SECTION 7. THEORETICAL MEDICINE

7.1 The mechanism of development of isoniazid and rifampicin induced hepatotoxicity in different age groups of rats

Multiple drugs, both prescription and over-the-counter, herbal products, or toxins can cause hepatotoxicity through a variety of mechanisms [407, 408]. According to World Health Organization statistics, drug-induced liver injury has risen as the 5th leading cause of death worldwide. Approximately 50% of liver failure patients receiving liver transplantation every year have drug-induced liver failure, which prompts the need for artificial liver and liver transplantation and even causes death [409]. However, the disease is often undiagnosed or misdiagnosed clinically because of diagnostic difficulties caused by lack of laboratory specific serological markers.

The probability of an individual drug causing liver injury ranges from 1 in 10,000 to 100,000, with some drugs reported as having an incidence of 100 in 100,000 (chlorpromazine, isoniazid) [410]. Drugs can cause liver cell necrosis as foreign antigens by activating the immune system and then mediating corresponding injuries. According to recent studies, microRNA (miR)-122, as a highly liver-specific miR, can be used as a molecular marker of hepatocellular injury [411], whose specificity and sensitivity are significantly higher than alanine aminotransferase in the early diagnosis of APAP-induced drug-induced liver injury [412]. Some pathological findings may be relatively specific. The common histopathological changes in the liver may include ballooning changes or microvesicular steatosis, eosinophil infiltration, epithelial cell granuloma and cholestasis, but pathological evidence makes sense only in combination with medical history and other clinical data.

There are various factors that enhance a person's susceptibility to a potentially hepatotoxic drug. Advancing age, gender, lifestyle factors, obesity, nutritional status, genetic background, dose, and duration of drugs may affect the risk of drug-mediated hepatotoxic reactions. Persons suffering from other diseases, such as, human

immunodeficiency virus, hepatitis C, rheumatoid arthritis, and systemic lupus erythematosus, are more prone to toxic drug reactions. Drug composition and drug-drug interaction may also be a reason for increased risk of drug-induced hepatotoxicity [413].

Tuberculosis is a leading public health problem worldwide, particularly in developing countries. In 2020, the largest number of new TB cases occurred in the WHO South-East Asian Region, with 43% of new cases, followed by the WHO African Region, with 25% of new cases and the WHO Western Pacific with 18% [414]. In 2020, 86% of new TB cases occurred in the 30 high TB burden countries. Eight countries accounted for two thirds of the new TB cases: India, China, Indonesia, the Philippines, Pakistan, Nigeria, Bangladesh and South Africa [414].

In 2020, an estimated 10 million people fell ill with tuberculosis (TB) worldwide. 5.6 million men, 3.3 million women and 1.1 million children. TB is present in all countries and age groups. Tuberculosis mostly affects adults in their most productive years. However, all age groups are at risk. Over 95% of cases and deaths are in developing countries [414].

Treatment for active TB infections comprises 4 months of rifampicin, isoniazid, pyrazinamide, and ethambutol. Hepatotoxicity decreases adherence to antitubercular treatment regimens and contributes to treatment failure, disease relapse, and drug resistance [415]. Hepatotoxicity is a leading cause of TB therapy treatment failure, with up to 28% of all anti-TB treatment failure attributed to toxicity [416].

Antituberculotics accounted for 17 % of all drug-induced liver injury, which is the type of Western drugs associated with the highest incidence of drug-induced liver injury. It is also one most common cause of discontinued antituberculosis therapy. During antituberculosis therapy, long-term use of antituberculotics is inevitable, thus almost all patients will experience varying degrees of adverse reactions at different frequencies [413].

Recent studies indicate the existence of a strong correlation between hepatic injury and oxidant stress in experimental animals treated with anti-tuberculosis drugs [417, 418]. Since all the drugs used in the treatment of tuberculosis are shown to have

hepatotoxic effects, studies have been performed to prevent or reduce the toxicity by the use of natural herbal drugs and/or synthetic compounds, without interfering with the therapeutic actions of the drugs.

Rifampicin, isoniazid, pyrazinamide and ethambutol are first line drugs used for the treatment of tuberculosis. According to the Centre of monitoring of adverse reactions of drugs (2007), isoniazid – 29.2%, rifampicin – 26.7%, capreomycin – 17.1%, ethambutol – 10.2% dominate among monopreparations in high incidence of the adverse reactions in world [419]. Thus, the risk of the development of hepatitis increases in patients who take rifampicin together with isoniazid. In this case hepatitis incidence is 5-8%. During the isoniazid monotherapy, the incidence of hepatitis is 1.2%, but during the rifampin monotherapy – 0.3% [419].

Isoniazid is the first-line treatment for tuberculosis; however, its use is limited by hepatotoxicity. Isoniazid, an anti-tubercular drug, is used alone or in combination with other drugs to eliminate the active bacteria. Usually, the therapy with isoniazid is continued for a longer time (6–12 months) since the bacteria may exist in a resting state for a longer period of time. The studies have indicated severe and fatal hepatitis with isoniazid therapy [420, 421]. In the liver, isoniazid is metabolized primarily by N-acetyl transferase 2 to acetyl-isoniazid, which subsequently is converted to mono-acetyl hydrazine and non-toxic diacetyl hydrazine, as well as other minor metabolites [420, 421]. Isoniazid inhibits glutathione biosynthesis, activities of antioxidant glutathione peroxidase and catalase activity in rats [422, 423]. Furthermore, acetyl-hydrazine, a metabolite of isoniazid, causes damage to hepatic cells by covalently binding to liver macromolecules [421].

Hepatotoxicity occurs in approximately 10% of patients treated with isoniazid with 1% developing clinical hepatitis [424]. One of the major risk factors for isoniazid-induced hepatotoxicity is old age [425, 426]. However, the reason for this observation is unclear. A better understanding of isoniazid pharmacokinetics and toxicity in old age is required to establish safer management and therapeutic dosing guidelines for older adults. In older people compared with young treated with the same single dose of isoniazid daily for 2 months, serum isoniazid concentrations were unchanged with age.

