

Protective effect of aminoguanidine against acute lung injury induced by influenza A(H1N1)pdm09 (mouse-adapted) virus in mice with diabetes mellitus

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ABSTRACT: Advanced glycation end products (AGEs) formation is a reason for protein dysfunction and inflammatory response during acute lung injury (ALI) and diabetes mellitus (DM). Previous studies showed the efficacy of AGEs blockers against inflammatory response and protein dysfunction. But the efficacy of AGEs blockers against ALI with combined pathology stays unclear. This study was performed on albino female mice with DM. Virus-induced ALI was induced by the pandemic strain of influenza virus A(H1N1)pdm09. Experimental DM was induced by a single subcutaneous injection of alloxan monohydrate (180 mg/kg). Aminoguanidine bicarbonate (25 mg/kg, subcutaneously) was administered during 7 days post-infection. In our research, we evaluated parameters of ALI during influenza infection such as survival rate, blood-oxygen saturation, levels of cytokines in lung tissue, specific hematological parameters, lung tissue morphology, and AGEs level in the lungs. The protective effect of aminoguanidine was confirmed by reduction mortality, decreasing of hypoxia and limitation of lung damage ($p < 0.05$). At the same time, a reduction of inflammation response and AGEs level in the lung was observed in treated infected mice with DM ($p < 0.05$). Based on obtained results, aminoguanidine showed efficacy against virus-induced ALI with DM.

KEYWORDS: advanced glycation end products; acute lung injury; aminoguanidine; diabetes mellitus; influenza.

1. INTRODUCTION

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are dangerous complications of respiratory viral infections [1-3]. Mortality ranges from 15 to 40% among hospitalized patients with ALI/ARDS [4-6]. A similar situation is being observed in patients with COVID-19. [7]. Lethal outcome risk is significantly higher among patients with comorbidity, especially those with diabetes mellitus (DM) [8, 9]. One of the reasons for these outcomes is the insufficient efficacy of treatment, especially the anti-inflammatory drugs. It makes a demand for finding new drugs against ALI/ARDS.

Various proteins are involved in the inflammatory process during influenza infection such as Toll-like receptors, adenosine receptors, sphingosine-1-phosphate receptor, platelet factor activation receptors [10-12]. At the same time, other studies showed a significant role of advanced glycation end products (AGEs) in inflammatory response for various pathologies [13-16]. AGEs are the products of amino acids glycation in biomolecules by reactive carbon compounds like methylglyoxal and 3-deoxyglucosone [17]. These products are involved in the inflammatory process via specific AGEs receptors in cell membrane. AGEs binding to these receptors leads to activation factor NF- κ B via the NADPH-oxidase/ROS/p21RAS/p38 signal pathway. NF- κ B acts as a transcription factor for translation genes of TNF- α , IL-1, IL-6, IL-8, INF- α , INF- γ , accumulation of reactive oxygen species and activation of the caspase pathway of apoptosis [18-20]. Despite a similar mechanism of inflammation, some aspects stay unclear for role AGEs in virus-induced ALI [21].

Previous studies showed several compounds with the ability to alter AGEs level. [22, 23]. Their mechanism of action linked to the prevention of AGEs formation via pre-AGEs molecules binding or destruction of the formed AGEs [22, 24]. Aminoguanidine, ALT-946, and LR-90 can bind free reactive carboxylic compounds (pre-AGEs) [25-27]. This interaction prevents glycation of amino groups in proteins, glyco- and lipoproteins and protects against dysfunctions. ALT-711 and TRC4149 are capable of breaking the

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bond in AGEs between amino groups of biomolecules and pre-AGE that leads to the restoration of functional activity [28, 29]. Previous studies showed the efficacy of aminoguanidine, AGEs blockers for the treatment of ALI induced by ionizing radiation and lipopolysaccharide [15, 16]. Recently we confirmed the efficacy of aminoguanidine against virus-associated ALI without combined pathology [30]. According of these results AGEs blocker has the perspective for the treatment of virus-induced ALI. However, the efficacy of this treatment for influenza-induced ALI with DM was not shown previously.

