium bromide (Sigma) in 50 mM potassium phosphate buffer, pH 6.0 and then homogenized for 1 min on ice. The homogenate was then centrifuged at $400 \times g$ for 30 min and the pellet was resuspended in 4 mL of the same buffer. These samples were freeze-thawed three times and sonicated by bath sonicator 50 (Ultra Autosonic, Maharashtra, India) on ice. Supernatants were taken for analysis. EPO activity assay was previously described.[20] EPO activity in the supernatant was assayed by monitoring O.D. changes at 490 nm using the substrate solution [0.1 mM O-phenylenediamine dihydrochloride in 0.05 M Tris-HCl (Sigma), pH 8.0 containing 2% Triton X-100 (Sigma) and 1 mM hydrogen peroxide (Sigma)].

Detection of Lung Tissue Eotaxin 2

Lung tissue was homogenized in PBS, pH 7.4 (100 mg/ mL), and stored overnight at -20°C. After two freeze-thaw cycles were performed to break the cell membranes, the homogenates were centrifuged for 5 minutes at 5000 x g at 4°C. The supernatants were removed, aliquoted, and stored at -80°C. Total mouse eotaxin2 in lung tissue homogenate was determined using a commercial kit (CUSABIO, Houston, USA). The procedure was performed according to the guidelines provided with the kit.

Detection of Rabbit Anti-BSA and DEC IgG Levels Against Tissue Homogenates by ELISA

The procedure was carried out as previously described with some modifications.[1,2] Briefly, Maxisorp flat-bottom 96-well microtiter plates (Nalge Nunc International, Denmark) were coated with 100 µL per well of coating buffer, pH 9.6, containing 5 µg/mL of control, Ova and Ova-DEC lung homogenates (n=10) and incubated overnight at 4°C. The antigen-coated plates were washed three times with wash buffer (PBS containing 0.1% Tween 20). The wells were blocked with 200 µL of blocking buffer (5% fetal calf serum in wash buffer) for 2 h at 37°C. After washing, the rabbit anti – BSA and anti – DEC polyclonal IgG were diluted at 1: 100 in wash buffer and added (100 µL/well) into coated wells. The first was tested against Ova in comparison to control homogenates while the second was tested against Ova-DEC in comparison to Ova. The incubation of sera was for 2 h at room temperature. After washing three times, the wells were incubated for 1 h at room temperature with 100 µL/well diluted peroxidase-labeled goat anti-rabbit IgG (KPL, Maryland, USA) at 1: 2000 in wash buffer. After three washes, 100 µL/well of appropriately diluted O-phenylenediamine (Sigma) in substrate buffer (pH 5) containing 0.04% hydrogen peroxide was added to the plate and left for 15-20 min till color development. The enzymatic reaction was stopped by 40 µL/well 3N H_2SO_4 and the optical density was read at 490 nm with ELx808 ELISA reader (BioTek). All the serum samples were tested in duplicates.

Statistical Analysis

SPSS (version 20) statistical program (SPSS Inc., Chicago, IL; IBM Corp., Armonk, NY, USA) was used to carry out a one-way analysis of variance (ANOVA). When significant differences by ANOVA were detected, analysis of differences between the means of the treated and control groups was performed by POST hoc *Dunnett's* with LSD a=0.05 was used to compare non-control groups. Finally, levels of rabbit anti-BSA and anti-DEC-IgG in tissue homogenates were compared by simple student *t*-test with the same version of the SPSS program.

Ethics Committee Approval

All animal procedures were conducted following the standards set in the guidelines for the care and use of

