Qualitative histological study of isoniazid-rifampicin induced liver injury in rats

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ABSTRACT

Objectives: Tuberculosis is still a major public health problem in developing world and in Bosnia and Herzegovina. Treatment of tuberculosis requires a “standard” combination of antituberculotics for a 6-month period. Prolonged use of isoniazid and rifampicin is associated with hepatotoxicity. The pathophysiology of hepatotoxicity is not yet elucidated, and suggested mechanism is oxidative stress. Isoniazid metabolite is considered to be responsible for the tissue damage through formation of free radicals.

Methods: Twenty one adult male Wistar rats (210-280 g) were randomized into two groups. In group I (14 rats) animals received rifampicin (50 mg/kg) and isoniazid (50 mg/kg) dissolved in 4 ml/kg isotonic saline. Group II (7 rats) served as control and the animals received 4 ml/kg isotonic saline. The administration was performed intraperitoneally, during 21 days. The animals were sacrificed at the end of that period. Blood samples were obtained for biochemical analysis and liver tissue was processed by histotechnological method. Liver tissue was stained with H&E and PAS method and qualitative histological analysis was performed using light microscopy.

Results: Our study revealed changes in liver tissue in the isoniazid-rifampicin treated group including enlargement and swelling of hepatocytes with vacuolization in centrilobular area, dilatation of sinusoids and mononuclear infiltration in portal space. Biochemical analysis of liver enzymes did not show significant difference between groups.

Conclusion: In the isoniazid-rifampicin treated group of animals qualitative histological analysis revealed mild changes in liver tissue.

Keywords: liver injury, isoniazid, rifampicin, rats, histology

INTRODUCTION

According to data of the World Health Organization the estimated incidence of tuberculosis in Bosnia and Herzegovina in 2013 was 46 per 100.000 population, while the prevalence was 69. The reported numbers are among the highest in the region, despite health services efforts for disease management [1].

The “cornerstone” of tuberculosis management is a 6-month regimen which includes isoniazid, rifampicin, pyrazinamide and ethambutol (2 months) followed by application of rifampicin and isoniazid (4 month) [2]. All of the listed drugs have some adverse effects but there are limited data for individual toxicity. Liver and kidney are the most exposed organs for damage induced by coadministration of antituberculosis drugs. Adverse effects associated with long-term use of antituberculosis drugs often require a change of treatment which itself has negative consequences (relapse or the drug-resistance) [3,4].

Drug-induced liver injury is a clinical diagnosis of exclusion. The incidence of antituberculosis drug-induced hepatotoxicity (ATDH) varies from 2% to 28% according to different reports [3,5]. Pathohistological evaluation of ATDH in several studies showed that changes in liver tissue were non-specific in both, human and animal liver specimen: hepatocellular steatosis and necrosis, hepatocyte vacuolation and spotty to diffuse necrosis [3,6].

The mechanism of ATDH still remains controversial. Previous studies suggest that main mechanism involved in hepatotoxic effects of rifampicin and isoniazid is peroxidation of endogenous lipids and formation of highly reactive oxygen species [3,6]. Isoniazid is metabolized mainly by liver N-acetyl transferase 2 (NAT-2) to acetyl-isoniazid and then to mono-acetyl hydrazine (MAH) and some non-toxic metabolites. MAH is considered to be responsible for the
tissue damage through free radical generation. Studies revealed that in rats isoniazid and its metabolites cause depletion of the free radical scavengers reserve such as glutathione-related thiols [6,7]. The other possible mechanism of isoniazid-induced liver injury is covalent binding to liver macromolecules and subsequent idiosyncratic reaction [6,8,9]. Rifampicin is widely used in treatment regimens for tuberculosis and some non-tuberculosis infections, especially serious Staphylococcal infection. Rifampicin occasionally causes hepatocellular injury but it is capable to potentiate hepatotoxicity of other medications. It increases production of toxic metabolites from isoniazid by induction of cytochrome P450. Rifampicin alone causes hyperbilirubinemia because of interference with bilirubin uptake and excretion [3,6].

The aim of this study is to determine changes in liver tissue induced by antituberculous drug.

**MATERIALS AND METHODS**

**Animals**

Twenty one adult male Wistar rats (210-280 g) used in this study were purchased from the Faculty of Veterinary Medicine in Sarajevo. The animals were housed in polypropylene cages and maintained in standardized laboratory conditions (12-hour light-dark cycle, temperature 23±2°C, humidity of 55±5%). Standard diet and water were provided ad libitum.