In this study, we examined the protective effects of aminoguanidine, as AGEs blockers, against virus-induced ALI with DM. We analyzed parameters of combined pathology: survival rate, blood-oxygen saturation, the lung injury score, and the degree of lung inflammation. In addition, we measured the lymphocyte-granulocyte index (LGI), thrombocyte number, proinflammatory cytokine level, and AGEs level in the lungs.

2. RESULTS

2.1. Confirmation of ALI model

The infected mice (group 2) showed clinical features of the ALI on 3 day post-infection (DPI). Clinical manifestations were similar for all infected mice: ruffled fur, suppression of locomotor activity, hunched back, decreased muscle tone, dyspnea and tachypnea, involved accessory muscles to breathe. Mortality was observed from 4 DPI to 13 DPI and reached 50%. At the same time, a progressive decrease of the saturation level reached $48.80 \pm 0.95\%$ on 7 DPI. Massive hemorrhages and structural changes in the lungs (0.61 ± 0.01 scores on 7 DPI) were shown. All results are given in Figure 1. These changes were accompanied by the presence of edema, leukocyte infiltration, hyaline membranes, and thickening of the alveolar septum. Alteration zones of respiratory epitheliocytes and atelectasis with the microcirculatory disorder have been shown in the lungs. At the same time, a plethora of capillaries with hemorrhages in alveolar space has been noted. Supporting histology is presented in Figure 2. Systemic inflammatory response syndrome had been developed during ALI, which was confirmed by next several parameters: increased proinflammatory cytokines concentration (TNF- α to 46.00 ± 2.21 pg/g; IL-6 to 806.80 ± 51.60 pg/g; IL-1 β to 501.80 ± 32.60 pg/g), hematological changes in the leukocyte formula (decreased LGI to 1.34 ± 0.08 scores) with thrombocytosis (increased thrombocytes concentration to $989.0 \pm 40.16 \times 10^9/l$). At the same time, noted changes were accompanied by the increasing AGEs concentration in the lung (to 42073 ± 1240 AU/g). All results are presented in Figure 3.

2.2. The aggravating role of DM during ALI

Alloxan-induced DM provoked intense accumulation of AGEs level in the lungs (to 50536 ± 1289 AU/g) with an increase of mortality rate to 80% and severity of hypoxemia to $40.80 \pm 0.80\%$ in infected mice with DM (group 3) as shown in Figure 1 and 3. An aggravation of ALI towards an inflammatory response was noted. An increase of proinflammatory cytokines levels was shown on 4 DPI (TNF- α to 49.40 ± 2.04 pg/g; IL-6 to 448.40 ± 14.15 pg/g; IL-1 β to 450.80 ± 25.49 pg/g). 1.5-2 times increase of these cytokine concentrations was indicated on 7 DPI (TNF- α to 61.40 ± 2.77 pg/g; IL-6 to 885.80 ± 29.34 pg/g and IL-1 β to 708.00 ± 30.58 pg/g). A decrease of LGI (to 1.08 ± 0.04 scores) and an increase of thrombocyte number (to $1027.00 \pm 36.07 \times 10^9/l$) were shown on 4 DPI. All results are given in Figure 3. Morphological changes in the lungs included an increase in the number of edematous alveoli, cellular infiltration and alteration zone in infected mice with DM as presented in Figure 2. The lung injury scoring and the degree of lung inflammation showed an aggravation of ALI (increased to 0.67 ± 0.01 and to 0.37 ± 0.01 scores, respectively), as shown in Figure 1.

2.3. Correction of ALI with DM by aminoguanidine

Experimental therapy by aminoguanidine of virus-induced ALI with DM (group 4) showed a correction of AGEs level in the lungs (to 36760 ± 1786 AU/g) as shown in Figure 3. These changes were accompanied by a decrease in the severity of pathology. The survival rate reached 65% in treated infected mice with DM (group 4). A decrease of hypoxemia (to $59.00 \pm 2.09\%$) and reduced incidence of hyaline membrane with alteration zones in lungs were shown during ALI with DM (group 4). All results are given in Figure 1 and 2. At the same time, correction of lung injury (to 0.58 ± 0.01 scores) with an inflammatory component of lung injury was indicated (to 0.45 ± 0.01 scores) as presented in Figure 1. System inflammatory response syndrome was decreased in treated infected mice with DM (group 4).