However, serum hydrazine levels were significantly increased in old age [427], which may imply older people could have increased susceptibility to hepatotoxicity caused by hydrazine.

Together with rifampicin, isoniazid is one of the most powerful drugs for the treatment of tuberculosis. These 2 drugs represent the cornerstone of the first-line treatment regimen recommended by WHO and used worldwide [428]. It is also a component of both shorter and longer second-line treatment regimens for rifampicin-resistant tuberculosis. Isoniazid is critical not only to the treatment of active tuberculosis, but it is also the most commonly used medicine for preventive therapy [429].

The recent systematic review of published literature revealed that treatment of isoniazid-resistant, rifampicin-susceptible TB with the standard first-line regimen for new TB patients resulted in 11% (95% CI 6%–17%) treatment failure, compared with 2% (95% CI 1%–3%) among drug-susceptible TB patients [430]. To increase the likelihood of long-term cure of TB patients, WHO released updated guidance for the treatment of patients with Hr-TB in 2018 [431]. The main regimen is a 6-month course of rifampicin, pyrazinamide, ethambutol, and levofloxacin. The addition of a higher dose of isoniazid may be considered if only low-level isoniazid resistance is present.

Rifampicin was then developed in 1966, proving highly effective combined with INH for treating drug-susceptible tuberculosis [432]. It is readily absorbed from the gastrointestinal tract (90%) and most of it is bound to plasma proteins in circulation. The involvement of oxidative stress in rifampicin-induced hepatotoxicity has been demonstrated previously in experimental rats [433]. Rifampicin is a potent inducer of cytochrome P450 action and enhances the covalent binding of reactive metabolites of acetyl hydrazine to the macromolecules of hepatocytes leading to hepatic cell damage [434]. Additionally, desacetyl rifampicin, another reactive metabolite of rifampicin, also contributes to some of its adverse effects. It has been reported to modulate the membrane permeability and cause membrane damage [435]. Its prolonged exposure significantly decreases glucose-6-phosphatase activity, which could be a reason for the increased level of lipid peroxidation [436].

Several in vivo studies found that rifampicin plus isoniazid induced hepatocyte apoptosis in rodent animals [437, 438]. The mechanism through which rifampicin induces liver injury remains obscure. An earlier study demonstrates that oxidative stress in the mitochondria is involved in the pathogenesis of rifampicin plus isoniazid-induced apoptotic liver cell injury in mice [439].

In patients, clinical data have elucidated the pharmacokinetics of isoniazid metabolism and rifampicin involved in the process of liver toxicity. Observational clinical studies are also confounded by age-associated factors that may impact susceptibility, which include increased polypharmacy and multimorbidities. Therefore, controlled models are required to understand the mechanism of isoniazid and rifampicin hepatotoxicity in the age aspect. The objective of the study was to investigate the mechanism of development of isoniazid and rifampicin intoxication in different age groups of rats.

The experiments were conducted on outbred white male rats of three age groups: the 1st group –immature (3-month-old animals, 90-110 g in weight); 2nd group –mature (6-month-old animals, 150-170 g in weight); and the 3rd group –senile (18-months-old animals, 280-300 g in weight). They were kept on a standard diet at the vivarium of Ternopil National Medical University.

The sustentation and manipulations of animals were carried out according to the "European Convention for the protection of vertebrate animals used for experimental and other scientific purposes" [440].

All experimental animals of each age group were divided into two groups: the control group – intact animals (injected with a physiological solution); the experimental group – animals that were administered isoniazid and rifampicin. In the each study group 6 animals were selected.

The experimental toxic affection of animals was simulated by combined effect of isoniazid and rifampicin. Isoniazid and rifampicin were administered intragastrically in aqueous solution to animals every day, 0.05 g/kg and 0.25 g/kg accordingly, during the 7th and 14th days. Euthanasia was performed by means of thiopental sodium (25 mg/kg) on the 7th and 14th day from the first day of anti-TB drugs administration.

The study of liver homogenate and blood serum was performed. The blood was taken from the heart of animals by centrifugation at 3000 rpm during 30 min. The obtained blood serum (a sedimentary liquid) was used for researches. Selected liver (250 mg) was used to obtain the homogenate by the method of differential homogenization; it was used after previous perfusion in physiological solution.

The activity of oxidative processes was evaluated by the determining the content of TBA-reactive products (TBA-RP) [441] and the content of oxidative modification of protein products (2,4-DNPhH) [442]. The state of the antioxidant system was evaluated by the antioxidant system indexes: by the determining the catalase activity [443] and the content of reduced glutathione (G-SH) [444]. The severity of endogenous intoxication syndrome was determined by the content of middle-weight molecules (MWM) [442]. The activity of liver enzyme markers was determined by the rate of aminotransferases (ALT and AST) [445].

The content of TBA-RP was calculated based on the molar extinction coefficient of the colored complex (malonic dialdehyde forms a red color complex with thiobarbituric acid), which is equal to 1.56×10^5 $cm^{-1}mol^{-1}$. The catalase activity was determined by the ability of hydrogen peroxide to form a stable colored yellow complex with ammonium molybdate. The content of reduced glutathione was determined by the method, the principle of which is the interaction of 5.5-dithiobis (2-nitrobenzoic) acid (Elman's reagent) with free SH-groups of reduced glutathione with formation of a yellow color of thionitrophenyl anion, the amount of which is directly proportional to the content of SH-groups. The content of G-SH was calculated based on the molar extinction coefficient for the thionitrophenyl anion, which is 1400 $mol^{-1}cm^{-1}$.