**Experimental design**

Animals were randomized into two groups: group I (14 rats) and group II (7 rats). In group I rifampicin (50 mg/kg) and isoniazid (50 mg/kg), dissolved in 4 ml/kg isotonic saline, were administered intraperitoneally (i.p.) daily, for 21 days. Group II served as control and the animals received 4 ml/kg isotonic saline i.p., daily, for 21 days. Both used chemicals, rifampicin and isoniazid, were of analytical grade.

**Histological analysis**

At the end of experimental procedure, 24 hours after the last administration of chemicals, rats were sacrificed using ketamine anesthesia. Liver tissue samples were quickly excised and fixed in 10% buffered formalin. Further tissue processing included carrying through fixation. Tissues were cut and mounted on glass slides and stained with hematoxylin and eosin (H&E) and periodic acid Schiff (PAS). Histological examination of slides was made under light microscope (Eclipse E400, Nikon) with X100 and X400 magnification and with digital camera (DN 100). The study was carried out at the Institute of Histology and Embryology, Faculty of Medicine, University of Sarajevo.

**Biochemical analysis**

Blood samples were collected by aortal puncture and centrifuged to obtain the serum for analysis. Blood glucose level (BGL), and total bilirubin serum concentration were assessed, while level of hepatocellular damage was estimated by alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Blood analysis was made in Biochemical-Immunological-Hematological laboratory “BIH medicinski laboratorij” Ilidža, Sarajevo.

**Statistical analysis**

All results were expressed as mean ±SD and statistically significant differences between groups were determined with Student’s t test. Analysis was performed using MS Excel 2013 for Windows.

**RESULTS**

Twenty-one day long antituberculous drug application was not associated with adverse effect in usual appearance and behavior of any of animals. Rats in both groups gained weight constantly, ate and drank regularly.

**Group I (isoniazid-rifampicin)**

Qualitative histologic study revealed alterations in liver tissue in all isoniazid-rifampicin treated animals. Most of the hepatic lobules were affected in the centrilobular region, although some lobules were diffusely affected. Centrilobular region showed uneven appearance of hepatocytes arranged in cords and separated by adjacent sinusoids flowing towards the central vein (Figure 1A). Hepatocytes were variable in size, shape and cytoplasm appearance. Most of enlarged hepatocytes were swollen and had abundant, eosinophilic cytoplasm with signs of vacuolization. Vacuoles appeared as very pale, small and round areas demarcated with eosinophilic strands of cytoplasm. Paranuclear region of the cell cytoplasm showed clearing. Some hepatocytes, angular in shape, had hypereosinophilic cytoplasm in particular in the marginal zone, and were scattered among hepatocytes with normal or swollen appearance. Nuclei were round and centrally positioned with excentrically placed one or more nucleoli. They showed variability in size and staining. Binuclear hepatocytes were frequent finding. Appearance of sinusoids varied among different zones. Sinusoids were dilated, with many erythrocytes in lumen, and rarely visible inflammatory cells. Liver sinusoidal endothelial cells were lined in thin layer that covered the sinusoid lumen, and had basophilic prominent nuclei. Central veins showed dilatation and congestion. Same findings were evident in larger blood vessels with thin walls, apparently veins. Presence of many cells with small, round basophilic nuclei surrounded with thin rim of cytoplasm, which corresponded those from lymphocytes, was evident in connective tissue of portal space (Figure 1B).
Figure 1A. Photomicrograph of liver in isoniazid-rifampicin group (H&E, X400)
Hepatic cords with uneven appearance of hepatocytes, separated with dilated sinusoids.

Figure 2A. Photomicrograph of liver in isoniazid-rifampicin group (PAS, X100)
Depletion of glycogen in centrlobular region.

Figure 3A. Photomicrograph of liver in control group (PAS, X100)
Regular distribution of glycogen in hepatic lobule.

Figure 1B. Photomicrograph of liver in isoniazid-rifampicin group (H&E, X100)
Periportal area showed moderate mononuclear infiltration, a sign of portal triaditis.

Figure 2B. Photomicrograph of liver in isoniazid-rifampicin group (PAS, X400)
Vacuolization of hepatocytes.

Figure 3B. Photomicrograph of liver in control group. (PAS, X400)
Normal width of sinusoids and plates of hepatocytes with abundant red stained glycogen particles.
Centrilobular area appeared pale, and PAS positive staining was present in perportal zone (Figure 2A). Higher magnification showed multiple paranuclear vacuoles in hepatocyte cytoplasm that appeared clear, suggesting the lack of glycogen. Minority of cells had normal PAS staining properties represented by small red particles of glycogen occupying the whole cell or paranuclear region (Figure 2B).

