METFORMIN'S IMPACT ON SERUM IMUNOGLOBULINS AND BRONCHOALVEOLAR LAVAGE CELLS IN MICE WITH COMPLICATED SEVERE ASTHMA

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METFORMIN'S IMPACT ON SERUM IMUNOGLOBULINS AND BRONCHOALVEOLAR LAVAGE CELLS IN MICE WITH COMPLICATED SEVERE ASTHMA (Abstract): **Background:** Severe asthma represents an important burden of mortality and morbidity in asthma being a clinical condition that has the risk of evolution to acute lung injury (ALI). Based on the anti-inflammatory and airway anti-remodeling effect of Metformin we presumed that it will promote beneficial effects in severe asthma complicated to ALI. Our study aimed to evaluate the effects of Metformin on a murine model of severe asthma complicated with ALI by assessing the modulatory effect of Metformin of plasma IgE levels and on differential cell count from bronchoalveolar lavage fluid. **Materials and methods**: There were three groups of BALB/c mice with ovalbumin (OVA)-induced asthma followed by ALI induction which consisted in OVA sensitization (days 0 and 7) and challenge (days 14, 15, 16 and 17) followed by lipopolysaccharide (LPS) intratracheal instillations (days 15 and 17) treated 30 min before OVA, in days 14-17: positive control group for disease (OVA+LPS), Dexamethasone treatment group (DEXA + OVA + LPS) and Metformin treatment group (OVA + LPS + METF). Measurement of OVA-specific IgE levels was made and bronchoalveolar lavage fluid (BALF) was collected in order to apply differential cell count. **Results**: administration of Metformin decreased the airway macrophage infiltration with no modulatory effect on airway neutrophilic infiltration. **Conclusions**: Metformin has the potential to influence the physio-pathological changes that occur in asthma followed by ALI. However, new studies to explore the specific mechanism by which Metformin influences the inflammatory and immune response in asthma/ALI overlap are needed. **Keywords**: METFORMIN, SEVERE ASTHMA, ACUTE LUNG INJURY, LIPOPOLY-SACCHARIDE.

INTRODUCTION

Asthma is a respiratory disease characterized by airway chronic inflammation and remodeling. It has a high prevalence being estimated that 334 million people worldwide are suffering from asthma (1). Severe asthma proposes a significant challenge in clinical practice considered to affect a small proportion of asthmatic patients but accounts for a significant burden of morbidity and mortality correlated with asthma (2). Available data show that severe asthma is involved in increasing the risk of pneumonia which is the most common cause for acute lung injury. Therefore, asthma is a condition that influences the risk of progression to acute lung injury (3, 4).

Acute lung injury (ALI) consists in a common respiratory disease defined by acute lung inflammation having as main pathological characteristics inflammatory cell infiltration and high permeability pulmonary edema (5). Although the therapeutic strategies of this disease are complex, the mortality and morbidity levels are high (6).

Metformin (METF), a drug used as first line agent in diabetes type 2 (7). Recent studies have highlighted its pleiotropic pharmacodynamic profile (8) and its beneficial involvement in both bronchial asthma and ALI based mainly on its anti-inflammatory and airway anti-remodeling effect (9-11).

The aim of our study was to evaluate the effects of Metformin on a murine model of severe asthma complicated with ALI by determining IgE plasmatic concentrations and evaluating the differential cell count from bronchoalveolar fluid (BALF).

MATERIAL AND METHODS

Study Design

The experiment was carried on 18 mice (females BALB/c, weighing 17-23g) from "Cantacuzino" Institute in Bucharest. Experimental site was in CEMEX facility of "Grigore T. Popa" University of Medicine and Pharmacy of Iasi, under normal conditions of temperature (22±3°C), humidity (55±5%), 12 hours day/night cycles of and with water and food *ad libitum*. Ethical approval (No. 335/17.07.2023) was obtained from The Research Ethics Committee of the "Grigore T. Popa" University of Medicine and Pharmacy of Iași, Romania.

Animals, divided into 3 groups of 6 animals each, were treated intraperitoneally, once a day, as follows: OVA+LPS group (positive control for disease) with saline solution (0.5 ml/mouse); OVA+LPS+DEXA group (DEXA group; positive control for treatment) with Dexamethasone (1 mg/kg); OVA+LPS+METFgroup (Metformin group) with Metformin (250 mg/kg).

Induction of severe allergic asthma and lung inflammation was realized with OVA(GRADE V, Sigma-Aldrich, St. Louis, MO, USA), through sensitization procedure on days 0 and 7 through intraperitoneal once a day administration of 0.2 ml/mouse OVA/aluminium hydroxide solution(Sigma-Aldrich Chemie GmbH, Deutschland) (12),and followed by the challenge procedure on days 14, 15, 16, 17, with OVA 1% aerosols (10 ml/mouse) and lipopolysaccharide (LPS) (lipopolysaccharide from Escherichia coli 0127: B8, Sigma-Aldrich, St.Louis, MO, USA) intratracheal administration on days 15 and 17 (13).