The principle of the determining method oxidative protein modification is based on the ability of radical residues of aliphatic amino acids to form aldehyde and ketone groups. They interact with 2.4-dinitrophenylhydrazine to form 2.4-dinitrophenylhydrazones, which have a characteristic absorption spectrum. Aldehyde and keto derivatives of the neutral character are registered at 370 nm, and of the basic character – at 430 nm.

The MWM content was calculated based on the precipitation of high molecular weight peptides and proteins of biological fluids with using trichloroacetic acid and quantification determining in the supernatant obtained by centrifugation of medium molecular weight peptides by absorption in monochrome light flux at 254 nm and 280 nm.

The evaluation of ALT rate was conducted by compound of 2-oxoglutaric acid and L-alanine, which under the influence of alanine aminotransferase formed L-glutamic and pyruvic acids. The interaction of pyruvic acid and 2.4-dynitrophenylhydrazine in alkaline medium formed 2.4-dynitrophenylhydrazones that had high coefficient of the molar extinction, so its optical density registered on the FEC was directly proportional to the activity of the enzyme. The enzyme activity was estimated by the calibration graph due to the content of pyruvic acid in μmol / (L · h).

AST rate was evaluated by optical density measuring of 2.4 nitrophenylhydrazones of 2-oxoglutaric and pyruvic acids in alkaline medium. Hydrazone of pyruvic acid has a higher coefficient of the molar extinction, so there is a directly proportional relationship of optical density of the reaction solution to the enzyme activity. The enzyme activity was evaluated by the calibration graph due to the content of pyruvic acid, mkmol / (L · h).

The processing of statistical data was carried out in the SPSS-22 software package. The distribution of data is analyzed according to Kolmogorov-Smirnov's criterion of normality. The obtained values had a nonparametric distribution, so the difference between the groups was analyzed according to the Student's t-criterion and the non-parametric Wilcoxon's criterion for the connected samples. The criterion $\chi 2$ was used to evaluate the difference between categorical data. The difference of probability values was $p \geq 0.95$ (P significance level). Differences were considered probable at $p < 0.05$ [446].

Lipid peroxidation can be described generally as a process under which oxidants such as free radicals or nonradical species attack lipids containing carbon-carbon double bond(s), especially polyunsaturated fatty acids (PUFAs) that involve hydrogen

abstraction from a carbon, with oxygen insertion resulting in lipid peroxyl radicals and hydroperoxides as described previously [447].

Glycolipids, phospholipids and cholesterol are also well-known targets of damaging and potentially lethal peroxidative modification. Lipids also can be oxidized by enzymes like lipoxygenases, cyclooxygenases and cytochrome P450. In response to membrane lipid peroxidation, and according to specific cellular metabolic circumstances and repair capacities, the cells may promote cell survival or induce cell death. Under physiological or low lipid peroxidation rates (subtoxic conditions), the cells stimulate their maintenance and survival through constitutive antioxidants defense systems or signaling pathways activation that upregulate antioxidants proteins resulting in an adaptive stress response [448].

By contrast, under medium or high lipid peroxidation rates (toxic conditions) the extent of oxidative damage overwhelms repair capacity, and the cells induce apoptosis or necrosis programmed cell death; both processes eventually lead to molecular cell damage which may facilitate development of various pathological states and accelerated aging. The impact of lipids oxidation in cell membrane and how these oxidative damages are involved in both physiological processes and major pathological conditions have been analysed in several reviews [447].

The extension of the oxidative catabolism of lipid membranes can be evaluated by several endpoints, including the measurement of exhaled alkanes (mainly ethane and pentane), determination of isoprostanes and of a variety of aldehydes in biological samples [449]. The most widely used method is the quantification of malondialdehyde (MDA), one of the stable aldehydic products of lipoperoxidation, present in biological samples, such as whole blood, plasma or urine [450]. Urinary lipid peroxidation products most probably reflect the global oxidative status of the whole body [451] and have been shown to be as informative as whole blood or plasma values.

MDA has been widely used for many years as a convenient biomarker for lipid peroxidation of omega-3 and omega-6 fatty acids because of its facile reaction with thiobarbituric acid (TBA) [452]. The TBA test is predicated upon the reactivity of TBA toward MDA to yield an intensely colored chromogen fluorescent red adduct; this test

was first used by food chemists to evaluate autoxidative degradation of fats and oils [447]. However, the thiobarbituric acid reacting substances test (TBARS) is notoriously nonspecific which has led to substantial controversy over its use for quantification of MDA from *in vivo* samples. Several technologies for the determination of free and total MDA, such gas chromatography-mass spectrometry, liquid chromatography-mass spectrometry and several derivatization-based strategies, have been developed during the last decade [448]. Because MDA is one of the most popular and reliable markers that determine oxidative stress in clinical situations [448], and due to MDA's high reactivity and toxicity underlying the fact that this molecule is very relevant to biomedical research community.

TBA-RPs are formed in the process of lipoperoxidation (POL) and allow to judge about the intensity of this process in the affected organism in blood serum. Results of the study of their content are given in Table 1. The content of TBA-RP in blood serum of all age groups was established in the organisms of rats. The content of TBA-RP in blood serum was increased by 267% in the immature animals, by 273% – in the mature animals and by 450% – in the senior animals in comparison with the intact control ($p<0.05$) on the 7^{th} day of the experiment.

Such a tendency in increase of the content of lipoperoxidation products can be explained by the fact that anti-tuberculosis drugs causes formation of the reactive oxygen species (ROS) in an organism. The latter interact with cells biopolymers, taking part in the reactions of POL, and stimulate the processes of free radical oxidation of biomolecules. The combined action of anti-tuberculosis drugs had led to an even greater increase of TBA-RP content already on the 14^{th} day of the experiment. It was 383% in animals of the immature age, in animals of the mature age – 442%, and in animals of the senior age – 445% (Table 1).