experimental animals by the Animal Ethics Committee of the Zoology Department in the Faculty of Science at Beni-Suef University, which was under an approval number: BSU/FS/2015/9.
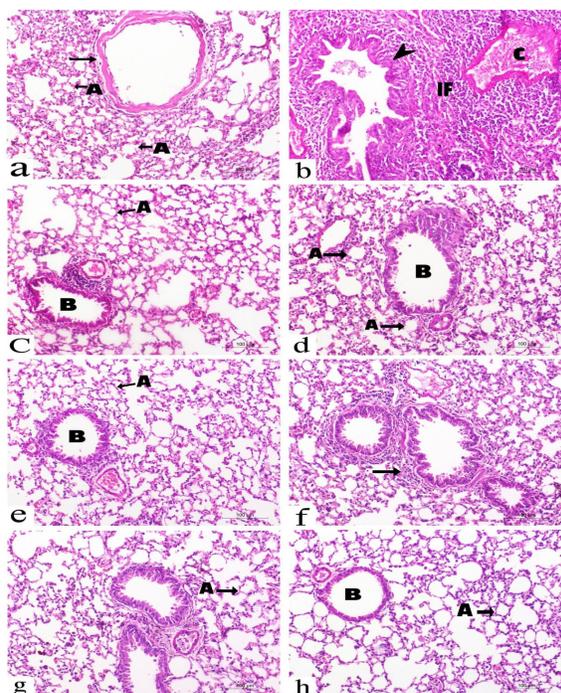
Figure 3. Representative photomicrograph depicts histopathological changes in lung sections sampled from experimental groups. (a): Control group showing normal histological architecture including blood vessels (arrow) and numerous clear alveoli with thin interalveolar septa. (b): Ova-group showing peribronchial hyperplasia (arrowhead), mononuclear leukocyte infiltration (if), thickening in the wall of alveoli, and congested blood vessels (C). (c and e): Ova- diethylcarbamazine citrate (DEC) and ova-αDEC-DEC groups, respectively showed normal structure except little aggregation of inflammatory cells. (d): ova-α bovine serum albumin (BSA)-DEC group indicated mild recovery of the structure except for the existence of inflammatory cell infiltration (arrow). (f): Quercetin (Qur)10 group showed aggregation of inflammatory cells (arrow) and thickening of the alveolar wall. (g): Qur20 showed less cell infiltration. (h): Qur40 showed a complete recovery of lung structure. Bronchiole (B); alveoli (A); H & E; scale bar=100μm.

RESULTS

Effect of anti-DEC Ab and Qur on DEC-induced Recovery of Histopathological Alterations

The control group indicated normal lung tissue architecture (Figure 3a). Ova group showed peri-bronchial hyperplasia, mononuclear leukocyte infiltration, thickening in the wall of alveoli, and congested blood vessels (Figure 3b). Ova-DEC (Figure 3c) showed less infiltration of inflammatory cells compared to the ova-αBSA-DEC group (Figure 3d). The Ova-αDEC-DEC group (Figure 3e) showed the same effect. Ova-αDEC-DEC did not show a potentiating effect when compared to ova-DEC. The reduction of inflammatory cells and thickened alveolar walls was dependent on the Qur dose (Figure 3f-h). Comparing ova-Qur40-DEC to ova-DEC indicated that Qur40 could potentiate the anti-allergic effect of DEC.
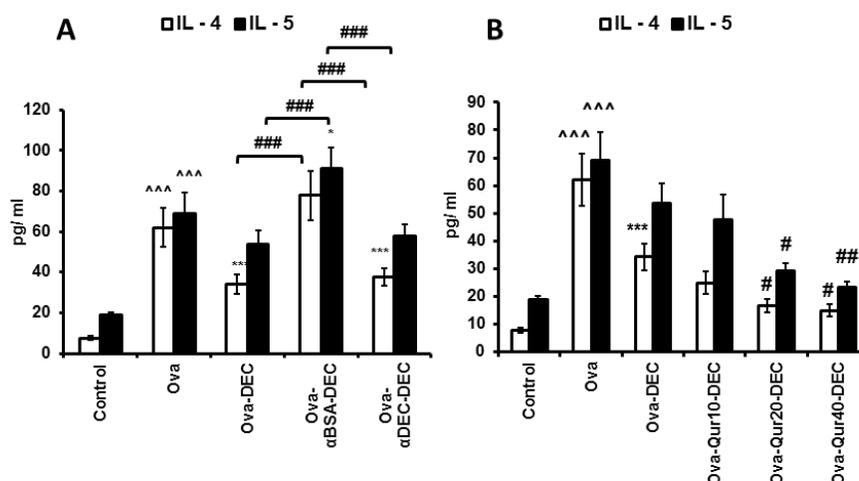
Effect of Anti-DEC Ab and Qur on DEC-induced Reduction in IL-4 and IL-5.