Measurement of plasma IgE levels

Blood was collected by intracardiac puncture in 0.5ml microcentrifuge tubes and the serum was separated from whole blood

by centrifugation at 3.000 rpm at 4°C for 10 min and immediately frozen at −80°C. Total IgE levels were measured with enzyme-linked immunosorbent assay (ELISA) using OVA-specific IgE using a Legend Max Mouse OVA Specific IgE ELISA kit (Biolegend, USA). ELISA plates were read with an Infinite 200 PRO M Plex Tecan 314 plate reader (Tecan, Austria) and Magellan 7.4 software (Tecan, Austria).

Bronchoalveolar lavage fluid (BALF) and differential cell count

BALF collection was through an incision in the trachea which was exposed and then cannulated and began 24 hours following the last challenge with OVA, under ketamine and xylazine general anesthesia. The BALF collection process involved three successive infusions-aspirations of 0.5 ml warm (37°C) PBS through the tracheal cannula, totaling 1.5 ml. The fluid was kept on ice until centrifuged at 250 g for 5 min at 4˚C. The supernatants were collected and transferred to Eppendorf tubes, centrifuged at 10,000 g for 15 min at 4˚C, aliquoted as 150 µl samples into other tubes and stored at -80˚C until assessment of cytokines. Following vortex mixing, 5 µl of the residual cell pellet from the 250 g centrifugation were used to prepare slides that were next stained with May-Grünwald (Sigma-Aldrich, 1.01424, USA) and Giemsa (Sigma-Aldrich, 1.09204, USA). Using an optical microscope (Leica DM6 B, Germany) with a dry objective lens (40x), ten microscopic fields were examined to determine the mean cell count per field. Additionally, in order to differentiate neutrophils, eosinophils, lymphocytes, and macrophages, 200 cells were examined with an immersion objective lens (100x), using the same optical microscope.

Statistical Analysis

Data is presented as means ± standard error (SE). The statistical analysis was done using *SPSS* software. The assessment of statistically significant differences between groups was made using parametric tests (One-Way Analysis of Variance (ANOVA) followed by the Holm-Sidak method for multiple parametric comparisons), or nonparametric tests (Kruskal-Wallis One-Way ANOVA on ranks). P-values less than 0.05 were statistically significant.

RESULTS

Plasma Ig E levels

The serum levels of OVA-specific IgE were evaluated in all three groups. The highest plasma IgE levels were detected in DEXA group followed by the levels induced by Metformin administration, but without significant statistically differences between the treated groups and the positive control group for disease (Kruskal-Wallis One-way ANOVA on ranks: H = 3.500 with 2 degrees of freedom; p=0.174; ns).

Analysis of cell count in Bronchoalveolar Lavage Fluid (BALF)

Figure 1 shows the microscopic characteristics of inflammatory cells from BALF.

Intratracheal instillation of LPS resulted in a significant predominance of neutrophilic infiltration observed in the OVA+LPS group, confirming the potency of the ALI-induction experimental model. Treatment with Metformin did not alleviate the neutrophilic infiltration, without no significant statistical differences between the OVA + LPS + METF group and the

OVA+LPS group (p>0.05). The treatment with Dexamethasone did not exhibit an effect on the neutrophilic infiltration either, with no statistically significant differences noted between the DEXA group and the OVA+LPS group (p>0.05). Treatment with METF resulted in a decrease in the total number of macrophages compared to OVA + LPS group (p<0.05), but did not induce a significant statistical difference compared to DEXA group (p>0.05). Compared to OVA + LPS group the number of lymphocytes from DEXA group and METF group was not significant statistically different (One-way ANOVA: F(2,15) = 0.162 p = 0.852; ns). The assessment of eosinophils in BALF did not provide any significant statistical differences between groups (One-way ANOVA: F(2,15) = 0.678 p = 0.525; ns).