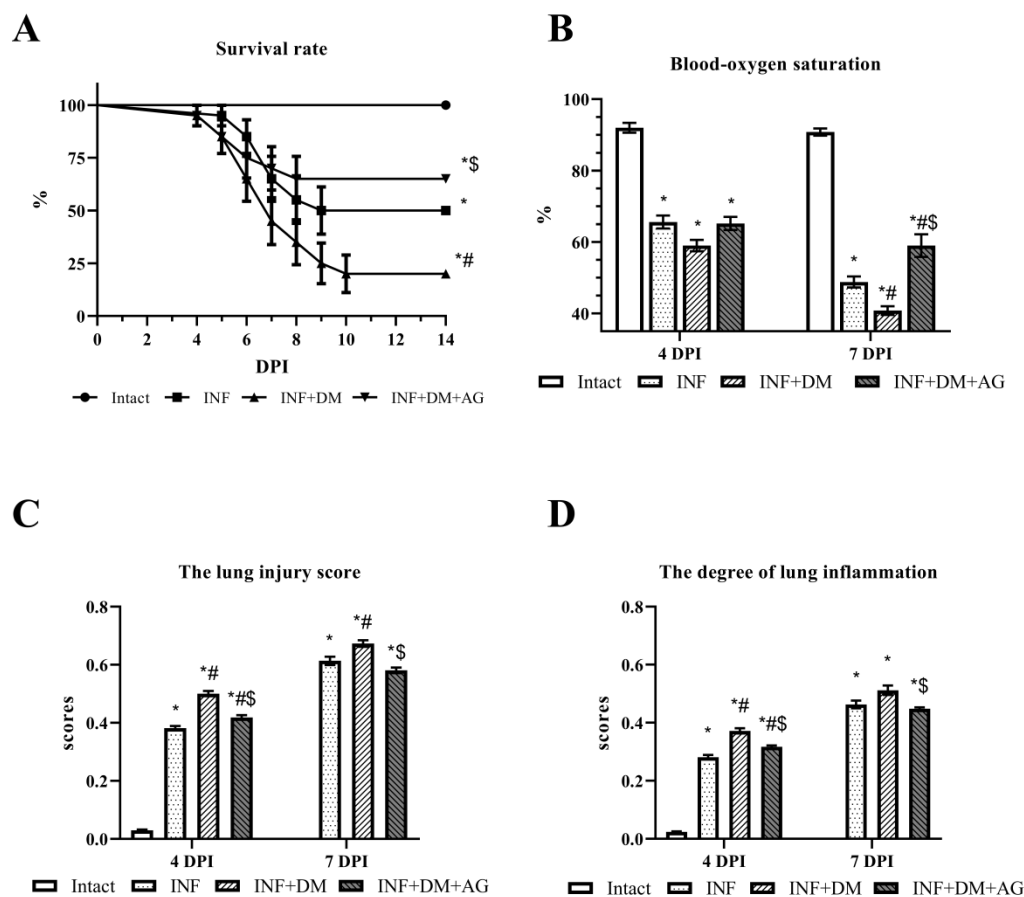


Figure 1. Parameters of virus-induced acute lung injury with experimental diabetes mellitus in mice on 4 day post-infection (DPI) and 7 DPI (A – Survival rate; B – Blood-oxygen saturation; C – The lung injury score; D – The degree of lung inflammation). Data are expressed as the Mean \pm SEM (n=5). Statistical significance was tested by Kruskal-Wallis and Tukey's post hoc analyses. Intact – intact mice, INF – infected mice without comorbidities and treated by placebo, INF+DM – infected mice with DM and treated by placebo, INF+DM+AG – infected mice with DM and treated by aminoguanidine. * – $p < 0.05$ compared to Intact; # – $p < 0.05$ compared to INF; \$ – $p < 0.05$ compared to INF+DM.

