# Histochemical changes of the lung tissue in experimental chronic alcoholic intoxication

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**Abstract**. Among organ systems in the human body affected by alcohol abuse, the lungs are particularly vulnerable to infections and injury. Chronic alcoholism causesalterations in host defence of the upper and lower airways, disruption of alveolar epithelial barrier integrity, alcohol-induced ciliary lesions and alveolar macrophages dysfunction. Currently with a spread of SARS-COV 2 infections which instantly destroys the lung tissue, the alcohol-induced lung damage issues acquire vital importance, as they might further increase severity of lesions of lung tissue in the infected alcohol abusers.Recent investigations suggest that the effect of the chronic excessive alcohol consumption and SARS-COV 2 infection on the lungs might have similar and thus synergizing mechanisms. Therefore the mechanism of the lung tissue lesions in chronic alcohol intoxication need to be scrutinized, including the time-line of their development, to be able to develop more effective preventive measures. The objective of the study is to assess histochemical changes in the lung tissue of laboratory animals with chronic alcohol intoxication of different duration. Total of 48 outbred male white mice weighing 18-22 g were enrolled in the study. The experimental animals were exposed to alcohol for 1, 2 and 3 months by the semi-voluntary intake, using 20% alcohol as the only source of fluid, while control animals were getting drinking water. At the end of experiment the lung tissue of the mice was processed histologically and histochemically for alcoholic dehydrogenase (ADH), glucose-6-phasphate-dehydrogenae (G6PDH), alkaline (ALP) and acidic (AP) phosphatases, nonspecific esterase (NE) and succinate dehydrogenase (SDH). Image analysis of the histological slides was performed using Image Pro Plus software. Statistical differences were assessed using paired t-test. Chronic alcohol consumption causes metabolic lesions in the alveolar epithelium and endothelium of alveolar capillaries revealed by an increase in the activity of ADH, G6PD and NE paralleled with a decrease in the total SDH activity of the respiratory portion of the lungs in a time-related pattern. High activity of alkaline phosphatase was noted in endothelial cells of lung capillaries. Thus, under conditions of chronic intoxication, ethanol disturbs cell metabolism, as evidenced by the changes of the enzymatic activity in the lung tissue which leads to inhibition of oxygen-dependent metabolic processes and activation of reserve mechanisms for compensating of energy deficits.

**Keywords:** lungs, chronic alcohol intoxication, alcohol dehydrogenase, succinate dehydrogenase, glucose-6-phosphate dehydrogenase, acid phosphatase, alkaline phosphatase, nonspecific esterases

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## 1. Introduction

Currently, chronic alcoholism is one of the most significant social problems of the modern world, which tends to deteriorate in many countries [1]. Binge drinking is associated with alcoholic cardiomyopathy, high blood pressure, increased risk of myocardial infarction, arrhythmias, and fatal cardiac arrest and stroke [2].

In 2016, the harmful use of alcohol resulted in almost 3 million deaths (5.3% of alldeaths) worldwide and 132.6 million disability-adjusted life years (DALYs), i.e. 5.1%of all DALYs in that year. Mortality resulting from alcohol consumption is higher thanthat caused by such diseases as tuberculosis, HIV/AIDS, and diabetes [1]. Many researchers studying the social, medical, and biological aspects of this topical problem show increased interest in this issue [3]. Clinicians have long observed an association between excessive alcohol consumption and adverse immune-related health effects, such as susceptibility to pneumonia. In recent decades, this association has been expanded to a greater likelihood of sepsis, alcoholic liver disease (ALD), and certain cancers; a higher incidence of postoperative complications; and slower and less complete recovery from infections and physical trauma, including poor wound healing [4].

Heavy alcohol use is a risk factor for acute respiratory distress syndrome (ARDS) [5], one of the most severe complications of COVID-19.

