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Understanding the relationship between lung GABA_A receptors and aquaporin-1: a new therapeutic approach for acute lung injury

Hamza Halici^{1*} , Zeynep Karakoy² , Elif Cadirci³ , Zekai Halici³ , Bengul Ozdemir⁴ and Erdem Toktay⁴

Abstract

Background This study aimed to investigate the effects of the GABA_A receptor and the potential relationship between GABA/ GABA_A receptor signalling and aquaporin-1 in acute lung injury (ALI).

Methods Fifty-six male Wistar albino rats (220–240 g) were divided into seven groups. Two groups received intraperitoneal diazepam at 5 or 10 mg/kg. In two other groups, 0.1 mg/kg flumazenil was administered five minutes after diazepam. Thirty minutes after the final treatment, all groups except the healthy controls were subjected to an LPS- induced ALI model. Lung tissue were collected 24 h after LPS administration and analysed using histopathological, molecular, and biochemical methods.

Results In the present study, it was found that aquaporin-1, which plays an important regulatory role in LPS-damaged lung tissue, decreased and the weight of lung tissue increased in wet/dry analysis of a murine model of ALI. The levels of IL-1 β , IL-6, and TNF- α were found to be elevated. Additionally, in groups treated with the GABA_A receptor modulator diazepam, the levels of these inflammatory cytokines were significantly reduced and in parallel a significant increase in aquaporin-1 expression was observed.

Conclusion Diazepam, acting as a GABA_A receptor modulator, may alleviate ALI by reducing inflammation and enhancing aquaporin-1 expression via GABA_A receptor activation.

Keywords Acute lung injury, Aquaporin-1, Diazepam, GABA_A

Introduction

Acute lung injury (ALI) is a respiratory disorder characterised by pulmonary oedema resulting from an imbalance of inflammatory and anti-inflammatory responses in the lungs and the disruption of alveolar-capillary physiology [1, 2]. This leads to the disruption of gas exchange and hypoxia in the lungs, causing significant morbidity and mortality. ALI is a major health problem worldwide. It is estimated that ALI and its more severe form, acute respiratory distress syndrome (ARDS), affect one in every 10 patients in intensive care and 200,000 cases are seen in the United States each year [2]. In recent studies, however, the effects of neuromuscular blocking

*Correspondence:

Hamza Halici
hamzahalici@atauni.edu.tr

¹Department of Hınıs Vocational Training School, Ataturk University, Yenikent District, Cevre Yolu 1017. Street No: 2 Hınıs, Erzurum 25600, Turkey

²Department of Pharmacology, Faculty of Pharmacy, Erzincan Binali Yıldırım University, Erzincan 24002, Turkey

³Department of Pharmacology, Faculty of Medicine, Ataturk University, Erzurum 25100, Turkey

⁴Department of Histology and Embryology, Faculty of Medicine, Kafkas University, Kars 36100, Turkey



agents (NMBA), phosphodiesterase inhibitors, steroids, and inhaled nitric oxide treatments on both side-effect profiles and mortality rates are reported inconsistently [3–6]. Similarly, although many different treatments including β_2 agonists, exogenous surfactants, anticoagulants, octreotide, betaine, ozone and nonsteroidal anti-inflammatory drugs have yielded favourable results in preclinical studies, clinical trials have failed [7]. In recent years, several preclinical studies investigating octreotide, betaine, ozone, and tocilizumab have reported that these agents may exert potential protective effects against lung injury; however, these findings still require confirmation through clinical studies [8–11]. The lack of a specific treatment for ALI makes it important to mechanistically investigate how the disorder develops.

Gamma-aminobutyric acid (GABA) is an important inhibitory neurotransmitter discovered in the mammalian central system in 1950 [12]. Two different GABA receptors, GABA_A and GABA_B, have been defined, which respond to the GABA neurotransmitter and show pharmacological, structural, and molecular differences [13]. GABA_A receptors are important target receptors for the treatment of many neuropsychological diseases, including anxiety, epilepsy, and insomnia, and for many drugs used for general anaesthesia in surgical operations [14]. Recent studies have confirmed that GABA_A receptors have very important functions and are found in many tissues and organs outside of the nervous system [15]. For example, recent studies have confirmed GABA_A receptor expression in the airways and alveolar epithelial cells [16, 17]. In a study conducted in rats, it was reported that GABA_A receptor expression increased significantly in lipopolysaccharide (LPS)-induced cases of ALI and that the GABA_A receptor signalling pathway may have a central role in the pathogenesis of ALI [18]. In another study, it was shown that propofol attenuated lung damage and increased lung function in ALI; this protective effect of propofol was suggested to be related to the upregulation of GABA_A receptors in alveolar epithelial cells [19]. GABA and GABA_A-positive allosteric modulators have been shown to reduce inflammation, increase alveolar clearance, and improve lung function in many different murine models of ALI [19–21]. In this point GABA_A receptor agonists such as diazepam, can be potential targets for ALI treatment.

Aquaporins are integral membrane proteins that mediate the transport of water across membranes in various systems. They are found in various cells and tissues, and they have important roles in the intracellular and extracellular transport of water under physiological and pathological conditions [22, 23]. Of the 13 aquaporins identified to date, aquaporin-1, -3, and -5 are found in the lungs [24]. In a rat model of lung injury, aquaporin-1 was shown to be highly influential in lung oedema

and inflammatory response [25]. In another study, it was emphasised that aquaporin-1 dysfunction may be an important cause of pulmonary oedema in ALI [26].

Our review of the literature suggested that the GABA/GABA_A receptor pathway and aquaporin-1 may be important in the pathology of ALI (Fig. 1). Previous studies have investigated the relationships between GABA receptors and aquaporins. GABA_A receptor activation was shown to be effective in interstitial fluid clearance in the brain in an aquaporin-4-dependent manner [27]. Such data suggest that aquaporin-1 may be associated with GABA_A receptors in the lungs, revealing the need for further research. In this study, we aimed to demonstrate the effects of the GABA_A receptor and the possible relationship between GABA/GABA_A receptor pathways and aquaporin-1 in ALI by histopathological, biochemical and molecular analyses.

Methods

Animals

In this study, 56 male Wistar albino rats weighing 220–240 g were used. The rats were obtained from the Atatürk University Medical and Experimental Application and Research Centre (ATADEM). The study was approved by the Ataturk University Local Animal Care Committee (Meeting Date: 26.03.2024; Meeting No. and Resolution No: 2024/3–80). Rats were housed in a room with a temperature of 22 ± 1 °C, 40–50% humidity, and a 12-h light/dark photoperiod for 1 week. During this period, they were given ad libitum access to food and water.

Drugs

LPS (Ref. No. L2880, Escherichia coli O55:B5) was obtained from Sigma-Aldrich Chemie GmbH (Munich, Germany), ketamine (Ketalar, 500 mg/10 mL) from Pfizer (Istanbul, Turkey), xylazine (Basilazine 2%) from Biotek (Ankara, Turkey), and diazepam (Diazem, 10 mg/2 mL) and flumazenil (Anesed-R) from Atatürk University Research Hospital (Erzurum, Turkey).