Serum IL-4 and IL-5 levels in the ova group were significantly ($p<0.001$) higher than control (Figure 4A). Ova-DEC group showed a significant reduction in IL-4 and IL-5 ($p<0.001$ and $p=0.04$, respectively) concerning the ova group. Passive immunization with anti-BSA Ab in the ova-αBSA-DEC group increased IL-4 and IL-5 ($p<0.001$) levels in comparison to the ova-DEC group.

However, immunization with anti-DEC Ab did not show significant changes but decreased ($p<0.001$) the levels of cytokines in comparison to the ova-αBSA-DEC group. Higher doses of Qur (20 and 40 mg/Kg) could potentiate the anti-allergic effect of DEC (Figure 4B). This appeared when levels of both IL-4 and IL-5 are downregulated in ova-Qur20-DEC and Ova-Qur40-DEC ($p<0.001$) in comparison to the ova-DEC group.

On the other way, one way ANOVA's data reported a significant level of change in the IFN-γ /IL-4 ratio between ova and control ($p<0.001$; Fig. 5.A). On the other hand; Qur (20 and 40 mg/ Kg) significantly enhanced the effect of DEC for reduction of IL-4 produced from the CD4^{+} Th lymphocytes ($p=0.01$ and $p=0.02$, respectively); therefore the ratio of IFN- γ /IL-4 increased and retained to the control level (Figure 5B).
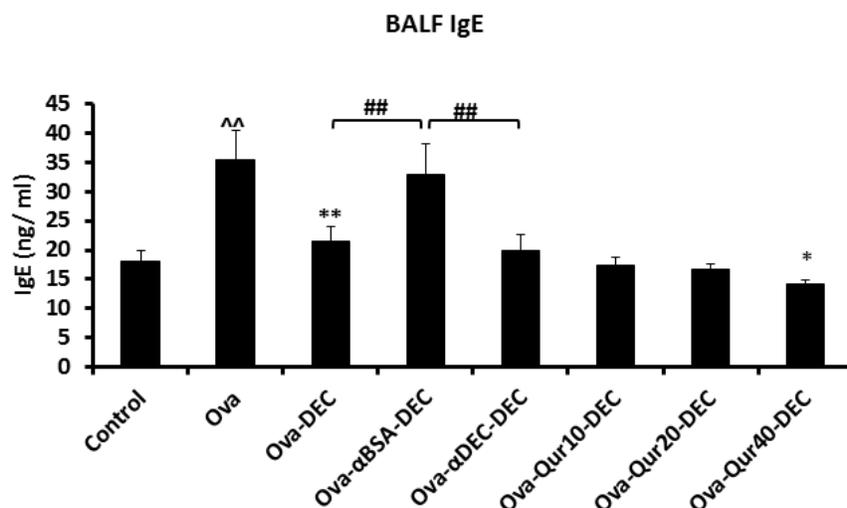
Figure 4. Effect of anti-diethylcarbamazine citrate (anti-DEC) Ab (A) and Qur (B) on DEC-induced reduction in IL–4 and IL– 5. Panel (A): Ova group showed increased (P < 0.001) serum levels of both IL – 4 and IL – 5. DEC reduced the levels of IL–4 and IL–5 significantly (P < 0.001 and *p*= 0.04, respectively). Ova-αDEC-DEC showed a significant reduction in the levels of both cytokines (P < 0.001) versus anti- ova-α bovine serum albumin (BSA)-DEC. Compared to the ova-DEC group, Ova-αBSA-DEC showed a significant increase (P < 0.001) of cytokines. (^) represents the significance level between ova and control, (*) represents significance level between ova and tested groups while (#) represents significance level between ova-αBSA-DEC and each of ova-DEC and ova-αDEC-DEC. Data were represented by mean ± SE. Panel (B): showed the Effect of Quercetin (Qur) at different doses (10, 20, and 40 mg/ Kg) on DEC-induced reduction of serum IL – 4 and IL – 5 levels. Qur at doses 20 and 40 potentiated the effect of DEC for the reduction of the cytokine levels. (^) represents significant differences between Ova and control, (*) represents significant differences between Ova and DEC groups, while (#) represents significant differences between DEC and Qur effects. Data were represented by mean ± SE. Values of *p*> 0.05 were considered non-significant; whereas others are significant with *p*-value < 0.05, *p*< 0.01 and *p*< 0.001
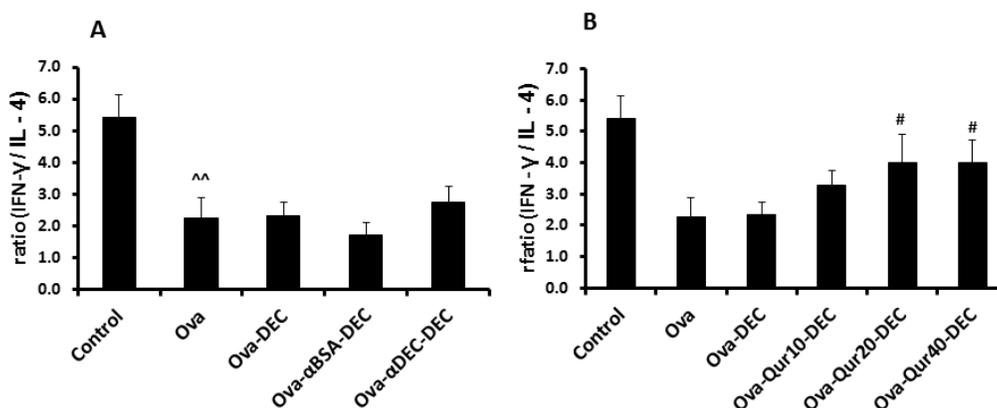
Figure 5: Effect of anti-diethylcarbamazine citrate (anti-DEC) Ab (A), and Quercetin (Qur) (B) on DEC-induced changes in IFN - γ /IL - 4 ratio in different groups. Ova showed a decrease in serum ratio of IFN- γ /IL - 4 (p< 0.001); while Qur (20 and 40 mg/ Kg) potentiated the effect of DEC for reduction of IL-4 produced from the CD4+ Th lymphocytes (p= 0.02 and P = 0.01, respectively); therefore the ratio of IFN-γ/IL-4 retained to the control level. (^) represents the significant differences between ova and control, (#) represents significance level between ova-α bovine serum albumin (BSA)-DEC and each of ova-DEC and ova-αDEC-DEC. Data were represented by mean ± SE, and # or ^ means p< 0.05, ## or ^^ or ** p< 0.01, and ### or ^^^ or ***p< 0.001). Values of p> 0.05 were considered non-significant; whereas others are significant with p-value < 0.05, p< 0.01 and p< 0.001