Table 1

The content of TBA-RP in blood serum (μmol / L) and liver (μmol / kg) of rats affected by isoniazid and rifampicin (M ± m)

Research material	**Group of animals**	**Age group of animals**					
		immature		mature		senior	
		Research duration, days					
		7th	14th	7th	14th	7th	14th
Blood serum	Control group	0.89±0.10		0.74±0.05		0.62±0.02	
	Experimental group	3.27± 0.19*	3.41± 0.18*	2.76± 0.15*	3.27± 0.19*	3.41± 0.18*	2.76± 0.15*
Liver	Control group	16.66±2.25		5.88±0.45		6.94±0.20	
	Experimental group	37.50± 1.77*	41.88± 2.14*	12.39± 0.46*	37.50± 1.77*	12.39± 0.46*	41.88± 2.14*

Note: here and in the following tables * – significant differences between the animals of the control group and the animals affected by toxicants, $p \leq 0.05$.

The most susceptible to isoniazid and rifampicin were senior rats; the content of TBA-RP in blood serum exceeded the level of the intact control ($p<0.05$) by 345% till the end of the experiment. It testifies the pronounced development of the oxidative processes in the organism of the senior animals and high permeability of the cell membranes, the violation of whose integrity may lead to activation of phospholipases and oxygenases. The increase of their activity leads to the formation of a significant amount of the free radicals [453] that provoke violations in the system of POL and affect the nature of flow of membrane-destructive processes in cells.

In liver of poisoned animals there was observed the similar pattern to increase of the content of POL products (Table 1). On the 7th day of the experiment in liver of the immature animals the content of TBA-RP increased by 125%; in the mature animals – by 111% and in senior animals – by 79% in comparison with the rats of the intact control ($p<0.05$). Obviously, the seventh day is a toxicogenic phase of the action of anti-tuberculosis drugs, since it is known that in the influence of the products of drugs metabolism, the activation of POL processes occurs most precisely in the first days after the affection.

We recorded the highest content of TBA-RP on the 14th day in experimental age groups of animals. It was 251% in the immature rats, in the mature rats – 638%, and in

the senior rats – 603% (Table 1). This, in our opinion, is related to the combined effect of isoniazid and rifampicin on the organism, which activate the processes of free radical oxidation, in particular, and create an additive effect on the organism.

It should be noted that in the blood serum of the affected animals there is a more significant accumulation of POL products than in the liver that may be a consequence of the damaging the hepatocyte membranes by anti-tuberculosis drugs, and as a consequence – the incidence of toxic factors in the blood.

There are reports in the literature indicating that not only lipids but also protein components of membranes are the subject to peroxidation processes [454,455]. Protein oxidative or redox modifications induced by reactive oxygen species (ROS) or reactive nitrogen species not only can impair protein function, but also can regulate and expand protein function under a variety of stressful conditions [455].

Protein oxidation is defined as the covalent modification of a protein induced either by the direct reactions with ROS or indirect reactions with secondary by-products of oxidative stress. ROS can cause oxidation in both amino acid side chains and protein backbones, resulting in protein fragmentation or protein–protein cross-linkages. Although all amino acids can be modified by ROS, cysteine, and methionine that are the most susceptible to oxidative changes due to high reaction susceptibility of the sulfur group in those amino acids [456]. Oxidative modifications of proteins can change their physical and chemical properties, including conformation, structure, solubility, susceptibility to proteolysis, and enzyme activities.

Based on the above, we studied the indicators of oxidative proteins modification in the organism of affected animals (Table 2). As can be seen from the results in Table 2, the content of 2.4-dinitrophenylhydrazones of neutral kind increased on the 7^{th} day of the experiment in rats affected by isoniazid and rifampicin. The oxidative proteins modification at 370 nm increased significantly by 69% and 68% in the immature animals in blood serum and liver respectively, by 70% and 71% – in the mature animals and by 68% and 126% – in the senior animals in comparison with the intact control ($p<0.05$)

Table 2

The content of 2.4-dinitrophenylhydrazine$_{(370\ nm)}$ in blood serum (μmol / L protein) and liver (μmol / g protein) of rats affected by isoniazid and rifampicin (M ± m)

Research material	**Group of animals**	**Age group of animals**					
		immature		mature		senior	
		Research duration, days					
		7th	14th	7th	14th	7th	14th
Blood serum	Control group	0.09±0.005		0.073±0.002		0.120±0.004	
	Experimental group	0.152± 0.004*	0.379± 0.007*	0.124± 0.002*	0.139± 0.006*	0.202± 0.003*	0.270± 0.005*
Liver	Control group	0.091±0.003		0.066±0.002		0.124±0.006	
	Experimental group	0.153± 0.005*	0.199± 0.001*	0.113± 0.002*	0.114± 0.004*	0.280± 0.007*	0.391± 0.005*

The content of 2.4-dinitrophenylhydrazine$_{370}$ in blood serum was increased by 321% in the immature animals, by 90% – in the mature animals and by 125% – in the senior animals in comparison with the intact control ($p<0.05$) on the 14th day of the experiment. In liver of poisoned animals there was observed the similar pattern to increase of the products of oxidative proteins modification (Table 2).

The increasing of 2.4-DNPhH$_{(370\ nm)}$ content in the immature and senior rats in comparison with the mature animals is due to low resistance to antioxidant protection, which leads to dysfunction of cytoplasmic enzymes and membrane ion pumps, followed by the launch of various rupture mechanisms of cells.

In blood serum of the experimental animals, affected by anti-tuberculosis drugs, the content of 2.4-DNPhH$_{(430\ nm)}$ was gradually increasing and reached the highest content at the end of the experiment by 475 % in the immature animals, by 63% – in the mature animals and by 291% – in the senior animals if compared to the control group of animals ($p<0.05$) (Table 3).

On the 14th day of isoniazid and rifampicin administration the content of 2.4-DNPhH$_{(430\ nm)}$ increased in liver and was 304% in the immature animals in comparison with the control group ($p<0.05$), 265% – in the mature animals and 372% – in the senior animals. The mature animals proved to be the least sensitive.