Experimental groups

Sample size calculation was performed using G Power version 3.1. Based on a 2-tailed alpha error of 0.05, a power of 0.8, and an effect size of 0.55, 8 rats for each group were calculated as the minimum sample size required to achieve statistical power. Seven experimental groups were randomly formed in this study with 8 animals in each group as follows:

Group 1: Healthy control group

Group 2: ALI group (LPS-induced acute lung injury model)

Group 3: ALI + Diazepam5 (LPS-induced acute lung injury model + 5 mg/kg diazepam)

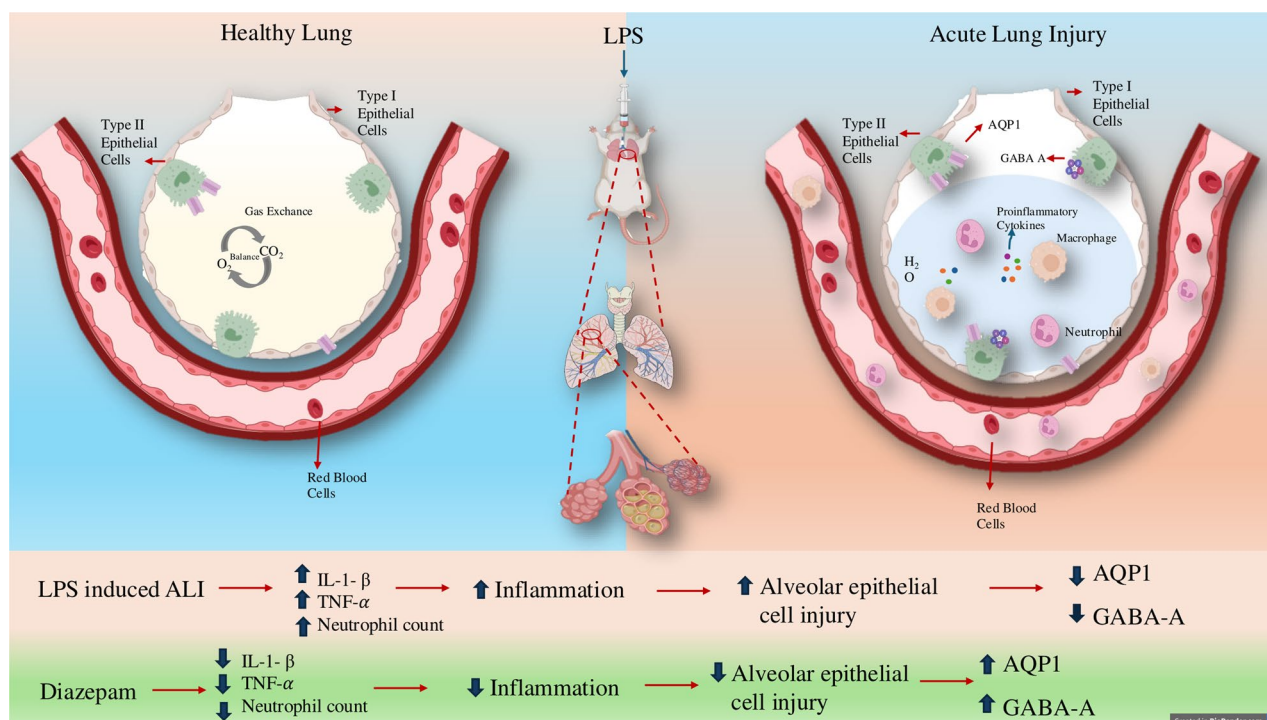


Fig. 1 Proposed involvement of the GABA/GABA_A receptor pathway and aquaporin-1 (AQP1) in the pathophysiology of acute lung injury (ALI). The graphical abstract illustrates how alterations in the GABA/GABA_A receptor pathway and AQP1 expression may contribute to inflammation and alveolar structural damage in LPS-induced acute lung injury

Group 4: ALI + Diazepam10 (LPS-induced acute lung injury model + 10 mg/kg diazepam)

Group 5: ALI + Flumazenil (LPS-induced acute lung injury model + 0.1 mg/kg flumazenil)

Group 6: ALI + Diazepam5 + Flumazenil (LPS-induced acute lung injury model + 5 mg/kg diazepam + 0.1 mg/kg flumazenil)

Group 7: ALI + Diazepam10 + Flumazenil (LPS-induced acute lung injury model + 10 mg/kg diazepam + 0.1 mg/kg flumazenil)

The diazepam groups received 2 different doses of diazepam (5 or 10 mg/kg) intraperitoneally. Five minutes after diazepam administration, 0.1 mg/kg flumazenil was administered intraperitoneally to the diazepam + flumazenil groups. Thirty minutes after the final administration for each group, the LPS-induced ALI model was applied to all groups except the healthy control group.

LPS-Induced acute lung injury model

Animals were anesthetised by the intraperitoneal administration of a combination of 90 mg/kg ketamine and 10 mg/kg xylazine. After the rats were placed in the supine position, cervical midline skin incisions were made. To minimise tissue destruction, the fascia, sternocleidomastoid muscle, and parathyroid glands were removed with cotton swabs and then the trachea was

surgically exposed. A 26G sterile insulin syringe was inserted into the intratracheal space through the tracheal cartilage. When the injector plunger was retracted and air entered the injector, 5 mg/kg LPS (Ref. No. L2880, Sigma-Aldrich Chemie) diluted with isotonic NaCl was slowly injected into the trachea. After the injection, the rats were rotated on three axes to evenly distribute the LPS throughout the lungs. The skin was closed using an Appose ULC AutoSuture Thin Body Skin Stapler (35 W, Covidien, Dublin, Ireland). Sodium fusidate was applied topically to the incision site. Animals were euthanised 24 h after intratracheal LPS administration with high-dose anaesthesia. For euthanasia induction, the ketamine/xylazine combination was administered ip to rats at four times the dose that produces anesthesia. Since the application required a surgical operation during the experiment, the practitioner was not blinded to the study, but the analyses (biochemical, immunohistochemical analyses) were performed by blinded investigators during the evaluation stages. Lung tissues were rapidly excised and fixed simultaneously under identical temperature and pressure conditions using a 3.7% formaldehyde solution, which is the most common fixative in histology laboratories. Lung tissue samples were obtained from all animals and stored under appropriate conditions for molecular, biochemical, histopathological, and lung water content evaluations.

Histopathological analyses

All histopathological evaluations were performed in the research laboratory of the Department of Histology and Embryology, Faculty of Medicine, Kafkas University, Kars, Turkey. All histopathological and immunohistochemical analyses were performed by two independent blinded investigators.

Tissue processing procedures and preparation of sections

After the completion of all surgical procedures, lung tissue pieces were placed in formaldehyde solution. They were fixed for 24–48 h and then placed in plastic tissue tracking cassettes. A routine tissue follow-up protocol was then initiated. After the tissue samples were washed under running water to remove the fixative, they were serially dehydrated in 70%, 80%, and 96% alcohol for 1 h each and then again in 96% alcohol for 1 night, followed by an absolute alcohol series of 2 repetitions for 1 h each. Subsequently, they were kept in pure xylene solutions (3 repetitions, 15 min each) for tissue transparency and then in a paraffin series (3 repetitions) in an oven at 60 °C for 1 h each. Upon completion of these processing steps, the tissue samples were embedded in metal blocking moulds and the blocking process was completed.