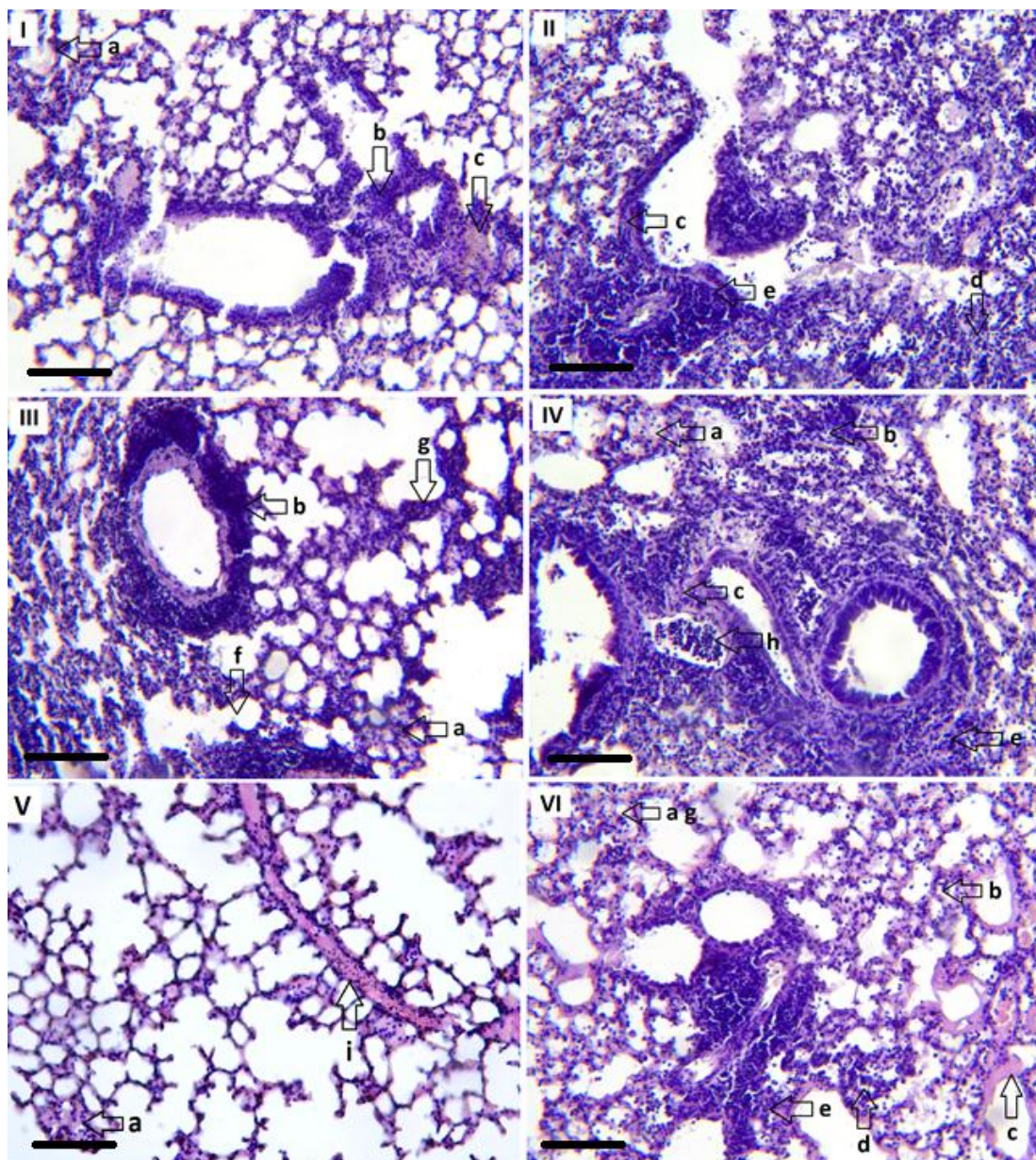


Figure 2. Histological features of the lung of mice on 4 day post-infection (DPI) and 7 DPI. Hematoxylin and Eosin stains. Magnification $\times 100$. The scale bar showed 200 μm in distance. I – infected mice without comorbidities and treated by placebo on 4 DPI; II – infected mice without comorbidities and treated by placebo on 7 DPI; III – infected mice with alloxan-induced DM on 4 DPI; IV – infected mice with alloxan-induced DM on 7 DPI; V – infected mice with DM and treated by aminoguanidine on 4 DPI; VI – infected mice with DM and treated by aminoguanidine on 7 DPI. a – alveolar edema; b – increased cellular infiltrate; c – hyaline membrane; d – atelectasis; e – “hepatization” pulmonary parenchyma; f – protein debris; g – thickening of the alveolar septum; h – hemorrhage; i – vascular plethora of the lungs.