The alcohol-related pathology of internal organs has been the subject of numerous experimental and clinical studies. They determined the mechanisms of tissue damage during long-term alcohol consumption for such vital organs as the liver [6], heart [7], brain [8], and gastrointestinal tract [9]. Similarly, COVID-19 mediates a damaging effect on organ systems through cytopathic effects and cytokine storm. Alcoholism potentially increases the risk of cardiac injury, pulmonary fibrosis, and liver damage in synergy with COVID-19; thereby worsening disease prognosis and outcome [10].

The study aims to identify the time-line of the morphofunctional changesin the lung tissue secondary to chronic alcohol intoxication.

## 2. Material and research methods

The experiment was carried out on 32 white outbred male mice weighing 18 to 22 grams. The experimental animals were subjected to 1, 2 and 3 months of alcoholisation by the semi-voluntary intake, using a 20% ethanol solution in water as the only source of fluid, because this concentration is optimal for simulating chronic alcohol intoxication in these animals [11]. Control mice were in similar conditions, but without access to alcohol. The experimental and control animals were sacrificed through decapitation with ether anaesthesia and according to the rules of euthanasia. The study was approved by the institutional ethical committee. Cryostat-microtome sections with a thickness of 10 μm were cut. To detect the activity of the studied enzymes, the sections were incubated in the appropriate substrates. Histochemical activity of ADH was detected G.B. Pierce method, G-6-PDH by the Nachlas-Seligman method in the modification of Gomori, SDH - by the Nachlas method; AP - by Burston reaction and alkaline ALP - by to Pearse method modified by Z. Loyd.

The histochemical intensity of the reactionwas assessed using RGB scale (0-255) by the Image Pro Plus software with five levels of activity. Paired T-test was used for statistical evaluation using SPSS, software with.

## 3. Results

In a histochemical study of lung tissue, ADH is determined in alveolocytes, macrophages, lung capillaries. Compared with the control, the enzyme activity in the lung tissues in experimental animals significantly increased (Fig. 1).

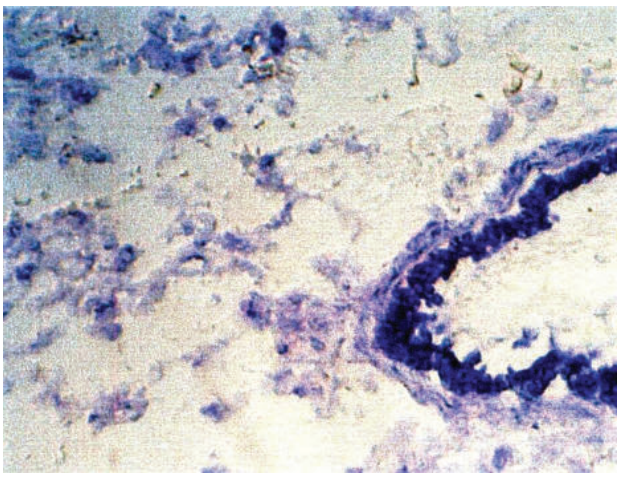

**Figure 1.** Mouse lung with chronic ethanol ntoxication. High activity of ADH in the epithelium of the ronchial tree. Pierce's reaction. x 200.

The highest activity of ADH was observed in smooth myocytes and epithelial cells of the bronchi, as well as in the cells of the secretory epithelium of the bronchial glands of the terminal sections of the bronchi. In the areas of purulent-necrotic inflammation of the walls of the bronchi, on the contrary, inhibition of enzymatic activity was noted. ADH activity was visualized by the uneven distribution of diformazan in the cells of the bronchial mucosa. The moderate activity of ADH was determined in alveolocytes and macrophages. In the epithelium of the alveolar septa, as well as in the walls of the vessels of the microvasculature, low activity of ADH was recorded. Image analysis showed that the overall activity of LDH was significantly increased in the lung tissue after 1 month of alcohol exposure ($p<0.05$); thereafter it started to decrease by the 2nd month and was further reduced after 3 months of experiment, still remaining significantly higher than in the control animals (Fig. 2).

In the cells of the alveolar epithelium, both in control and in experimental animals, the activity of SDH was not always determined. Mononuclear phagocytes with hemosiderin in the cytoplasm had an extremely low SDH activity, and epithelial cells of the bronchial glands, as a rule, had a negative reaction to this enzyme.