From the obtained blocks, sections of 5 µm in thickness were obtained with a microtome device. The sections were then placed in hot water and mounted on poly-L-lysine slides [28]. Subsequently, the slides were subjected to a pre-treatment protocol before the administration of colouring solution.

Haematoxylin and eosin staining protocol

Lung tissue sections on slides were kept in an oven at 60 °C for 20 min to fix the tissue on the slide and remove the paraffin. Deparaffinised slides were then kept in a xylol series with 2 repetitions for 20 and 10 min, respectively. They were passed through a series of alcohols of decreasing strength (99%, 96%, 80%, 70%) for 3 min each, followed by distilled water for 5 min. This was followed by haematoxylin staining for 5 min. Tissues were then washed under running water to remove excess stain and, after staining with eosin stain for 5 min, they were rapidly passed through an alcohol series of increasing strength (70%, 80%, 96%, 99%). Finally, the tissues were placed in a xylol series with 3 repetitions and respectively held for 5, 10, 15 min and then covered with Entellan. In our study, lung tissues obtained from each animal were evaluated using a random sampling method. The sizes of the alveoli and the septal thicknesses between these alveoli were measured in at least five different fields per animal lung tissue block using ImageJ software, and the results were compared.

Immunohistochemical staining method

Sections on poly-L-lysine slides were passed through xylol and alcohol series and then washed in distilled water for 5 minutes. They were subsequently held in sodium citrate solution for 1 minute under high pressure to expose the antigen surfaces blocked as a result of fixation. Thereafter, the slides were washed with phosphate-buffered saline (PBS, pH 7.2) for 5 minutes and kept in 3% H₂O₂ for 10 minutes to inactivate endogenous peroxidases. After washing the surfaces of the slides 3 times with PBS, they were incubated with a protein blocking solution for 5 minutes. The excess protein blocking solution remaining on the slides was then poured off, the primary antibody was added, and slides were kept at 4 °C for 1 night. The slides were then washed 3 times for 5 minutes each with PBS. This was followed by incubation with a biotinylated secondary antibody (Anti-AQP1 primary antibody (polyclonal; BT-Lab, Cat. No: BT-AP00552) for 10 minutes at room temperature, washing again with PBS, incubation with a streptavidin-peroxidase solution for 10 minutes, and washing again with PBS. Subsequently, 3–3' diaminobenzidine (DAB) chromogen was added. After the reaction was observed, the slides were washed again. For background staining, they were kept in Mayer's haematoxylin for 1 min and then washed with tap water. Finally, the slides were passed through alcohol and xylol series and covered with coverslips. The immunopositivity of the sections was evaluated as absent (-), mild (+), moderate (+++), or severe (++++). All sections were scored by two independent investigators and means of their scores were presented.

Molecular analyses

RNA extraction from rat lung tissue

TissueLyser II (QIAGEN, Hilden, Germany) was used for homogenisation of lung tissue samples. The samples were weighed individually. Total RNA isolation was performed using the QiaCube RNA isolation device and the RNeasy Mini Kit (QIAGEN, Germany). An EPOCH NanoDrop spectrophotometry device and Take3 plate (Bio-Tek, Santa Clara, CA, USA) were used and RNA purity and total RNA amount were determined with measurements performed at 260/280 nm.

Reverse transcriptase reaction and cDNA synthesis

Production of cDNA from RNA was achieved with the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher, Waltham, MA, USA). cDNA synthesis was performed using a Veriti 96-Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA) with 10 µL of total RNA, 2 µL of 10× RT buffer, 0.8 µL of 25× dNTP mix, 2 µL of 10× RT random primer, 1 µL of MultiScribe reverse transcriptase, and 4.2 µL of diethylpyrocarbonate H₂O. cDNA was quantified using the EPOCH NanoDrop

spectrophotometry device and Take3 plate (Bio-Tek, USA) and stored at -20 °C.

Quantitative detection of GABA_A and aquaporin-1 mRNA expression by real-time PCR

GABA_A (α subunit) and aquaporin-1 mRNA expression levels were analyzed using the LightCycler TaqMan Master (2X) (Roche, Basel, Switzerland) with β-actin serving as a reference gene. Amplification and quantification were performed with the StepOnePlus Real-Time PCR system (Applied Biosystem, USA). Ct values were automatically converted to ΔΔCt [29]. For amplification, a primer–probe set specific to the Rat_Gabra1 gene was used; the forward primer was 5'-TGACTATCTTTGGGCCTGGA-3', the reverse primer was 5'-GACAGTGGTGTTCCTTAAGTT-3', and the FAM-labeled hydrolysis probe was FAM-TGAGCACTCTCTCGGAAGAAGCTA-BHQ1. For the amplification of the Rat_Aqp1 gene, a gene-specific primer–probe set was employed; the forward primer was 5'-CCTGCTCGAGAACTCACTTG-3', the reverse primer was 5'-AGAACGCACAGCACCAG-3', and the FAM-labeled hydrolysis probe was FAM-AAATGACCTGGCTCGAGGTGTGAA-BHQ1. All results were statistically analyzed using GraphPad Prism 5.03.

Measurements of IL-1β, IL-6, and TNF-α levels by ELISA

Lung tissue samples obtained at the end of the experiment were ground in liquid nitrogen using the TissueLyser II grinding jar set (QIAGEN, Germany). Approximately 100 mg of ground tissue was homogenised in TissueLyser II with the addition of PBS homogenate buffer according to the procedure recommended by the kit's manufacturer. The samples were then centrifuged at 5000 × g for 5 min at 4 °C and supernatants were obtained. The interleukin (IL)-1β, IL-6, and tumour necrosis factor alpha (TNF-α) levels of the supernatants were measured with enzyme-linked immunosorbent assay (ELISA) kits using the EPOCH NanoDrop spectrophotometry device and Take3 plate (Bio-Tek, USA). The ELISA procedure was performed according to the steps described in the kit's instructions. Total protein amounts were measured using the Lowry method [30].

Lung wet-to-dry weight ratios

The weight of the upper lobe of the left lung, removed at the end of the experiment, was weighed before drying (wet weight). These samples were then incubated in an oven at 72 °C for 48 h. At the end of the incubation, the tissues were weighed again (dry weight). The wet-to-dry weight ratio was calculated as wet weight divided by dry weight.

Statistical analysis

GraphPad Prism 5.03 and IBM's 22.00 SPSS package program was used to compare the data. The data were calculated and shown as mean and standard deviations. The Shapiro-Wilk test was applied to each group to test the compliance of normal distribution (Gaussian), and a p value greater than 0.05 was accepted as a normal distribution. A skewness test was also used to test normal distribution, and the Levene's test was used to evaluate the homogeneity of variances. It was assumed that the variances of data with a p value higher than 0.05 were homogeneous. Statistical analyses of data other than immunochemical scoring data were performed using one-way ANOVA and if the ANOVA test is statistically significant, Tukey's multiple comparison tests were applied. Statistical analyses of immunochemical scoring data that did not comply with normal distribution were performed using the Kruskal-Wallis test with post hoc analysis (Dunn's test). Values of $p < 0.05$ were considered significant. All results are expressed as mean ± standard deviation for rats in each group.