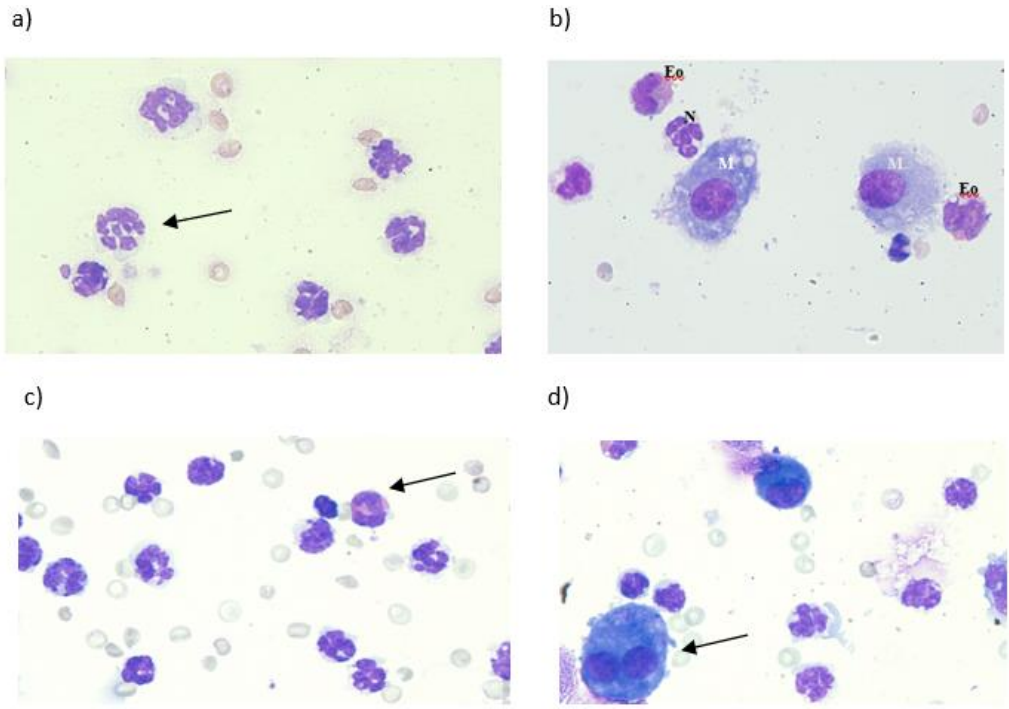

Fig. 1. Differential cell counts on optical microscopy, x100 immersion objective a) OVA + LPS group: neutrophils, hyper-segmented neutrophil (arrow); b) OVA + LPS +DEXA group: alveolar macrophages (M), neutrophils (N) and eosinophils (Eo); c) OVA + LPS +METF group: neutrophils and an eosinophil (arrow); d) OVA + LPS +METF group: neutrophils and two alveolar macrophages, one binucleated (arrow); OVA + LPS: positive control for disease; OVA + LPS + DEXA: Dexamethasone treated group; OVA + LPS + METF: Metformin treated group.

In each group, the differential number of cells represented by eosinophils, neutrophils, lymphocytes and macrophages was counted (fig. 2)