Table 3

The content of 2.4-dinitrophenylhydrazine$_{(430\ nm)}$ in blood serum (μmol / L protein) and liver (μmol / g protein) of rats affected by isoniazid and rifampicin (M ± m)

Research material	**Group of animals**	**Age group of animals**					
		immature		mature		senior	
		Research duration, days					
		7th	14th	7th	14th	7th	14th
Blood serum	Control group	0.024±0.001		0.030±0.002		0.053±0.002	
	Experimental group	0.042± 0.001*	0.138± 0.003*	0.039± 0.002*	0.049± 0.003*	0.153± 0.005*	0.207± 0.005*
Liver	Control group	0.027±0.002		0.023±0.002		0.094±0.006	
	Experimental group	0.062± 0.002*	0.082± 0.003*	0.049± 0.003*	0.061± 0.003*	0.205± 0.004*	0.350± 0.005*

The 2.4-dinitrophenylhydrazones level of basic and neutral kinds was very high in blood serum and liver of the affected animals. Apparently, this caused the interaction of membranes protein components with reactive oxygen species (ROS), and as a consequence, the entry of major components of cells into the blood and changes in the activity of many membrane-dependent enzymes.

From received results, we can assume that the combined effect of isoniazid and rifampicin leads to the activation of free radical oxidation with enhanced reactions of peroxidation of lipids and proteins, destruction of cell membranes and accumulation of deoxidized products, peroxides, aldehydes and hydroperoxides, which cause the secondary endogenous intoxication of the organism.

Toxic products formed during the anti-tuberculosis drugs input in rats caused the activation of free radical oxidation processes, which led to violations of the protective and compensatory forces. There are systems in the organism that their act to neutralize free radicals and toxic products. Such systems include the antioxidant protection system [457]. To protect the cells and organ systems of the body against ROS, humans have evolved a highly sophisticated and complex antioxidant protection system. It involves a variety of components, both endogenous and exogenous in origin, that function interactively and synergistically to neutralize free radicals [458]. Both enzymatic and non-enzymatic antioxidant system are essential for cellular response in

order to deal with oxidative stress under physiological condition. Therefore, antioxidant enzyme such as CAT, SOD, and GSH-Px and non-enzymatic electron receptors such as GSH are affected and used as indexes to evaluate the level of oxidative stress [459, 460]

Antioxidant enzymes are capable of stabilizing, or deactivating free radicals before they attack cellular components. They act by reducing the energy of the free radicals or by giving up some of their electrons for its use, thereby causing it to become stable. In addition, they may also interrupt with the oxidizing chain reaction to minimize the damage caused by free radicals.

The liver is the most frequently targeted organ in terms of drug toxicity. The production of radical species has been proposed as an early event of drugs hepatotoxicity and as an indicator of hepatotoxic potential [461]. It has been discovered that a lot of drugs could induce oxidative stress including increase of cellular oxidants and lipid peroxidation, depletion of antioxidants in the liver, such as anti-inflammation drugs, anti-analgesic drugs, anti-cancer drugs and antidepressants. Anti-tuberculosis agent isoniazid resulted in both oxidative and nitrosative stress [462].

We have studied the catalase activity in blood serum and liver of animals after their affection isoniazid and rifampicin. Catalase (H_2O_2 oxidoreductase) is composed of four polypeptide chains, each chain is over 500 amino acids long, and contains four porphyrin heme (iron) groups allowing the enzyme to react with the H_2O_2[463].
It serves to protect the cell from toxic effects of high concentrations of hydrogen peroxide by catalyzing its decomposition into molecular oxygen and water, without the production of free radicals. It is important to measure catalase levels because oxidative stress is inherent in pathological conditions such as cancer, diabetes, cataracts, atherosclerosis, neurodegenerative disease, aging, and nutritional deficiencies [463].

From the data in Table 4, it follows that the catalase activity in blood serum of the experimental groups (7^{th} day) was decreased by 21% in the immature animals, by 33% – in the mature animals and by 12% – in the senior animals compared with the animals of the intact control group ($p<0.05$). Reducing of the enzyme activity in blood serum of animals in this experimental group leads to the accumulation of H_2O_2,

destruction or modification of biological molecules, and, as a consequence, the collapse of cell structures that causes the death of cells.

Table 4

Catalase activity in blood serum (μcat / L) and liver (μcat / kg) of rats affected by isoniazid and rifampicin (M ± m)

Research material	**Group of animals**	**Age group of animals**					
		immature		mature		senior	
		Research duration, days					
		7th	14th	7th	14th	7th	14th
Blood serum	Control group	11.70±0.67		21.16±0.28		27.06±1.19	
	Experimental group	9.21± 0.83*	8.30± 0.83*	14.27± 0.44*	12.70± 0.28*	23.78± 0.29*	20.14± 0.41*
Liver	Control group	10.55±0.88		14.23±0.33		15.38±0.60	
	Experimental group	5.19± 0.29*	4.86± 0.35*	8.99± 0.53*	6.70± 0.34*	10.15± 0.38*	7.30± 0.25*

The maximum decrease of the catalase activity in blood serum was registered in mature rats on the 14th day of the research by 40% compared with animals of the intact control ($p<0.05$), which testifies the low level of neutralization of toxic products by the antioxidant system of the mature organism.

Toxic effects of our drugs are related to their hepatotoxicity that led to the decrease of the catalase activity in the animals' liver in three age groups (Table 4). In the immature animals the activity of this enzyme decreased by 51% on the 7th day of research, in the mature animals – by 37% and in the senior animals – by 34%. It is known that at the chemical affection of the liver its protein synthesizing function is violated. The latter caused the inhibition of the catalase's synthesis in this organ throughout the experiment. The maximum of enzyme activity was decreased by 54% in liver of the immature animals in comparison with the control group ($p<0.05$).