BALF IgE

Figure 6. Effect of anti-diethylcarbamazine citrate (anti-DEC) Ab and Quercetin (Qur) (10, 20, and 40 mg/Kg) on DEC–induced reduction of total bronchoalveolar lavage fluid (BALF) IgE. Ova group showed a significant increase ($p=0.002$) in total IgE concerning the control group, while the ova-DEC group showed a significant reduction ($p=0.007$) concerning the ova group. Qur at dose 40 mg/Kg potentiated the effect of DEC significantly ($p=0.036$). (^) represents significant differences between Ova and control, (*) represents a significant level between Ova and DEC groups, while (#) represents significant differences between DEC and Qur effects. Data were represented by mean±SE. Values of $p>0.05$ were considered non-significant; whereas others are significant with p-value<0.05, $p<0.01$ and $p<0.001$.

Detection of BALF IgE Level

Specific anti-ova IgE in BALF was significantly higher in ova than the control group ($p<0.001$). The level of IgE in ova-DEC significantly decreased ($p<0.001$) in comparison to the ova group, while ova-αBSA-DEC and ova-αDEC-DEC did not show any changes in comparison to ova-DEC indicating no potentiating effect (Figure 6). Higher doses of Qur (20 and 40 mg/Kg) showed a decrease ($p<0.001$) in comparison to ova-DEC (Figure 6). Total BALF IgE increased significantly ($p<0.001$) in ova in comparison to control, while ova-DEC showed a significant decrease ($p< 0.001$) concerning the ova group. Like serum IL-4 and IL-5, BALF total IgE was significantly higher ($p<0.001$) in ova-αBSA-DEC when compared to both ova-DEC or ova-αDEC-DEC (Figure 6). The maximum dose of the Qur (40 mg/Kg) could potentiate the effect of DEC significantly (Figure 6).