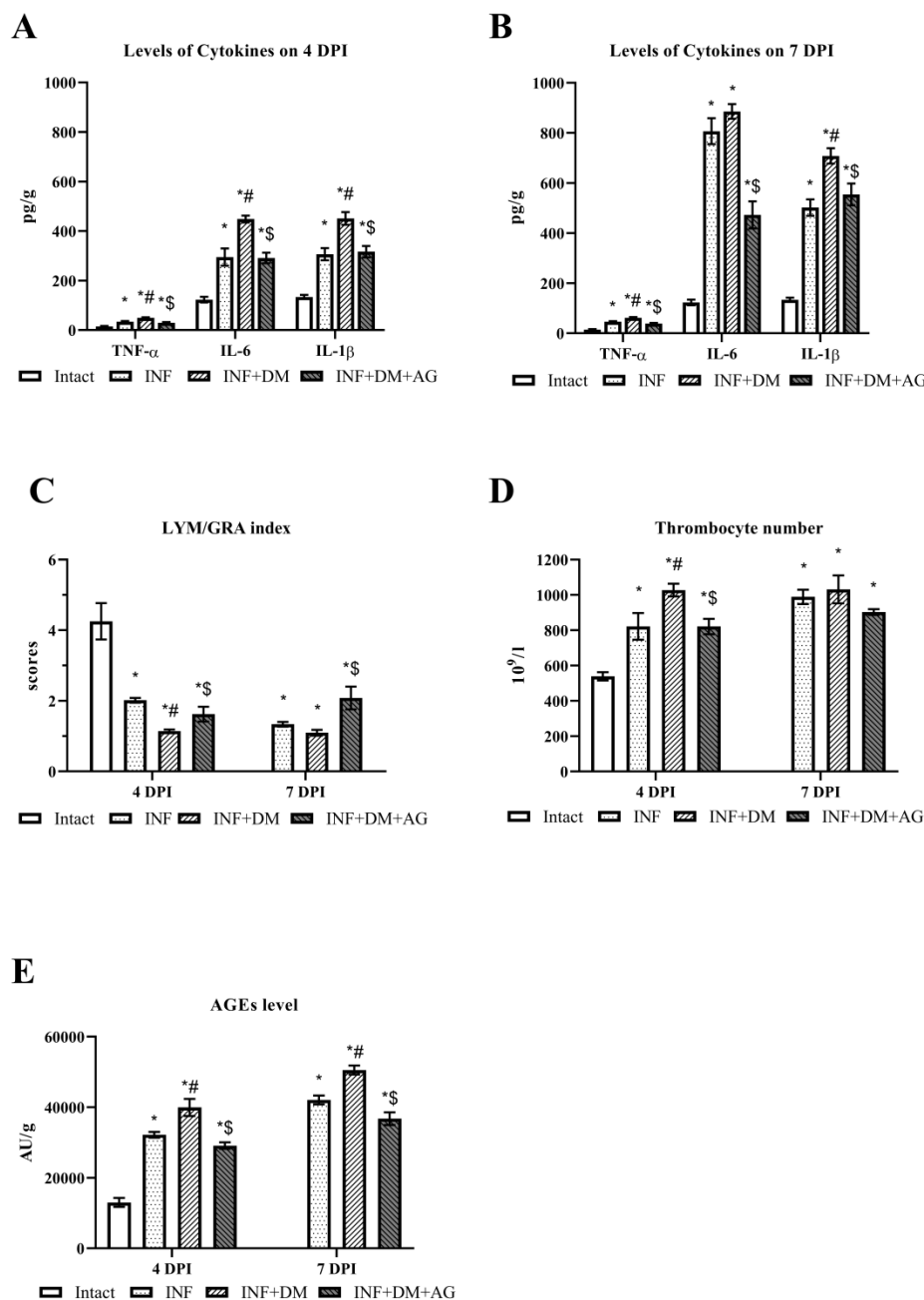


Figure 3. Additional parameters of virus-induced acute lung injury with experimental diabetes mellitus in mice on 4 day post-infection (DPI) and 7 DPI (A - Levels of cytokines in lung on 4 DPI and B - 7 DPI; C- Lymphocyte-granulocyte index (LYM/GRA index); D-Thrombocyte number; E-Advanced glycation end-products (AGEs) level). Statistical significance was tested by Kruskal-Wallis and Tukey's post hoc analyses. Intact - intact mice, INF - infected mice without comorbidities and treated by placebo, INF+DM - infected mice with DM and treated by placebo, INF+DM+AG - infected mice with DM and treated by aminoguanidine. Data are expressed as the Mean \pm SEM (n=5). * - p<0.05 compared to Intact; # - p<0.05 compared to INF; \$ - p<0.05 compared to INF+DM.

Decreasing of proinflammatory cytokines concentration was shown in the lungs (TNF- α to 38.20 ± 2.87 pg/g; IL-6 to 472.40 ± 54.43 pg/g; IL-1 β to 554.00 ± 43.69 pg/g. An increase of LGI (to 2.08 ± 0.32 score) and a correction of thrombocytes number to $821.00 \pm 43.40 \times 10^9/l$ on 4 DPI were demonstrated. All results are given in Figure 3.

3. DISCUSSION

The current study showed a protective effect of AGEs blocker against ALI, induced by influenza virus A(H1N1)pdm09 in mice with DM.

Similar to DM, ALI is accompanied by an accumulation of reactive carbonyl species. An increase AGEs level is associated with respiratory failure, provoked by alveolar damage and disruption of gaseous exchange. A disruption contributes to the progressive development of hypoxia and provokes metabolic alterations with affecting Krebs cycle. The