Along with the catalase activity studying, we determined the content of reduced glutathione in the organism of animals, which is a structural component of the glutathione antioxidant system.

Reduced glutathione (GSH), a ubiquitous tripeptide thiol, is a vital intracellular and extracellular protective antioxidant, which plays a number of key and/or crucial

roles in the control of signalling processes, detoxifying certain xenobiotics and heavy metals, as well as other functions. It is a tripeptide composed of cysteine, glutamic acid and glycine. Intracellular and whole blood concentrations of GSH are in the milimolar range, while the plasma concentration is in the micromolar range and accounts for approximately 0.4% of total blood GSH [464]. GSH is synthesised in the cell by γ-glutamylcysteine synthetase (γ-GCS) and glutathione synthetase [465]. The γ-GCS-catalysed formation of γ-glutamylcysteine is the first and rate-limiting step in *de novo* GSH synthesis and is feedback-inhibited by GSH, a mechanism that is central to the regulation of cellular GSH concentrations [465].

Within cells, total GSH exists free and bound to proteins. Since the enzyme glutathione reductase, which reverts free glutathione from its oxidised form (GSSG) is constitutively active and inducible upon oxidative stress; free glutathione exists almost exclusively in its reduced form. The ratio of reduced to oxidised glutathione within cells is often used as a marker of cellular toxicity [466].

The content of G-SH decreased by 26% in blood serum in the immature animals after 7 days, it was lower by 29% in comparison with its indicator at the end of the experiment in the intact animals ($p<0.05$) (Table 5).

Table 5

The content of G-SH in blood serum (mmol / L) and liver (mmol / g) of rats affected by isoniazid and rifampicin (M ± m)

Research material	**Group of animals**	**Age group of animals**					
		immature		mature		senior	
		Research duration, days					
		7^{th}	14^{th}	7^{th}	14^{th}	7^{th}	14^{th}
Blood serum	Control group	3.67±0.17		3.40±0.06		6.60±0.15	
	Experimental group	2.70± 0.05*	2.55± 0.22*	2.26± 0.11*	1.88± 0.09*	4.39± 0.09*	4.26± 0.12*
Liver	Control group	2.78±0.12		3.27±0.10		5.51±0.11	
	Experimental group	1.75± 0.10*	1.48± 0.11*	1.52± 0.10*	1.24± 0.12*	3.88± 0.13*	3.67± 0.16*

The critical decrease of G-SH content in blood serum may be due to exhaustion in the first days of an affection of one of the most powerful antioxidant systems in the

organism – glutathione. The content of G-SH decreased by 34% on the 7th day of the experiment in the mature animals, and 33% – in the senior animals from the level of the intact animals ($p<0.05$). The lowest content of G-SH in blood serum was observed on the 14th day of research in the mature age animals and it was 55% (Table 5).

In liver of the experimental group, this indicator decreased in the immature rats by 37% on the 7th day of the experiment and by 47% on the 14th day in comparison with the control group ($p<0.05$), in the mature – by 54% and 66 %, in the seniors – by 30% and 33%, respectively (Table 5). The maximum decrease of G-SH content in liver was recorded in the senior animals on the 14th day of the research. It was 34% in comparison with the control group ($p<0.05$). The decrease of G-SH content is associated with the decrease of glutathione reductase activity, which is capable to restore its disulfide form with help of $NADFN_2$.

Significant disturbances in functioning of the antioxidant system have been observed in the animals, which affected by isoniazid and rifampicin. The decrease of catalase activity observed in both blood serum and liver of experimental animals of all age grops that indicates a violation of the protein-synthesizing function of the liver and degradation of protein molecules at conditions of toxicosis. This is confirmed by the decrease of reduced glutathione content in the researched material after the affection of an organism, which may be the result of destruction and changes the permeability of hepatocyte membranes under the influence of toxic factors.

Increased activity of POL processes and oxidative modification of proteins in the poisoned organism, and also destruction of membrane proteins causes the accumulation of middle-weight molecules that are markers of endogenous intoxication.

Endogenous intoxication is a polyethiological polipatogenny syndrome characterized by the accumulation in tissues and biological fluids of the endogenous toxic substances, which are excess products of normal or distressed metabolism [467]. Several mechanisms of endogenous intoxication can be distinguished, including excessive production of endogenous toxic products, resorption of toxic substances, accumulation of products of lipid peroxidation and other inflammatory mediators, primarily tumor necrosis factor, translocation of the vital products of microorganisms

and microbial bodies themselves, with subsequent autoimmune processes, and violation of the release of endogenous toxic products from the body by the natural detoxification organs [468].

Endogenous intoxication is considered as one of the universal mechanisms of the pathogenesis of various diseases, which includes the release of toxic products into the blood from the pathological focus, their distribution throughout the body with blood flow, and effects on other organs and tissues. The development of pathological disorders in the body during intoxication depends on the balance of two opposite processes – the rate of formation and release of endotoxins into the blood, on the one hand, and the elimination (detoxification) of these substances, carried out by the body's protective systems, on the other. The development of a methodology for evidence-based biochemical assessment of the severity of the condition of patients in the development of endotoxemia is one of the urgent problems of modern medicine, clinical biochemistry. [469]

The results of research of $MWM_{(254\ nm)}$ content in blood serum of all age rats' groups are presented in Table 6.