Detection of Lung Tissue EPO and Eotaxin 2

Levels of lung EPO and eotaxin2 increased significantly ($p<0.001$) in ova in comparison to the control (Figs. 7 and 8, respectively). They were decreased significantly ($p=0.003$ and $p=0.02$, respectively) in the ova-DEC group in comparison to the ova group. Levels of EPO and eotaxin2 in passively immunized groups did not show a change in comparison to the ova-DEC group indicating no potentiating effect. EPO and eotaxin2 levels in the ova-Qur40-DEC group showed a significant ($p=0.02$ and $p=0.01$, respectively) reduction effect in comparison to the ova-DEC group indicating a potentiating effect.

Detection of Rabbit Anti-BSA and DEC IgG Levels against Tissue Homogenates

When rabbit anti – BSA IgG was tested against control and ova tissue homogenates (n=10), IgG level was significantly higher ($p<0.001$) against Ova concerning control (Figure 9A). Likewise, anti–DEC IgG was significantly higher ($p=0.03$) against Ova-DEC concerning Ova (Figure 9B).
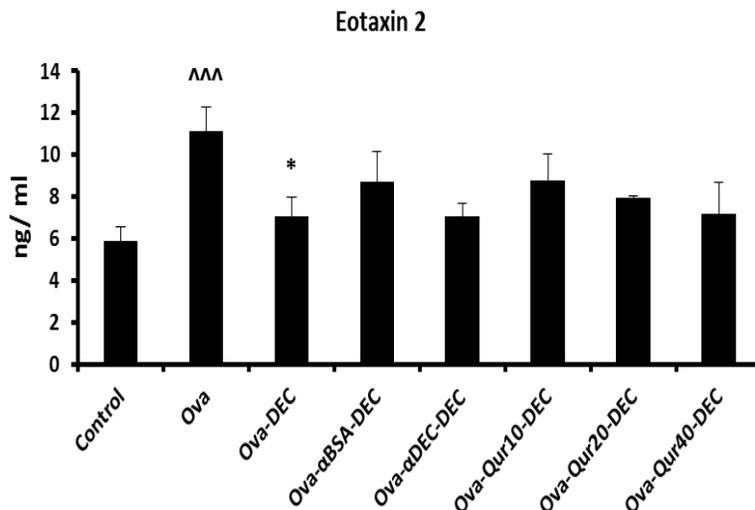
Eotaxin 2

Figure 8. Effect of anti-diethylcarbamazine citrate (anti-DEC) Ab and Quercetin (Qur) (10, 20, and 40 mg/Kg) on DEC – induced reduction of total lung tissue eotaxin 2. Ova group showed a significant increase ($p<0.001$) in eotaxin 2 concerning the control group, while the ova-DEC group showed a significant reduction ($p=0.02$) concerning the ova group. Ova-α bovine serum albumin (BSA)-DEC and ova-αDEC-DEC did not show a significant change concerning the ova-DEC group. Qur at dose 40 mg/ Kg could reduce ($p=0.01$) tissue eotaxin 2 in comparison to ova-DEC group. (^) represents significant differences between Ova and control, while (*) represents significant differences between Ova and DEC groups. Data were represented by mean±SE, while (*) represents the significance differences (whereas, *$p<0.05$, **$p<0.01$ and ***$p<0.001$). Values of $p>0.05$ were considered non-significant; whereas others are significant with p-value < 0.05, $p<0.01$ and $p<0.001$.
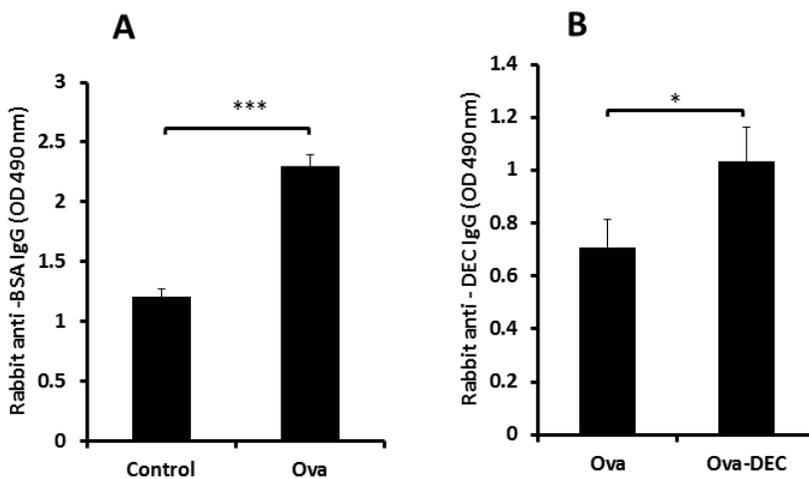
Figure 9. Detection of rabbit anti-bovine serum albumin (anti-BSA) (A) and diethylcarbamazine citrate (DEC) (B) IgG levels against tissue homogenates. Rabbit anti–BSA IgG was significantly higher ($p<0.001$) against Ova with respect to control. Likewise, anti–DEC IgG was significantly higher ($p=0.03$) against Ova-DEC with respect to Ova. Data were represented by mean±SE, while (*) represents the significance differences (whereas, *$p<0.05$, **$p<0.01$ and ***$p<0.001$). Values of $p>0.05$ were considered non-significant; whereas others are significant with p value < 0.05, $p<0.01$ and $p<0.001$.