Results

Haematoxylin and eosin staining results

In the histopathological examination of the lung tissues of the healthy group (Fig. 2A), terminal bronchioles, alveolar sacs, alveolar walls, and vascular structures were examined. No pathological features were observed in these lung tissues. In the ALI group (Fig. 2B), areas of oedema and numerous inflammatory cells were observed in the interalveolar areas and around the vessels. Inflammatory cell infiltration was observed from the vessel into the tissue. Due to oedema in the alveolar walls, thickening of the alveolar septum and shrinkage of the alveolar sacs were observed. In the ALI + Diazepam5 group (Fig. 2C), the inflammatory cells observed in the ALI group were again observed. However, alveolar septum thickening was less prominent and alveolar sacs were larger compared to the ALI group. In the ALI + Diazepam10 group (Fig. 2D), the alveolar septum thickening was decreased. Although leucocyte infiltration from the vessel lumen into the tissue was still present, the extent of the infiltration had decreased. The thickness of the alveolar septum and the size of the alveolar sacs were closest to the values obtained for the healthy group. In the ALI + Flumazenil group (Fig. 2E), advanced cellular infiltrations and alveolar septal thickening similar to those observed in the ALI group were noted. In the ALI + Diazepam5 + Flumazenil (Fig. 2F) and ALI + Diazepam10 + Flumazenil (Fig. 2G) groups, advanced cellular infiltrations and alveolar septal thickening were again similar to those of the ALI group. Alveolar wall thickening and alveolar dimensions were measured and compared between the groups (Fig. 3A and B).

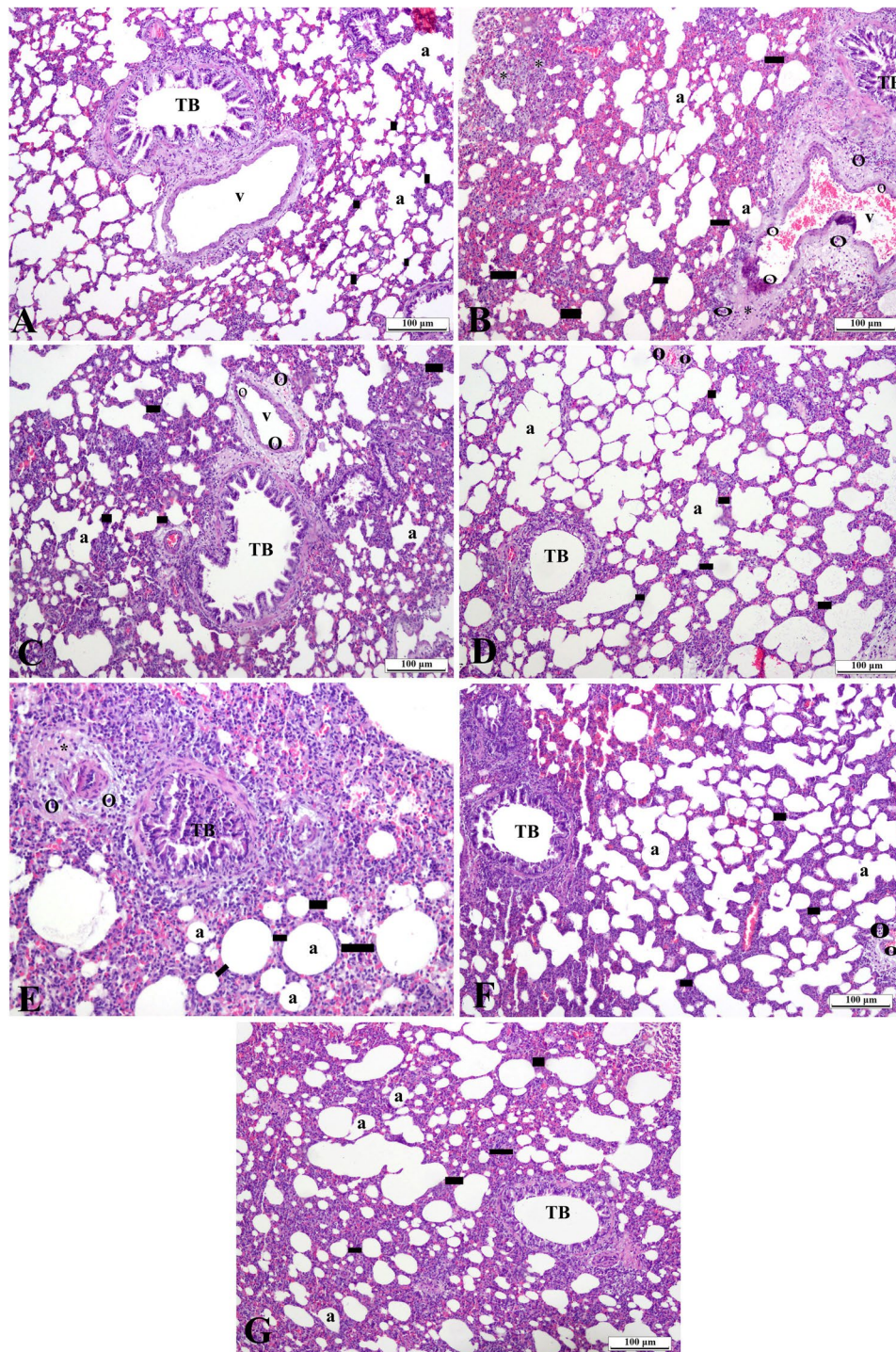


Fig. 2 Lung Tissue-Hematoxylin Eosin Staining Findings. (TB: terminal bronchioles, v: vessel, a: alveolus, thick bars: alveolar septum, o sign: leukocytes, * sign: oedema), **A** HEALTHY, **B** ALI, **C** ALI+Diazepam5, **D** ALI+Diazepam10, **E** ALI+Flumazenil, **F** ALI+ Diazepam5 + Flumazenil, **G** ALI+ Diazepam10 + Flumazenil)

Immunohistochemical staining results

Immunopositive cells and the degree of immunopositivity in five different areas in the lung tissue were scored semiquantitatively for each group and compared between the groups. Immunopositivity is reported as - for immunonegative results (0–1%), + for mild immunopositivity

(10–33%), ++ for moderate immunopositivity (33–66%), and +++ for severe immunopositivity (66–99%).