Table 6

The content of $MWM_{(254\ nm)}$ in blood serum (conventional units / L) and liver (conventional units / kg) of rats affected by isoniazid and rifampicin (M ± m)

Research material	**Group of animals**	**Age group of animals**					
		immature		mature		senior	
		Research duration, days					
		7^{th}	14^{th}	7^{th}	14^{th}	7^{th}	14^{th}
Blood serum	Control group	0.63±0.05		1.31±0.05		1.41±0.02	
	Experimental group	0.79± 0.05	0.88± 0.04*	1.75± 0.05*	2.16± 0.10*	2.98± 0.1*	3.06± 0.04*
Liver	Control group	0.46±0.05		1.35±0.06		1.34±0.02	
	Experimental group	0.72± 0.05*	0.76± 0.02*	1.95± 0.08*	2.20± 0.05*	2.21± 0.10*	2.66± 0.03*

Middle-weight molecules were defined as toxins in the molecular weight range of 500-60,000 Da, which exceeds the molecular weight of 2000 Da defined in the

original middle molecule hypothesis. Under this new proposed definition, most of these middle molecules are low molecular weight peptides and proteins.

On the 7th day of isoniazid and rifampicin administration $MWM_{(254\ nm)}$ content increased in blood serum and was 125% in the immature animals, 134% – in the mature animals and 211% – in the senior animals in comparison with the control group ($p<0.05$). We evidenced the highest content of MWM in blood serum at the end of research in the senior animals, which was 217% in comparison with intact animals that is by 35% and 24% higher than in the immature and mature animals, respectively. On the 7th day of the experiment in liver of the immature animals the content of $MWM_{(254\ nm)}$ increased by 125%; in the mature animals – by 111% and in senior animals – by 79% in comparison with of the control group ($p<0.05$).

One of the reasons for their increased content may be intensified proteolysis in damaged tissues, and also in blood serum during the release of proteolytic enzymes into the blood. The hydrophobic toxins formed under these conditions, in particular, protein products degradation, which are considered the most toxic. They bind very quickly to cell membranes and intracellular proteins and most modify their structure, leading to increased membrane permeability and inhibition of enzyme activity.

The content of $MSM_{(280\ nm)}$ (aromatic amino acids predominant) was studied and its growth was found in all researched tissues after simultaneous affection by isoniazid and rifampicin (Table 7).

Table 7

The content of $MWM_{(280\ nm)}$ in blood serum (conventional units / L) and liver (conventional units / kg) of rats affected by isoniazid and rifampicin (M ± m)

Research material	**Group of animals**	**Age group of animals**					
		immature		mature		senior	
		Research duration, days					
		7th	14th	7th	14th	7th	14th
Blood serum	Control group	0.61±0.05		1.02±0.06		1.35±0.05	
	Experimental group	0.78± 0.05	0.81± 0.04*	1.62± 0.04*	1.76± 0,09*	2.93± 0.23*	3.13± 0.06*
Liver	Control group	0.45±0.05		1.15±0.05		1.39±0.05	
	Experimental group	0.70± 0.05*	0.72± 0.02*	1.82± 0.06*	1.91± 0.07*	2.20± 0.08*	2.69± 0.04*

As can be seen from the results in Table 7, the content of $MWM_{(280\ nm)}$ increased on the 7th day of the experiment in rats affected by isoniazid and rifampicin. The middle-weight molecules content at 280 nm increased significantly by 29% and 56% in the immature animals in blood serum and liver respectively, by 59% and 58% – in the mature animals and by 117% and 58% – in the senior animals in comparison with the intact control ($p<0.05$).

In liver of the experimental group, this indicator increased in the immature rats by 56% on the 7th day of the experiment and by 60% on the 14th day in comparison with the control group ($p<0.05$), in the mature rats – by 58% and 66 %, in the senior rats – by 58% and 94%, respectively (Table 7). The maximum increased of the $MWM_{(280\ nm)}$ content in liver was recorded in the senior animals on the 14th day of research. It was 194% in comparison with the control group ($p<0.05$).

Toxic effects of isoniazid and rifampicin on animals of all age groups led to increased MWM content in blood serum and liver, which are dominated by chain and aromatic amino acids. This may be due to direct hepatotoxic effects of researched tuberculostatics.

According to the researches [427, 433], the hepatotoxicity of isoniazid may be developed in two ways: 1. The accumulation of free radicals with activation of lipid peroxidation and the formation of reactive metabolites: acetylisoniazid, hydrazine, monoacetylhydrazine. 2. The increased activity of N-acetylisoniazid by N-hydroxylation and formation of acetyl radical and acetyl carbonium ion.

The metabolism of acetyl hydrazine and microsomal monooxygenases cause hepatotoxic effect as a result of the covalent addition of acetyl groups to the liver proteins [427], which manifest as temporary asymptomatic increase of transaminases activity. The hepatotoxicity of rifampicin is also due to the formation of toxic metabolites as a result of its deacetylation in the liver that leads to hepatocytes dystrophy [433, 439]. It was established that hepatotoxicity of the metabolites of isoniazid and rifampicin caused the lipid peroxidation of hepatocyte biomembranes [437, 438].

Aminotransferases or transaminases are a group of enzymes that catalyze the interconversion of amino acids and oxoacids by transfer of amino groups. Aspartate aminotransferase (AST), formerly termed glutamate oxaloacetate transaminase, and alanine aminotransferase (ALT), formerly termed glutamate pyruvate transaminase, are the two aminotransferases of greatest clinical significance [470]. Aminotransferases catalyze the redistribution of nitrogen between amino acids and corresponding oxoacids participating in both protein metabolism and gluconeogenesis. They are ubiquitous in their cellular distribution.

Tissue activity for AST is as follows in decreasing concentration: heart, liver, skeletal muscle, kidney, pancreas, spleen, lung, and erythrocyte. Two distinct forms have been identified: a cytoplasmic, or soluble isoenzyme, and a mitochondrial isoform. Selective measurement of these isoenzymes has no currently demonstrated clinical application [470].

The distribution and relative tissue concentration of ALT is similar but importantly different. Highest activity is found in the liver, followed by kidney, myocardium, skeletal muscle, pancreas, spleen, lung, and erythrocyte. ALT activity is found in the cytosol; organ- or organelle-specific isoenzymes have not been demonstrated. The concentration of ALT in hepatic cell cytoplasm is comparable to AST. Comparable elevations of both AST and ALT are highly characteristic of acute viral, toxic, or no ethanol drug-induced hepatitis [470]. Due to this thesis, we studied aminotransferase rate in blood serum and liver of the rats of different age groups affected by xenobiotics.