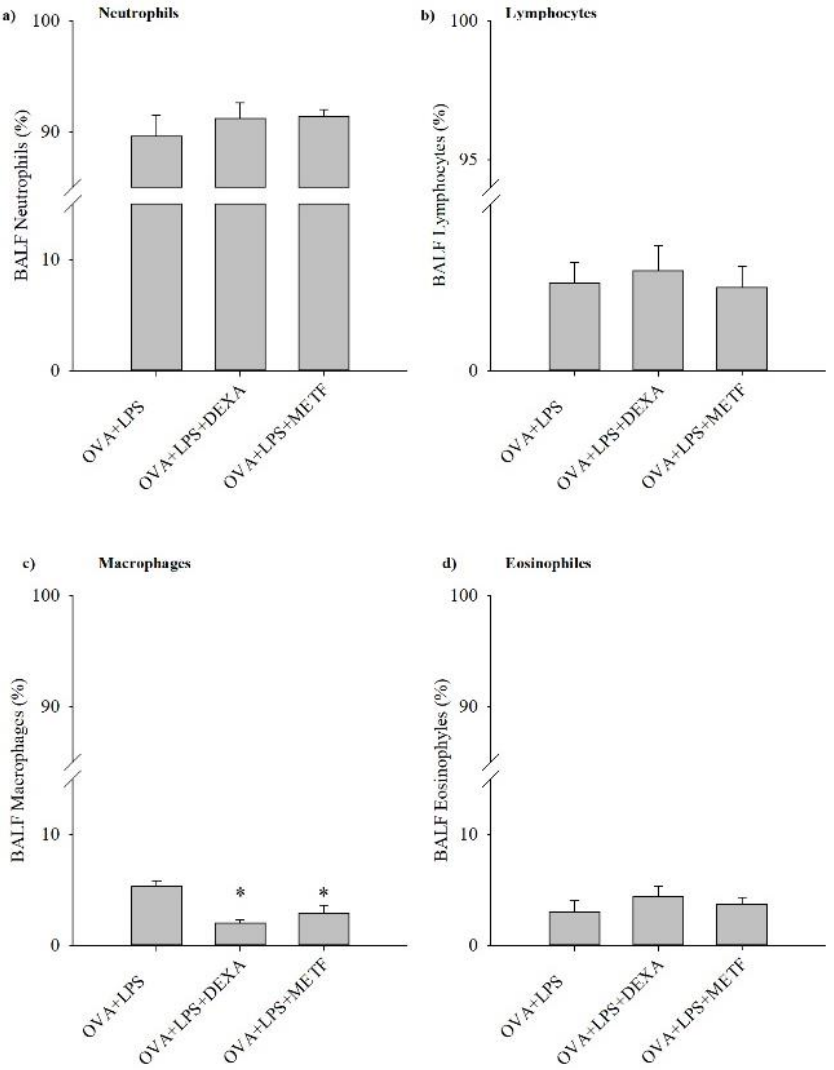

Fig. 2. Cell differential count in bronchoalveolar lavage fluid in percentages for (a) neutrophils; (b) lymphocytes; (c) macrophages; (d) eosinophils. Values are expressed as mean ± standard error; n=6. One-way ANOVA: * $p < 0.05$ *vs.* OVA+LPS group; followed by the Holm-Sidak method for multiple comparisons. OVA + LPS: positive control for disease; OVA + LPS + DEXA: Dexamethasone treated group; OVA + LPS + METF: Metformin treated group.

DISCUSSION

The results of our research highlighted the rich predominant neutrophilic airway infiltration which confirms the authenticity of the experimental model. It is considered that the neutrophilic airway infiltration

represents a defining feature of ALI which promotes epithelium and endothelium lesions through the release of cytotoxic molecules (14). Our study shows that the total neutrophils count induced by LPS was not influenced by the administration of Metformin nor by Dexamethasone. The results showing the eosinophilic and lymphocytes airway infiltration in all three experimental groups are sustaining the potency of the induced experimental model.

Data from literature shows the alleviating effect of Metformin on the airway neutrophilic infiltration in a mice model of ALI. The research published by Yu L *et al.*, points that Metformin is efficient in decreasing neutrophilic airway infiltration through inhibiting myeloperoxidase activity (15). Thus, our study presents contradictory results, this aspect could be interpreted in the context of the complexity of the animal model, being a severe asthma ALI overlap which leads to the idea of other possible underlying mechanisms of Metformin in this respiratory pathology. However, another study indicates that Metformin is efficiently in reducing neutrophilic infiltration in LPS-induced ALI in diabetic mice exposed to methylglyoxal MGO, being proposed the scavenger MGO activity of Metformin. The particular point of this study is that Metformin by itself in diabetic mice which were not exposed to MGO did not influence the neutrophilic infiltration induced by LPS (14).

Our statistical analyze on BALF cell count revealed that Metformin was efficient in decreasing the macrophage airway infiltration induced by LPS administration. Our results are confirmed by another study which points the effect of Metformin in reducing macrophage infiltration in an experimental model of ALI by suppressing LPS-induced endothelial cell pyroptosis mediated by SIRT-1 activation (16). Data show the essential role of macrophage in inflammatory respiratory diseases like allergic asthma, ALI and chronic obstructive pulmonary by initiating immunity and host defense (17, 18). In ALI the macrophages are involved in the modulation of the inflammatory response and also in the process of repairing damaged tissue (19). Based on the important role of macrophages in alveolar and lung environment the regulation process of macrophage function is proposed as therapeutic target in inflammatory respiratory disease (18).

Regarding the role of Metformin in modulating Ig E levels in respiratory diseases the published data show that Metformin has the ability to reduce OVA-specific Ig E in chronic asthma in mice (9). Our study consisting in severe asthma complicated with ALI revealed no modulatory effect of Metformin nor Dexamethasone on OVA-specific IgE levels.

Our research did not show significant differences between the potency of Metformin and Dexamethasone treatment. An interesting result is the rich neutrophilic infiltration in DEXA group. These findings can be approached based on the fact that in steroid resistant asthma phenotype Dexamethasone proven to have an impaired function in inhibiting IL-8 production which leads to persistent neutrophilic inflammation (20). Also, our results are consistent with another study that reveals that Dexamethasone treatment fails to reduce neutrophilic airway inflammation in a murine model of asthma (21).

The research direction of our study is valuable considering the clinical studies

which aim to explore the potential of Metformin treatment in reducing asthma exacerbations and acute lung severity in diabetic patients. The clinical data have controversial results, being shown the positive effect of Metformin treatment in reducing the risk of asthma exacerbation in diabetic patients (22), but also there are studies that reveal that Metformin treatment increased the risk of exacerbation asthma in diabetic patients (23).

CONCLUSIONS

Our research revealed the potency of Metformin in reducing the airway macrophage infiltration, a therapeutic target that could be influenced in severe asthma complicated with ALI. The results open the necessity of following experimental research in order to assess the therapeutic effect of Metformin in asthma-ALI overlap.

CONFLICT OF INTEREST AND FUNDING

The authors declare no conflict of interest.

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