Accordingly, as a result of immunohistochemical staining with the aquaporin-1 antibody, the healthy control group (Fig. 4A) and ALI+Diazepam10 group (Fig. 4D) showed severe (+++) immunopositivity, while

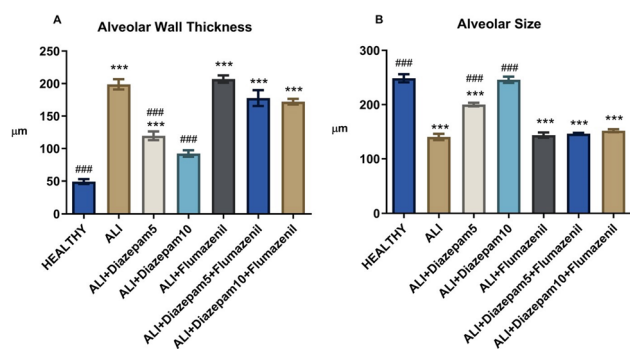


Fig. 3 **A** Alveolar Wall Thickness **B** Alveolar Size * Comparison relative to healthy, # comparison relative to ALI group (difference between groups according to One Way ANOVA-Tukey test *** and #### indicates $p < 0.001$)

the ALI + Diazepam5 group (Fig. 4C) showed ++ immunopositivity and the ALI + Flumazenil (Fig. 4E), ALI + Diazepam5 + Flumazenil (Fig. 4F), ALI + Diazepam10 + Flumazenil (Fig. 4G), and ALI (Fig. 4B) groups showed mild (+) immunopositivity. Figure 4H presents the results for the negative control with no immunopositivity (-) (Table 1; Fig. 4). These scoring data are presented together in Fig. 5, where a significant decrease ($p < 0.05$) can be observed in the ALI and ALI + Flumazenil groups compared to the healthy control group. Furthermore, compared to the ALI group, a significant increase was observed in the ALI + Diazepam10 group ($p < 0.05$).

Molecular results

GABA_A receptor mRNA expression in lung tissue

Evaluation of GABA_A receptor mRNA expression in lung tissue revealed a significant reduction in the ALI ($p < 0.001$), ALI + Diazepam5 ($p < 0.05$), ALI + Flumazenil ($p < 0.001$), ALI + Diazepam5 + Flumazenil ($p < 0.001$), and ALI + Diazepam10 + Flumazenil ($p < 0.01$) groups compared with the healthy control group. In contrast, relative to the ALI group, GABA_A receptor mRNA expression was significantly higher in the ALI + Diazepam5 ($p < 0.05$) and ALI + Diazepam10 ($p < 0.001$) groups (Fig. 6A).

Aquaporin-1 mRNA expression in lung tissue

Aquaporin-1 mRNA expression levels in lung tissue were markedly reduced in the ALI, ALI + Flumazenil, ALI + Diazepam5 + Flumazenil, and ALI + Diazepam10 + Flumazenil groups compared with the healthy control group ($p < 0.001$). However, when compared with the ALI group, aquaporin-1 mRNA expression levels were significantly elevated in the ALI + Diazepam5 and ALI + Diazepam10 groups ($p < 0.001$) (Fig. 6B).

IL-1 β , IL-6, and TNF- α ELISA results

ELISA analysis demonstrated that IL-1 β levels were markedly higher in the ALI ($p < 0.001$), ALI + Flumazenil ($p < 0.001$), ALI + Diazepam5 + Flumazenil ($p < 0.001$), and

ALI + Diazepam10 + Flumazenil ($p < 0.05$) groups compared with the healthy control group. In contrast, IL-1 β levels were significantly lower in the ALI + Diazepam5 ($p < 0.05$) and ALI + Diazepam10 ($p < 0.01$) groups compared with the ALI group (Fig. 7A).

A similar pattern was observed for IL-6 levels. The ALI, ALI + Flumazenil, ALI + Diazepam5 + Flumazenil, and ALI + Diazepam10 + Flumazenil groups exhibited markedly higher IL-6 levels than the healthy control group ($p < 0.001$). However, treatment with diazepam (5 or 10 mg/kg) significantly reduced IL-6 levels compared with the ALI group ($p < 0.001$) (Fig. 7B).

Likewise, TNF- α levels were significantly elevated in the ALI, ALI + Flumazenil, ALI + Diazepam5 + Flumazenil, and ALI + Diazepam10 + Flumazenil groups compared with the healthy control group ($p < 0.001$). Compared with the ALI group, TNF- α levels were significantly reduced in the ALI + Diazepam5 and ALI + Diazepam10 groups ($p < 0.001$) (Fig. 7C).

Lung wet-to-dry weight ratio

Analysis of lung wet-to-dry weight ratios demonstrated markedly higher values in the ALI, ALI + Flumazenil, ALI + Diazepam5 + Flumazenil, and ALI + Diazepam10 + Flumazenil groups compared with the healthy control group ($p < 0.001$). In contrast, the wet-to-dry ratios in the ALI + Diazepam5 and ALI + Diazepam10 groups were significantly lower than those observed in the ALI group ($p < 0.001$) (Fig. 8).

Discussion

In this study, we investigated the effects of GABA_A receptor agonist diazepam and GABA_A antagonist flumazenil in rats with LPS-induced ALI. We aimed to clarify the relationship between GABA_A receptors and aquaporin-1, which has been shown to play a significant role in ALI. Before discussing the findings of the present study, it is necessary to provide clarification regarding the diazepam doses used. Although diazepam exerts various therapeutic effects in humans, it is also associated with several potential adverse effects. The diazepam doses administered in the experimental protocol (5 and 10 mg/kg, i.p.) were determined based on the pronounced pharmacokinetic differences between rats and humans. The elimination half-life of diazepam in rats (~ 1 h) is approximately 60-fold shorter than that observed in humans (~ 56 h) [31, 32]. Moreover, the extremely high systemic clearance in rats (255 mL/kg/min) necessitates the use of these doses in order to achieve plasma concentrations comparable to the therapeutic range reported in clinical settings in humans (150–300 ng/mL) [32]. In a study by Zomorodi et al., doses up to 10 mg/kg in rats were shown to exhibit linear pharmacokinetics without causing metabolic saturation [33]. Furthermore, from a safety