We noted the increased ALT rate in the blood serum of all age animals (Table 8). On the 7^{th} day of anti-tuberculosis drugs administration ALT rate increased in blood serum by 120% in immature animals in comparison with the control group ($p<0.05$), by 30% – in mature animals and by 90% – in senior animals. The mature animals proved to be the least sensitive. We evidenced the highest ALT rate in blood serum at the end of research in the senior animals, which was 281% in comparison with intact animals that is by 13% and 19% higher than in the immature and mature animals, respectively.

Table 8

Alanine aminotransferase rate in blood serum (mkmol / L·h) and liver (mkmol / kg·h) of rats affected by isoniazid and rifampicin (M ± m)

Research material	**Group of animals**	**Age group of animals**					
		immature		mature		senior	
		Research duration, days					
		7th	14th	7th	14th	7th	14th
Blood serum	Control group	0.83±0.05		2.96±0.18		2.34±0.15	
	Experimental group	1.83± 0.11*	2.03± 0.20*	3.84± 0.24*	6.74± 0.26*	4.44± 0.32*	6.58± 0.30*
Liver	Control group	5.40±0.12		8.30±0.21		6.08±0.33	
	Experimental group	3.68± 0.20*	2.83± 0.11*	6.48± 0.27*	5.39± 0.19*	3.68± 0.25*	3.01± 0.16*

In liver of the experimental group, this indicator decreased in the immature rats by 32% on the 7th day of the experiment and by 48% on the 14th day in comparison with the control group ($p<0.05$), in the mature rats – by 22% and 35%, in the senior rats – by 39 % and 50%, respectively (Table 8). The maximum decrease of ALT rate in liver was recorded in the senior animals on the 14th day of research. It was 50% in comparison with the control group ($p<0.05$).

In liver of the rats after combined administration of anti-tuberculosis drugs we evidenced decrease in this enzyme rate, which indicated the cytolysis of hepatocytes and liver protein synthesis dysfunction in cases of affection by isoniazid and rifampicin. It was determined that the increase of ALT rate in blood serum was higher than in liver. It could be caused by toxic influence of isoniazid- rifampicin affection of liver. The damaged liver cannot synthesize this enzyme because hepatocytes damaged and their release in blood serum; so we evidenced increased rate of this enzyme.

Because the determination of aminotransferases activity in blood serum is a subtle indicator of severity and activity of a pathological process in liver that why we investigated the activity of another enzyme, it was AST (Table 9).

In blood serum of the experimental animals, AST rate was gradually increasing and reached the highest rate at the end of the experiment by 163% in the immature animals, by 89% – in the mature animals and 224% – in the senior animals in

comparison with the control group ($p<0.05$). The most vulnerable age group to the anti-tuberculosis drugs action was senior rats, which indicates the deepest violation of their protein-synthesizing function of liver.

Table 9

Aspartate aminotransferase rate in blood serum (mkmol / L·h) and liver (mkmol / kg·h) of rats affected by isoniazid and rifampicin (M ± m)

Research material	**Group of animals**	**Age group of animals**					
		immature		mature		senior	
		Research duration, days					
		7^{th}	14^{th}	7^{th}	14^{th}	7^{th}	14^{th}
Blood serum	Control group	0.70±0.06		0.64±0.04		1.05±0.20	
	Experimental group	1.05± 0.05*	1.84± 0.10*	1.10± 0.05*	1.21± 0.06*	2.61± 0.09*	3.40± 0.14*
Liver	Control group	1.21±0.11		2.66±0.06		2.48±0.12	
	Experimental group	1.02± 0.04	0.86± 0.03*	1.66± 0.10*	1.34± 0.07*	1.45± 0.04*	1.18± 0.03*

During research the decrease of AST rate in liver of the affected animals of all age groups was determined (Table 9). In the immature animals the activity of this enzyme decreased by 16% on the 7^{th} day of research after the administration of isoniazid and rifampicin, in the mature animals – by 38% and in the senior animals – by 42%. The maximum decrease of AST rate in liver was recorded in the senior animals on the 14^{th} day of research. It was 48% in comparison with the control group ($p<0.05$). The decrease of AST rate in liver proved the transamination of aspartate according to breaking of the Citric acid cycle and negligible release of protein enzyme from tissue cells into blood.

Increased activity of aminotransferases in blood serum and their decreased in liver is a confirmation of the toxic effect of isoniazid and rifampicin on animals of different ages, accompanied by impaired permeability of plasma hepatocytes membranes and causes a significant amount of enzymes in blood.

The isoniazid–rifampicin administration to all age animals caused the activation of free radical processes, which led to accumulation in the organism of large amounts of ROS, which cause oxidative stress. The latter is accompanied by activation of POL

processes, that is confirmed in experimental research, TBA-RP content increased in blood serum and liver of affected animals. The formed free radicals and ROS lead not only to changes of lipid components but also protein components of membranes, as evidenced by increased of 2.4-DNPhH content .

Oxidative stress has caused changes in an antioxidant system. There was a decrease of catalase activity in experimental animals of all ages, which indicates a violation of the protein-synthesizing function of liver. This is confirmed by a decrease of reduced glutathione content in studied materials. The above changes in the organism of affected animals led to deepening of endogenous intoxication, which was confirmed by the increase of MWM.

The syndrome of endogenous intoxication may be due to the accumulation in organism of degradation products of lipid and protein molecules. There is a hepatocytes cytolysis in liver by isoniazid and rifampicin. This is indicated by an increase of aminotransferases activity in blood serum of poisoned animals of all ages. The most pronounced changes were observed in senior animals.