Overall activity of the SDH in the lung tissue of the experimental animals was highly significantly lower than in the control animals, with the inhibition reaching its peak by the end of the 3rd month of alcoholisation ($p<0.01$), (Fig. 2).

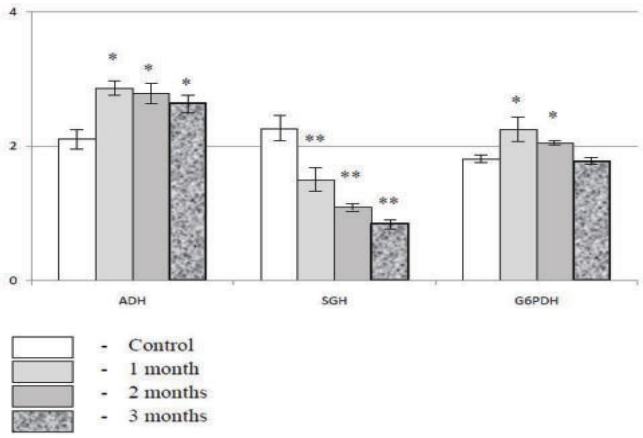


**Figure 2.** Activity of dehydrogenases in mice with chronic alcohol intoxication, M+/-SE.

The histochemical activity of G6PDH was visualized in the alveolar epithelium and macrophages (Fig. 3). In alveolocytes of experimental animals, the enzyme activity was significantly increased compared to the control, in macrophages of experimental animals, the enzyme activity was also increased in comparison with the control. Siderophages exhibited high enzyme activity. In the cells of the secretory epithelium of the bronchial glands, the moderate activity of G-6-FDG was noted. As follows from Figure 2, in the alveolocytes of experimental animals, the enzyme activity is significantly increased after 1 month of experiment compared with the control ($p<0.05$), thereafter it continued to decrease by the end of the 2nd month still remaining significantly higher than in the control ($p<0.05$) while by the end of the 3rd month it almost reached the level of the control.

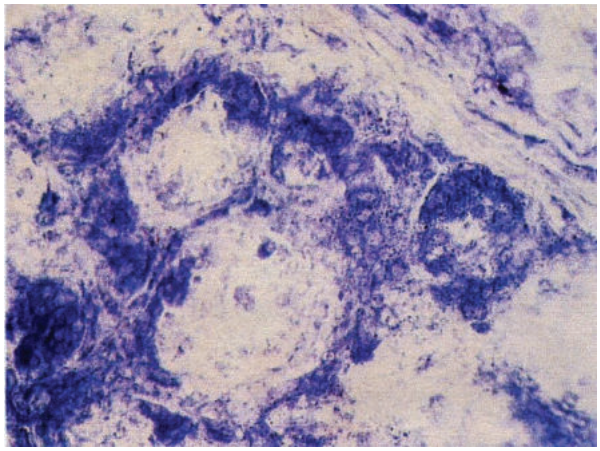

**Figure 3.** A mouse lung with chronic ethanol intoxication for 3 months. The moderate activity of G-6-FDG in the epithelial cells of the bronchial glands. Hess reaction. x 400.

In alveolar phagocytes, a high activity of acid phosphatase was found in the form of a diffuse distribution of the reaction product throughout the cytoplasm. At the same time, the response to AP was insignificant in the cells of the epithelial lining of the alveoli (Fig. 4).

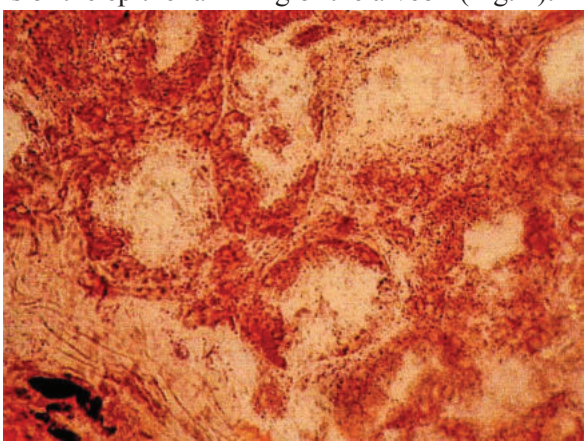

**Figure 4.** Mouse lung against the background of chronic ethanol intoxication. The moderate activity of AP in epithelial cells of bronchial glands. Burston reaction. x 400.