DISCUSSION

In the current study, the induced model of lung asthma in mice by ova was successful as the levels of Th2 cytokines (IL-4 and IL-5) and IgE (ova-specific and total) were increased. IL-4 and IL-5 are responsible for IgE class switching.[22] Moreover, increased leukocyte infiltration/ thickening of the alveolar wall and lung tissue EPO/eotaxin2 were observed. DEC showed an anti-allergic effect against induced murine lung asthma through decreased cellular infiltration, cytokines, IgE, eosinophil peroxidase (EPO) and eotaxin2.[6] Notably, IL-4, IL-5, and eotaxin were reported before to play a great role in eosinophil trafficking to the lung tissue during lung asthma.[23-25] Anti-DEC Ab was tested as a potentiating agent for DEC pharmacological effect in allergy. This test was conducted to answer the question about the possibility of these antibodies to delay the clearance of DEC and hence potentiate its action against bronchial allergy. This had been found when this Ab was tested as a potentiating agent for the microfilaricidal effect of the drug.[9] Our test did not show a potentiating effect of anti-DEC Ab for the anti-allergic effect of DEC. However, the ELISA test could show the specific reaction to accumulated DEC in DEC-Ova concerning Ova. Thus, the potentiating effect of this Ab for the microfilaricidal effect of DEC could be a result of Ab cross-reaction with microfilariae antigens rather than a reaction to accumulated DEC in microfilariae. This could lead to the rapid clearance of microfilariae without delaying drug clearance. Nevertheless, anti-BSA Ab in the ova-αBSA-DEC group was used as a control to anti-DEC Ab in the ova-αDEC-DEC group. The increased serum levels of IL-4, IL-5, and total BALF IgE in the Ova-αBSA-DEC group concerning ova-DEC and ova-αDEC-DEC groups indicated that this Ab could abrogate the anti-allergic effect of DEC. Furthermore, histological examination showed the same effect of anti-BSA Ab as illustrated by increased leukocyte infiltration. Indeed, the secretion of Th2 cytokines (IL-4 and IL-5) and increased infiltration of leukocytes in the tissue were found to associate with increased IgE.[26] Passive injection with anti-bovine gamma globulin serum was used previously in animals for sensitization.[27] Furthermore, current vaccines like measles which contain BSA were found to induce sensitization and allergic reactions.[28] This had been

observed when rabbit anti-BSA IgG was tested against Ova tissue homogenates in comparison to control. The IgG level was significantly higher against Ova lung homogenates as a result of IgG cross-reaction with the accumulated Ova in lung tissues. This could explain the inability of anti-DEC Ab to potentiate DEC action as a result of the preparation method. The prepared Ab to DEC contains a fraction that is specific for the carrier protein (BSA). This fraction could be responsible for attenuating the effect of anti-DEC Ab to potentiate the DEC effect. Thus, future tests should principally depend on the complete adsorption of produced anti-DEC Ab on BSA before applications. However, passive immunization with allergen-specific IgG (αBSA IgG) was found before to prevent the activity of allergen-specific IgE (ova-specific IgE).[14] This is why the level of ova-specific IgE in ova- αBSA-DEC in comparison to ova-DEC and ova- αDEC-DEC groups was different from total IgE. In the former case, it was not significantly higher than in other groups.