tricarboxylic acid cycle plays a major role in the oxygen-dependent catabolism of glucose. A disruption of this cycle changes glucose metabolism to an alternative pathway, without oxygen-dependent enzymes [17, 31]. As a result, this shift provokes the accumulation of reactive carbonyl species and AGEs.

High AGEs level is associated with a disruption of cellular glucose transport during DM. Insufficient level of insulin (DM first type) or altered insulin-dependent glucose transport (DM second type) is the reason for these pathologic changes [32]. Dramatically increased free glucose concentration in the blood leads to increasing the role of non-enzymatic metabolism of these carbohydrates and the accumulation of AGEs and their precursors [33]. Therefore, increased AGEs level influences the severity of influenza infection during combined pathology and was confirmed by our results.

The ability of aminoguanidine to block AGEs accumulation has been confirmed in other studies. This AGEs blocker prevents an increase of AGEs level in the left ventricular in rats with streptozotocin-induced DM and in the heart, aorta and kidneys in rat during aging [23, 34]. Aminoguanidine decreased inflammatory response to parasite infection model in mice and improved an anti-inflammatory effect of meloxicam on the rat model of carrageenan-induced paw edema [35, 36]. The mechanism of this AGE blocker is based on the binding of active carbonyl forms. This reaction prevents forming unstable Schiff bases and the subsequent Amadori rearrangement. This transformation is named the Maillard reaction, the main way of AGEs formation [37].