As follows from Figure 5, in comparison with the control group, a significant increase in the activity of AP in the experimental group of animals was recorded in the secretory epithelium of the bronchial glands by the end of the 1st month of alcoholisation ($p<0.05$) and continued to increase progressively towards the end of the 3rd month. The increased activity of acid phosphatase was observed mainly in neutrophilic leukocytes in the areas of purulent inflammation of the walls of the

bronchi and lung parenchyma. In the walls of blood vessels in animals, both in the control and in the experimental groups, the activity of AP was practically not determined.

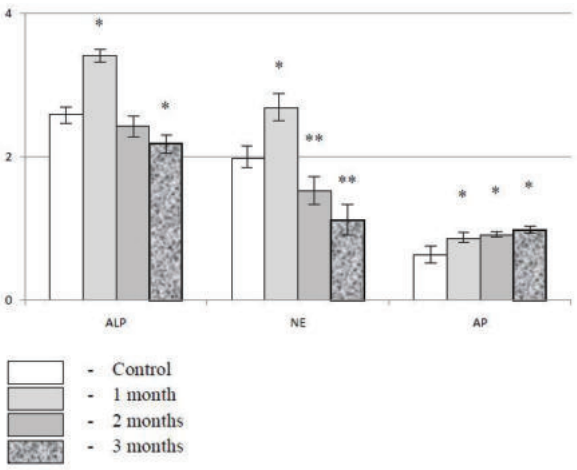


**Figure 5.** Activity of hydrolases in mice with chronic alcohol intoxication, M+/-SE.

The histochemical activity of alkaline phosphatase was detected in the vascular endothelial cells of the microvasculature of the alveolar septa (Fig. 6).

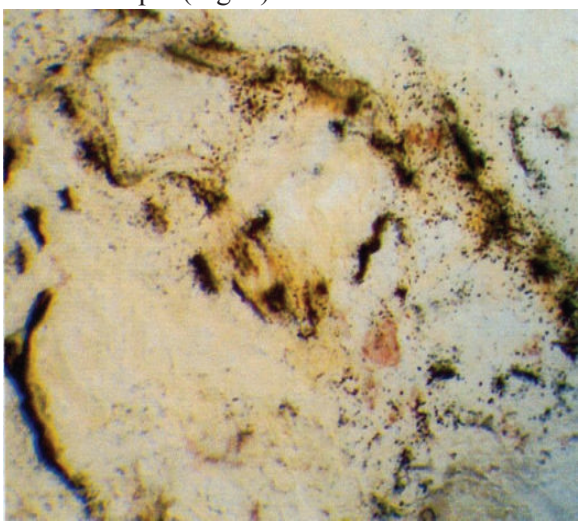

**Figure 6.** High ALP activity in the walls of the vessels of the microvasculature of the lungs. Pierce's reaction. X200.

The enzyme activity is represented by dark brown azo dye granules, which diffusely stain the walls of blood vessels. In the alveolar epithelium, the walls of large vessels and the epithelium of the bronchial glands, the histochemical activity of alkaline phosphatase was not recorded. High activity of ALP was noted in the cellular elements of inflammatory exudates located in the walls of the bronchi and alveoli, as well as in the areas of growth of connective tissue.

As follows from the Figure 5, the activity was statistically significantly higher after 1 month of alcoholisation than in the control ($p<0.05$), thereafter it started to decrease, almost reaching the control level by the end of the 2nd month and further reducing by the end of the 3rd month, when it becomes significantly lower than in the control ($p<0.05$).