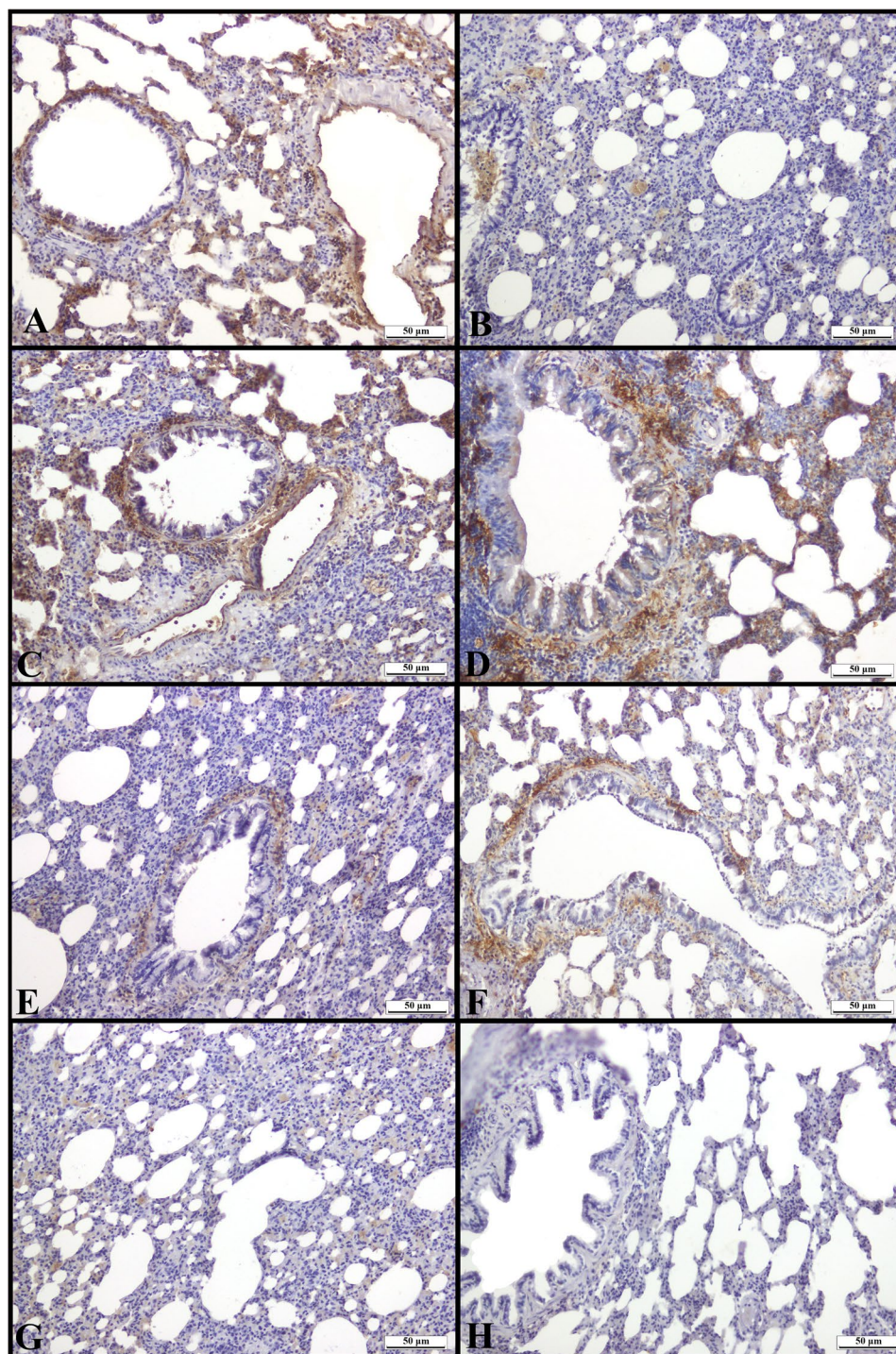


Fig. 4 + Immunohistochemistry staining results. **A** HEALTHY, **B** ALI, **C** ALI+Diazepam5, **D** ALI+Diazepam10, **E** ALI+Flumazenil, **F** ALI+ Diazepam5 + Flumazenil, **G** ALI+ Diazepam10 + Flumazenil, **H** Negative Control

perspective, a dose of 5 mg/kg has been reported not to impair the righting reflex and to suppress central apnea by stimulating inspiratory minute ventilation [34], supporting the appropriateness of the selected doses.

We found that GABA_A receptor expression decreased significantly in parallel with aquaporin-1 expression in

LPS-induced ALI. In the diazepam-treated groups, the GABA_A receptor expression increased significantly in a dose-dependent manner and, in parallel, aquaporin-1 expression increased significantly. Significant histopathological improvement was detected in these groups. The effect of diazepam was eliminated by the administration

Table 1 Aquaporin-1 immune Positivity in lung tissues

Groups	Degree of Immune Positivity
Healthy	+++
ALI	+
ALI+Diazepam5	++
ALI+Diazepam10	+++
ALI+Flumazenil	+
ALI+Diazepam5 + Flumazenil	+
ALI+Diazepam10 + Flumazenil	+

The immunopositivity of the sections was evaluated as absent (-), mild (+), moderate (+++), or severe (++++)

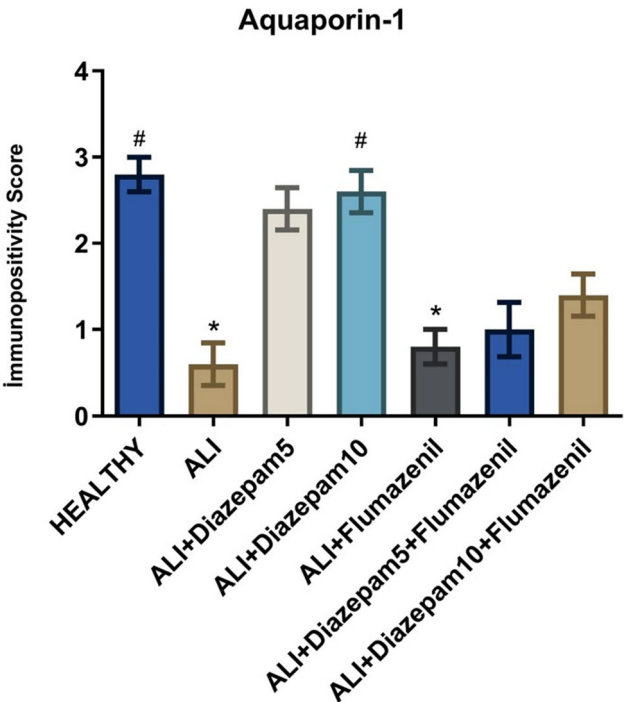


Fig. 5 Aquaporin-1 Immunopositivity Score. * Comparison according to healthy, # comparison according to ALI group (difference between groups according to Kruskal Wallis test with post hoc analysis (Dunn's test) * and # indicates $p < 0.05$)

of GABA_A receptor antagonist. Therefore, the obtained results suggest that the GABA–GABAA receptor–mediated signaling pathway may play a role in the regulation of aquaporin-1 expression, and that this mechanism may potentially contribute to the development and progression of acute lung injury.

GABA is a crucial compound for neuronal activity and cell metabolism in the central nervous system. It has two different receptors, GABA_A and GABA_B [35], which play important roles in neural tension and neurotransmission in association with the central nervous system [36, 37]. Beyond the central nervous system, GABA receptors have been detected in many peripheral systems and particularly the immune system [35]. It was previously found that GABA_A receptors exerted an anti-inflammatory effect by inhibiting T-cell

proliferation, which raises the question of whether GABA receptor agonists and antagonists could be used outside the central nervous system [38]. In lung studies, both GABA_A and GABA_B receptors have been shown to be present in human and rodent lung epithelia [39–41]. GABA and GABA_A receptor-positive allosteric modulators have been shown to reduce inflammation, increase alveolar fluid, and improve lung function [19, 41, 42]. Clinical studies on GABA_A receptors have confirmed that these compounds reduce pulmonary inflammation [43, 44]. The positive allosteric modulators of GABA_A receptors have also been shown to reduce the number of macrophages and inflammatory cytokine levels in the lungs and improve the macrophage-dependent inflammatory response [45, 46]. However, it is also necessary to discuss the relationship between GABA_A and aquaporins. In the literature, activation of GABA_A receptors has been shown to promote interstitial fluid clearance in the brain through an Aquaporin 4 dependent mechanism [27]. The similar relationship observed in lung tissue in the present study suggests that Aquaporin 1 may not merely function as a passive water channel, but may also act as a dynamic protective factor regulated through GABA_A receptor-mediated signaling pathways. This finding suggests that GABAergic modulation may potentially contribute to the maintenance of lung fluid homeostasis. However, given that AQP4 is predominantly expressed in astrocytes and has a specialized role in brain water homeostasis, whereas AQP1 exhibits a broader tissue distribution and may undertake tissue-specific functions, direct mechanistic comparisons between AQP4 and AQP1 are limited.