Obtained results showed the efficacy of AGEs blocker for reduction inflammatory response and a decrease in the severity of the structural injury of respiratory epitheliocytes during virus-induced ALI with DM. Correction of the inflammatory response included a decrease proinflammatory cytokine level in the lung, a change of level of blood elements, and the degree of lung inflammation. These changes can be explained by the decreased role of AGEs in inflammation. Well known that AGEs play a pivotal role in inflammatory response via receptor of AGEs, also known as RAGE. The binding of AGEs with RAGE induces NADPH-oxidase/ROS/p21RAS/p38 signaling pathway that promotes the production of pro-inflammatory cytokines [18-20]. Aminoguanidine suppressed uncontrolled inflammatory response, which was confirmed by obtained results and other studies. [35, 36]. Respiratory tract injury was decreased and was confirmed by prevention of severe hypoxia in the acute phase of virus-induced ALI and correction of lung damage evaluated by lung injury score. Numerous studies suggest a link between AGEs level and protein dysfunction with cell damage [38, 39]. This process is based on uncontrolled post-translational modification of biomolecules with unlinked amino groups by precursors of AGEs. This modification leads to the formation of covalent bonds in molecules that affect their transformation during their functioning. The AGEs blocker decreased cell destruction during virus-induced ALI with DM and was confirmed in the current study using histological analysis and the lung injury score.

Interestingly, treated infected mice with DM showed a similar disease state to untreated infected mice without comorbidity. Comparative analysis of the inflammatory response and the lung structural changes didn't show a significant difference between mice in these groups. At the same time, untreated infected mice with DM showed an increase in the severity of virus-induced ALI comparative with infected mice without comorbidity. These changes could be linked to a negative action of dramatically increased AGEs level during combined pathology. Aminoguanidine reduced the aggravating effect of diabetes on virus-induced ALI and was confirmed in the current study.

4. CONCLUSION

Herein, aminoguanidine showed efficacy against ALI induced by influenza virus A(H1N1)pdm09, in mice with alloxan-induced DM, via the prevention of AGEs formation. This effect has been shown by decreased inflammatory response and lung injury during the experiment. Obtained results showed the perspective of AGEs blockers for the treatment of influenza-induced ALI with DM.

5. MATERIALS AND METHODS

5.1. Animals and Experimental design:

8-week-old female albino mice from the NRC "Kurchatov Institute" – "PLZH" Rappolovo" (Saint-Petersburg) were used in the experiment. All experimental procedures were carried out in accordance with the principles of laboratory animal care the 2010/63/EU Directive of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes and the guidelines of the bioethical committee of the Smorodintsev Research Institute of Influenza. Mice were kept on a 12-h light-dark cycle with a free access to food and water supply. Animals were housed in the standard conditions with temperature: $21 \pm 2^\circ\text{C}$ and humidity: $55 \pm 15\%$.

Animals were divided on 4 groups: group 1 – intact mice, $n = 15$, group 2 – infected mice without comorbidities and received placebo, $n = 40$; group 3 – infected mice with alloxan-induced DM and received placebo, $n = 60$; group 4 – infected mice with DM and received aminoguanidine, $n = 60$. Biomaterials were taken at the end of the experiment from intact mice. Animals were divided into two equal subgroups in the remaining groups. Survival rate and clinical manifestation were evaluated in the first subgroups during 14 DPI. Biomaterials from infected mice were taken on 4 DPI and 7 DPI in the second subgroups ($n = 5$).

5.2. Materials:

Aminoguanidine bicarbonate (Sigma-Aldrich, USA) was administered at the dose of 25 mg/kg by subcutaneous rouse during 7 DPI [40]. First injection took place 30 min after infecting. Alloxan monohydrate (Acros organic, USA) was administered at the dose of 180 mg/kg by subcutaneous rouse at once in the experiment.

5.3. DM and ALI model:

Experimental DM was modeled by a single subcutaneous injection of alloxan for 10 days before infection. Animals with 2-time increased blood glucose level were taken in an experiment [41]. Blood glucose level was measured by a glucometer (Accu-Chek, Germany).

Influenza virus A/California/7/09MA (mouse-adapted) (H1N1)pdm09 was used for virus-induced ALI model. The viral material was inoculated intranasally at the dose of 1 LD₅₀ (3.4 lg EID₅₀, 50 µl). The dose was calculated by the Reed and Muench method [42]. The mouse-adapted pandemic strain of influenza virus was obtained from the collection of the laboratory of chemotherapy of viral infection FSBI "Smorodintsev Research Institute of Influenza" Ministry of Health of Russia. Viral etiology of ALI was confirmed by the hemagglutination assay in the lung's supernatant on 4 DPI and 7 DPI [43].