NE were detected in the cells of the alveolar epithelium and phagocytes containing hemosiderin. In the alveolocytes located in the area of the interconnections of the interalveolar septa, a high

histochemical activity of NEwasdetermined. The activity of these enzymes was revealed in the form of a diffuse arrangement of azo dye granules throughout the cytoplasm of cells.

NEs were absent in epithelial cells of bronchial glands in both groups of animals. The moderate activity of these enzymes in the cells of the stromal elements of the bronchial glands was observed only in experimental animals.The total activity of NE in the tissues of the lungs of experimental animals wassignificantly increased by the end of the 1st month of the experiment ($p<0,05$) as compared to the control (Fig. 5). Thereafter it started to decrease and after the 2nd month onwards it was highly significantly lower than in the control ($p<0.01$).

The results of the study showed that in experimental animals, against the background of chronic intoxication with a 20% ethanol solution, metabolic disorders occur in the epithelial cells of the respiratory parts of the lungs and alveolar macrophages, and a decrease in the level of metabolic processes is also observed.

## 4. Discussion

In our study, we propose several possible mechanisms through which chronic alcohol abuse and SARS CoV2 infection synergize to exacerbate detrimental processes with inflammation as a key common factor in underlying both pathogeneses. Understanding of the synergetic mechanisms will also aid in the development of new therapeutic strategies to prevent alcohol-mediated effects in SARS CoV2 -infected population.

In the histochemical study of lung tissue, alcohol dehydrogenase(ADH) was determined in alveolocytes, macrophages, and capillaries of the lungs. ADH-oxidation of ethanol generates reactive metabolite acetaldehyde, which readily forms adducts with proteins and causes oxidative stress[13]. Once formed, acetaldehyde is rapidly absorbed through the lungs [14]. Biological consequences of acetaldehyde exposure include a reduced phagocytotic index of lung macrophages[15]. According to Abassi Z. et al. [16], while macrophages play an important role in antiviral defence mechanisms, in the case of SARS-CoV, they may also serve as a Trojan horse, enabling viral anchoring specifically within the pulmonary parenchyma. Moreover, enhanced activity of pro-inflammatory macrophages in part of the COVID-19 patients leads to accelerated production of inflammatory cytokines and chemokines, and among them is CXCL10, which leads to cytokine storms associated with poor prognosis [17, 18]. Although the authors are not intended to promote alcohol consumption whatsoever, we assume that control of the phagocytotic activity of lung macrophages may be an important goal in future research to decrease unfavourable outcomes of SARS-CoV2 infection.

The high activity of ADH in the cells of the epithelium of the bronchi in animals of the experimental group, compared with the control was established which contributes to the accumulation of excess acetaldehyde in the tissues. Acetaldehyde converts angiotensinogen to angiotensin I in rat plasma in vitro [31]. The biological effects of angiotensin II depend on its interaction with specific angiotensin II receptors. Of the angiotensin II receptors, the type 1 receptor (AT1) and the type 2 receptor (AT2) have been best characterized. The AT2 receptor is present in a few tissues during adulthood, whereas it is abundantly expressed during embryogenesis and in response to injury [32]. Stimulation of the AT2 receptor inhibits cell proliferation and leads to apoptosis, actions directly opposed to the proliferative responses that often follow AT1 activation [30]. Experimentally, chronic alcohol ingestion markedly increases the relative expression of the AT2 receptor within the alveolar epithelium and in parallel renders these cells susceptible to apoptosis when exposed to oxidative stress or proinflammatory cytokines [33].

In the same manner, AT2R may be re-expressed in COVID-related lung injury and hence the use of AT2R agonists may also be explored to elicit their anti-inflammatory and anti-fibrotic effects [32].