LPS-induced injury models are most commonly utilised in studies of ALI because they are low-cost and clinically relevant. In addition, it is important to examine hemodynamic and pulmonary mechanistic parameters [47, 48]. The model applied in the present study is the most popular model for research with relevance to ALI in humans [48]. In this model, primary disruption of the alveolar epithelium is the main mechanism. The migration of inflammatory cells begins immediately after LPS administration. The binding of LPS to TLR4 (toll-like receptor-4) receptors in lung epithelial cells leads to the expression of various inflammatory cytokines such as TNF and nuclear factor kappa B (NF-κB) [49]. The arrival of inflammatory cytokines in the region subsequently causes significant damage in the lung tissue. This leads to alveolar oedema via pulmonary capillary endothelial damage [50]. The lungs contain two important compartments: vascular and airway compartments. While the epithelium of the vascular compartment is responsible for immune responses against environmental damage, the airways

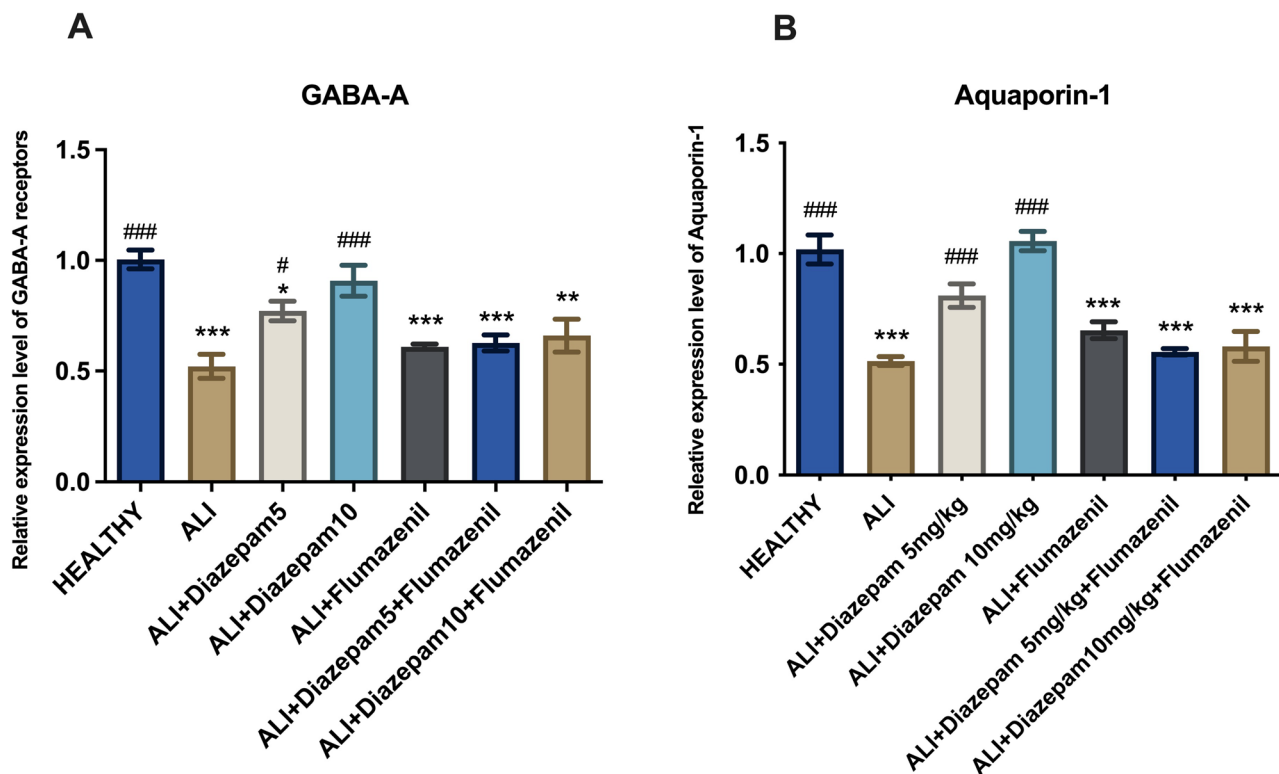


Fig. 6 **A** Relative expression level of GABA_A **B** Relative expression level of Aquaporin-1. * Comparison with respect to healthy, # comparison with respect to ALI group. (According to the One Way ANOVA-Tukey test, *** and ### denotes $p < 0.001$, ** denotes $p < 0.01$, * and # denotes $p < 0.05$)

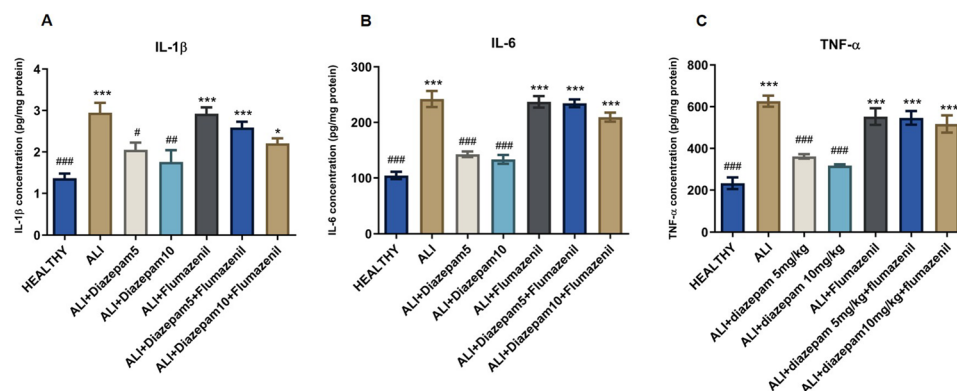


Fig. 7 **A** IL-1 β concentration (pg/mg protein) **B** IL-6 concentration (pg/mg protein) **C** TNF- α concentration (pg/mg protein). * Comparison with respect to healthy, # comparison with respect to ALI group (According to One Way ANOVA-Tukey test, *** and ### denotes $p < 0.001$, ## denotes $p < 0.01$, * and # denotes $p < 0.05$)

ensure gas exchange. Epithelial damage plays the most important role in inflammatory lung diseases [51]; it activates the NF- κ B pathway, leading to inflammatory systemic stimulation [52, 53]. Epithelial damage also decreases pulmonary surfactant function [54]. LPS damage increases the number of neutrophils, encourages the secretion of adhesion molecules, and causes more intense inflammatory cells to migrate to the damaged region [55]. The activation of alveolar macrophages causes the levels of TNF- α and other similar inflammatory cytokines to increase significantly

[56]. Considering the basic immune mechanisms in LPS-induced ALI, we see similarities with the possible anti-inflammatory effects of GABA_A receptor agonists as described above. In LPS-induced cases of ALI and many other relevant clinical studies, there are important differences in the characteristics of the lungs. The structure of the lungs is unique among organs; the epithelium is specialised to ensure ventilation and liquid-gas transfer is another important variable. Damage to this region has important consequences. Aquaporins were previously detected in cells where liquid-gas