The potentiation of the DEC anti-allergic effect by the Qur was found to be dependent on its dose (10, 20, and 40 mg/Kg body weight). Higher doses (20 and 40 mg/Kg) could show a potentiation effect on DEC for the reduction of IL-4, IL-5, IgE, EPO, and eotaxin 2. It was also postulated that Qur at dose 20 mg/Kg is enough to act as a bronchodilator in experimental asthma.[29] Previous studies confirmed the anti-allergic effect of the Qur through decreased levels of IL-4 and IL-5 versus increased levels of IFN-γ.[13] Other allergic reactions were found to decrease after the Qur treatment. For histopathological examination, Qur at dose 40 mg/Kg could recover the inflammatory reactions in the lung tissue in comparison to the ova-DEC group. This dose also showed significant potentiation for the DEC effect to reduce tissue EPO and eotaxin2 which are diagnostic markers for tissue eosinophilia. Similarly, Qur was found to reduce the recruitment of eosinophils and neutrophils during allergic reactions.[30]

Based on the fact that IFN-γ displays and presents more immunomodulatory properties than the type I interferons,[31] the present changes in levels of IFN-γ /IL-4 ratios indicated that the classical Th2 pattern of cytokine production and CD4+ Th lymphocytes are closely associated and contribute to the pathogenesis of allergic inflammation.[32] High levels of IL-4, IL-5, IL-9, and IL-13 are produced in the bronchoalveolar lavage

(BAL) fluids and airway biopsies, which orchestrate the recruitment and activation of effector cells related to allergic responses, such as eosinophils and mast cells. Moreover, the present data illustrated the potential effects of Qur different doses with DEC for reduction of the IL-4 cytokine produced from the CD4+ Th lymphocytes; therefore the ratio of IFN-γ/IL-4 retained to control level effect. IFN- γ plays a crucial role in inhibiting Th2 responses. Loss of IL-4 receptor responsiveness may be another mechanism that suppresses Th2 development in polarizing Th1 cells in case of asthma inflammation.[33] The present data proved to counteract the roles of IFN-γ in the Th2 differentiation and proliferation processes.[34] The present data came following others like Teixeira et al[33] and Bocek et al[35] who assumed that the precise amount of cytokines may be the key for the dominant effect of IFN- γ to enhance or suppress Th2 priming. IFN-γ had shown an induction for apoptosis in T cells and eosinophils through caspase and CD95/Fas-mediated mechanisms, respectively.[36,37] This represented an alternative explanation for the suppressive effects of IFN- γ directly on the local recruitment of these cells in allergic situations.

Following the present changes of IgE levels after potentiation with Qur, data supported the critical role of IFN-γ in allergic reactions and proved its ability to inhibit immunoglobulin class switching to IgE, which is an important mediator of allergic pathologies induced by Th2 cytokines as discussed before.[33] The potentiation of drug anti-inflammatory effects by other drugs was previously reported. Generally, it showed that another one that may be synergistic to manage the complex inflammatory reactions could potentiate the anti-inflammatory effect of the drug. Qur was used to potentiate the cytotoxic activity of luteolin to cancer cell lines.[38] In addition, it was used to strengthen the ability of neural growth factors for cell growth.[39] The current study introduced a novel approach to improve the anti-allergic effect of DEC in lung asthma by one of the flavonoids which are existed naturally in some vegetables and fruits. It was previously recommended as a potential therapeutic choice against lung asthma.[40] The limitations of the current study represented the necessity of using BSA as a conjugate to induce the production of anti-DEC IgG antibodies. These polyclonal antibodies showed a cross-reaction to ovalbumin to produce inflammatory responses which could have led to a reduction in the potential effect of anti-DEC antibodies on the DEC anti-allergic effect.

Passive immunization with anti-DEC Ab did not potentiate the anti-allergic effect of DEC. This can be referred to as the presence of a reactive fraction of this Ab with BSA which increased the allergic reactions. This could imply the necessity of adsorption techniques before Ab applications. On the other hand, Qur at 40 mg/ Kg could be recommended to potentiate the DEC effect suggesting its potential as a novel approach for treatment.

CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

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