In our study, inhibition of the total activity of SDH in the cells of the respiratory parts of the lungs of experimental animals compared with the control group was revealed. As it was established earlier inhibition of the total activity of succinate dehydrogenase in a mouse model leads to increased levels of succinate[18]. Succinate activates hypoxia-inducible factor (HIF) 1α in a normoxic manner in alveolar epithelial cells [19] facilitating the adaptation of these epithelial cells to mechanical stress by

increasing the glycolytic capacity of the cells, tricarboxylic acid flux and mitochondrial respiration, thus increasing in the amount of ATP produced by alveolar epithelial cells. Thus pharmacological targeting of glycolysis in alveolar epithelial cells in COVID-19 during the ARDS stage may be a useful strategy to promote disease tolerance during infection [20] as well as against the collateral lung damage caused by the use of mechanical ventilators, which can contribute to lung injury.

The study also identified the histochemical activity of G6PDH in the alveolar epithelium and macrophages. It is tempting to assume that just like macrophage G6PD stimulates proinflammatory responses with oxidative stress in adipocytes [21] it is also true for alveolar macrophages, and pharmacological control over G6PD may lessen poor outcomes in some patients with SARS-CoV-2 infection preventing cytokine storm development [22].

In alveolar phagocytes, a high activity of AP was found in the form of a diffuse distribution of the reaction product throughout the cytoplasm. As it was established earlier, reductions in lysosomal acid phosphatase coincide with impairment of the bactericidal activity of alveolar macrophages [23]. Theoretically, it is possible to use of acid phosphatase activity levels as a marker of phagocytic activity of alveolar macrophages against SARS-CoV2 in bronchoalveolar lavage to predict unfavourable outcome in some COVID-19 patients.

NE were detected in cells of the alveolar epithelium and phagocytes especially in the alveolocytes located in the area of the interconnections of the interalveolar septa, where a high histochemical activity of nonspecific esterases is revealed.

Changes in esterase activity might reflect physiological and immunological stimulation of monocytes [25] and probably their progeny - including alveolar macrophages since macrophages are capable of activation by environmental factors including viruses which is crucial for the initiation of immune responses against invading respiratory viruses [26] since this esterase is an ectoenzyme which may function as a mediator of cell response to injurious agents from the outside [27]. Pharmacological interventions including steroids (dexamethasone) aimed to decrease phagocytosis [28] by alveolar macrophages while stabilization of phagosomes and reduced secretion by alveolar macrophages may have beneficial effects in circumstances of lung injury and alveolitis [29] seen in some pulmonary pathologies including COVID 19 [28]. This correlates with increased intracellular levels of NE probably indicative of lower phagolysosome formation and lower enzyme secretion in alveolar macrophages [30].

Thus, the identification of lysosomal enzyme content of alveolar macrophages in bronchoalveolar lavage may serve as a predictive tool of phagocytic activity of alveolar macrophages.

Ethanol promotes the expression of ALP activity in human vascular smooth muscle cells (HSMCs) [34]. The histochemical activity of ALP was detected in the vascular endothelial cells of the microvasculature of the alveolar septa as well as in the cellular elements of inflammatory exudates located in the walls of the bronchi and alveoli, as well as in the areas of the proliferation of connective tissue.

ALP is a marker of tissue damage and type II cell proliferation. Type II pneumocytes are extensively involved in fibrosis [24]. We assume that identifications of ALPactivity has in the bronchoalveolar lavage fluid obtained from patients infected by COVID-19 can be used as a predictive tool of pulmonary fibrosis following SARS-CoV-2 infection.

The high activity of ALP in the vascular endothelium of the lungs in experimental animals contributes to increased vascular permeability, which ultimately may lead to diapedesis of erythrocytes, their haemolysis, and subsequent haemosiderosis of the lungs. Likewise, conditions that affect alveolar-capillary integrity/permeability (e.g., Goodpasture's disease) [35] can lead to haemorrhage into the alveolar space. These patients show alterations in lung iron homeostasis with increased iron levels in alveolar macrophages, likely resulting from haemoglobin-derived iron from phagocytosed RBCs. Excessive accumulation of erythrocyte destruction products by macrophages may contribute to the weakening of their phagocytic function, in particular, to microorganisms.

## AUTHOR'S CONTRIBUTION

Alexey Lazko – 40%.
Larisa Udochkina – 30%.
Nina Losovskaya – 30%.

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