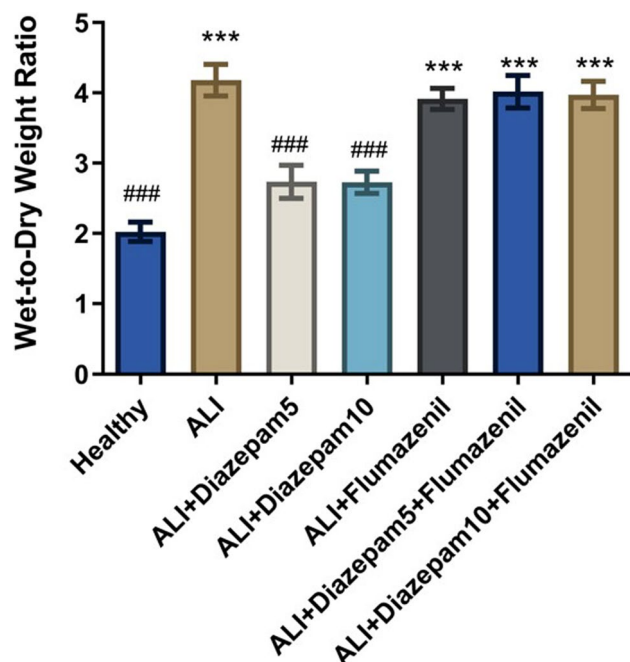


Fig. 8 Wet-to-Dry Weight Ratio. * Comparison with respect to healthy, # comparison with respect to ALI group (According to One Way ANOVA-Tukey test, *** and ### denotes $p < 0.001$)

exchange had taken place and where fluid passage was important, suggesting the need for further research [57]. Aquaporins have important roles in intracellular and extracellular water transfer. In LPS-induced ALI, disruption of the epithelial structure leads to increased extracellular fluid and lung damage. In such cases, the role of aquaporins cannot be overlooked. Aquaporin-1 has been shown in many studies to be important in lung injury [58, 59]; more specifically, it has been reported as an important diagnostic marker for ALI [60, 61].

The main role of aquaporin in the lungs is to ensure the water balance of the blood vessels and the surrounding peripheral tissues. Fluid secretion in this region is necessary to keep the airways moist and to ensure uninterrupted transport [57]. Studies have shown that aquaporin-1 plays an especially important protective role in ALI [62, 63]. In the present study, it was found that aquaporin-1, which plays an important regulatory role in LPS-damaged lung tissue, decreased and the weight of lung tissue increased in wet/dry analysis of a murine model of ALI. The levels of IL-1 β , IL-6, and TNF- α were found to be elevated. In the groups treated with diazepam, however, the levels of these inflammatory cytokines significantly decreased and, in parallel, a substantial increase in aquaporin-1 expression was observed. In line with the studies cited here, GABA_A expression was found to be significantly increased. The relationship between GABA_A

and aquaporin-1 was revealed in this study. When the GABA_A antagonist flumazenil was administered, the opposite effect was observed and no improvements occurred. The LPS-induced effect did not improve when the agonist and antagonist were administered, confirming that the healing effect occurred via GABA_A. These findings suggest that GABA_A receptor-mediated signaling may contribute to the amelioration of LPS-induced lung injury, potentially through the regulation of aquaporin-1 expression.

In a study by Huang et al., propofol was shown to reduce lung injury by GABA_A receptor upregulation [19]. In line with the present findings, it was shown by molecular, biochemical, and histopathological analyses that propofol decreased the levels of inflammatory cytokines and thus attenuated lung injury in the treatment groups. Considering the results obtained for GABA_A receptor expression in the present study, it can be concluded that GABA_A receptor expression increased significantly in the treatment groups compared to the control group. Even in the groups with LPS damage plus ventilator-induced lung injury, GABA_A receptor expression increased significantly compared to the control group. Our findings regarding GABA_A receptor mRNA expression revealed an increase in the treatment groups and significant improvement was observed in these groups when histopathological data and other results were compared. In the LPS-induced ALI group, there was a significant decrease in GABA_A receptor expression compared to the healthy group, contrary to the findings of Huang et al. Among the possible reasons for the differences between these two studies, differences in models can be noted. More importantly, the termination of the experiment in the present study occurred at the 24th hour, whereas this period was 5 h in the study by Huang et al. Five hours is a very short period of time for studies of lung injury and defence, particularly as receptor adaptation may require more time.

Conclusion

This study has revealed that diazepam, a GABA_A receptor modulator, may attenuate ALI by increasing aquaporin-1 expression through the upregulation of GABA_A receptor expression. Further clarification of this mechanism, which may play a key role in the pathogenesis of lung injury, might support the development of new therapeutic targets.

Limitations

The study was conducted using only male rats. Considering that sex-related differences in immune and inflammatory responses have been reported in experimental models of lung injury, the findings may not

fully reflect possible sex-dependent variations in the response to GABA_A receptor modulation. Also the present study focused on an acute LPS-induced lung injury model evaluated at a single time point (24 h). While this model is widely used and clinically relevant for studying inflammatory lung injury, it may not fully represent the complex and heterogeneous pathophysiology of human ALI/ARDS. Moreover, longer observation periods could provide additional insights into the temporal dynamics of GABA_A receptor and aquaporin-1 regulation during the progression and resolution of lung injury. Future studies are therefore required to clarify the mechanistic link between GABA_A receptor activation and AQP1 regulation. In particular, investigations focusing on intracellular signaling pathways such as NF- κ B, MAPK, cAMP/PKA, or other inflammatory signaling cascades may help to determine how GABA_A receptor modulation influences AQP1 expression and lung fluid homeostasis under inflammatory conditions. Furthermore, the detailed investigation of the potential roles of different GABA_A receptor subtypes in this process represents an important area for future research.

Abbreviations

ALI	Acute lung injury
AQP1	Aquaporin-1
AQP4	Aquaporin-4
GABA	Gamma-aminobutyric acid
GABA _A receptor	Gamma-aminobutyric acid type A receptor
IL-1 β	Interleukin-1 beta
IL-6	Interleukin-6
i.p.	Intraperitoneal
LPS	Lipopolysaccharide
TNF- α	Tumor necrosis factor- α
W/D	Wet-to-dry weight ratio

Supplementary Information

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Supplementary Material 1.

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Not applicable.

Authors' contributions

E.C. and H.H. conceptualized and designed the study. The methodology was developed by B.O., E.T., and Z.K. Formal data analyses were performed by Z.K., Z.H., E.C., and H.H. The investigation was carried out by H.H., Z.K., and Z.H. Z.K. and H.H. provided the study resources, while data curation was performed by B.O. and E.T. The original draft of the manuscript was prepared by H.H., Z.H., E.C., and Z.K., and the manuscript was reviewed and edited by H.H., Z.K., and Z.H. Visualization was carried out by E.C. and Z.K., and the study was supervised by Z.H. and H.H.

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Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

This study is not a clinical trial. It is an experimental animal study. The study was approved by the Ataturk University Local Animal Care Committee Meeting Date 26.03.2024 Meeting No and Resolution No 2024380.

Consent for publication

Not applicable. This study does not include any individual person's data in any form.

Competing interests

The authors declare no competing interests.

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