5.4. Evaluation of ALI:

Blood-oxygen saturation was assessed using the Jonxis method [44]. Blood was taken from the buccal sinus. The optical density of hemoglobin solutions was measured using photometer ABchPhc-02 NPP-TM (Technomedica, Russia).

The lungs were fixed in 10% phosphate-buffered formalin. Histological sections were stained with hematoxylin and eosin. The lung injury score was evaluated using the American Thoracic Society method [45]. This method is based on the counting of morphological changes in the lung histological section in 20 fields (magnified 400 times) and assigning scores. The next parameters of morphological changes are counted: A – neutrophils in the alveolar space (0 score – absence, 1 score – from 1 to 5 neutrophils, 2 scores – more than 5 neutrophils); B – neutrophils in the interstitial space (0 score – absence, 1 score – from 1 to 5 neutrophils, 2 scores – more than 5 neutrophils); C – hyaline membranes (0 score – absence, 1 score – 1 zone, 2 scores – more than 1 zone); D – proteinaceous debris filling the airspaces (0 score – absence, 1 score – 1 zone, 2 scores – more than 1 zone); E – alveolar septal thickening (0 score – less than 2 times, 1 score – from 2 to 4 times, 2 scores – more than 4 times).

The lung injury score is calculated using the formula:

$$\text{(Eq.1)} \quad \text{Score} = \frac{(20 \times A) + (14 \times B) + (7 \times C) + (7 \times D) + (2 \times E)}{\text{number of fields} \times 100}$$

The degree of lung inflammation was evaluated via calculation without the presence of hyaline membranes and proteinaceous debris filling the airspaces, according to the following formula:

$$(Eq.2) \quad \text{Score} = \frac{(20 \times A) + (14 \times B) + (2 \times E)}{\text{number of fields} \times 100}$$

5.5. Evaluation of inflammatory response:

The level of proinflammatory cytokines (IL-1 β , IL-6, TNF- α) were evaluated in lung tissue homogenates using BioLegend's ELISA MAXTM (Biolegend, USA) with spectrophotometer EPOCH2TC (BioTek, USA) and calculated per gram of protein. 100 μ l of mouse-specific rat monoclonal antibody was first coated on a 96-well plate which incubated overnight between 2 °C and 8 °C. After that 100 μ l standards and samples of the supernatant of the lung's homogenate were added to the wells for 2 hours. Next, 100 μ l biotinylated rat monoclonal anti-mouse antibody was added to each well. Avidin-horseradish peroxidase was subsequently added, followed by 3,3',5,5'-Tetramethylbenzidine substrate solution, producing a blue color in proportion to the concentration of cytokine present in the sample. The protein concentration was determined using the biuret method on a KeyLab Automatic Analyzer (BioSed, Italy) [46].

The leukocytes level was measured using Abacus JuniorVet automatic hematology analyzer (Diatron, Austria) in blood. Lymphocyte-granulocyte index was calculated using the following formula:

$$(Eq.3) \quad LGI = \frac{LYM\%}{GRA\%}$$

5.6. Evaluation of AGEs level:

The AGEs level was measured and calculated per gram of protein in lung tissue homogenates. The concentration of AGEs was measured by fluorescence analysis with λ_{ex} = 360 nm (extinction wavelength) and λ_{em} = 440 nm (emission wavelength). Fluorescence level was evaluated using fluorescence module of microplate reader CLARIOstar (BMG LABTECH, Germany) [47].

5.7. Statistical analysis:

Statistical analyses were performed with GraphPad Prism (GraphPad Software, Inc., version 8). Means were compared using Kruskal-Wallis and Tukey's post hoc analyses. All variables are reported as mean \pm standard error of the mean (SEM). The dynamics of mortality was showed by the Kaplan-Meier curves. Log-rank test was used for comparing these data.

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