**Group II (control group)**

Control group showed normal liver morphology. Hepatocytes, arranged in hepatic cords, were regular in shape, size and cytoplasmic staining properties. It was evident that glycogen particles were abundant in most of the hepatocytes, clearly visible as intense red particles in the PAS stain slides (Figure 3A). Glycogen particles occupied the whole cytoplasm and not only the paranuclear region. Sinusoids and other blood vessels showed no congestion (Figure 3B). Portal spaces with interlobular artery, vein and bile duct, that were regular in appearance, showed no signs of mononuclear cell infiltration.

**Biochemical analysis**

Biochemical analysis of BGL, total bilirubin, AST and ALT showed no statistically significant differences between control and isoniazid-rifampicin group. However, ALT showed trend to higher and total bilirubin to lower serum activity in control group of rats than in experimental group (Table 1).

**Discussion**

Our study revealed mild alterations in liver tissue of isoniazid-rifampicin treated group including swelling of hepatocytes and vacuolization, dilatation of sinusoids and mononuclear infiltration in portal space. Regardless of study protocols several studies showed that combined application of isoniazid and rifampicin produces hepatic cell injury in animal models [10-13]. Although the majority of the previous studies used intragastric application [10-14], some authors gave priority to intraperitoneal route [11,15,16]. We used intraperitoneal route because it provides needed technical accuracy of administered dose and is relatively simple to perform.

Rana *et al.* [10] demonstrated that hepatic alterations induced with combined application of rifampicin and isoniazid were dose dependent. They found that even lower doses (25 mg/kg) of isoniazid could produce focal lobular inflammation, and higher doses (50 mg/kg) portal triaditis and piecemeal necrosis. They concluded that 50 mg/kg of rifampicin and 50 mg/kg of isoniazid was the minimum dose with maximum hepatotoxicity in Wistar rats. This is in accordance with our study, although we did not find piecemeal necrosis.

Lian *et al.* [12] showed that even higher doses of combined antituberculosis therapy (150 mg/kg of isoniazid and 300 mg/kg of rifampicin) caused only moderate alterations in mouse liver tissue: hepatocellular swelling, vacuolization and fatty degeneration, without necrosis. In study reported by Metushi *et al.* [14] no evidence of severe liver injury were shown, even when rats were treated with higher doses of INH (150 mg/kg/day for up to 4 weeks). This can be explained with different immune response which is involved as possible mechanism of INH-induced hepatotoxicity [17]. It is also suggested that animals that developed severe liver injury after antituberculosis drug administration, had concomitant undetected viral infection [14]. Other pro-oxidant therapeutics used for inducing hepatotoxicity in rats, caused similar changes in liver cells. Potent cytotoxic agent methotrexate caused abnormal histological appearance in liver tissue with marked eosinophilic hepatocytes in portal area, mononuclear inflammation, blood vessel congestion and hydropic degeneration (swelling) of hepatocytes [18,19]. Histological assessment of gentamicin induced liver injury showed similar alterations with associated fatty degeneration of hepatocytes [20].

Our study showed that glycogen content in hepatocytes was lower in rifampicin-isoniazid group, and vacuoles with pale appearance in PAS staining were still present in their cytoplasm. This finding excludes glycogen as potential accumulating material, which is seen in car-

![Table 1. Comparison of biochemical results between control and isoniazid-rifampicin group](image-url)
bon tetrachloride damaged hepatocytes, as a cellular adaption to toxic injury [21]. Other studies also showed deprivation in glycolytic content in liver of animals treated with different pro-oxidant therapeutics [19,22]. Vacuolization in hepatocytes is a regular finding in toxic liver studies, although it could be misinterpreted with postmortem changes when time interval between animal death and necropsy is prolonged [23]. There is lack of evidence for vacuole content in isoniazid-rifampicin induced hepatotoxicity. Cytoplasmic vacuolization could be sign of increase in intracellular water content because of impaired cellular membrane permeability, which is known as hydropic degeneration or swelling [24,25]. Hepatocyte swelling is a typical oxidative injury, associated with stress-proteins accumulation that is accompanied by increase in cell hydation [26]. Significance of hepatocyte vacuolization and vacuole content in toxic liver injuries are not yet clear and further investigations are needed to clarify their importance. Although comparison between two groups: control and experimental, showed no statistically significant difference in average values of biochemical parameters, these results led us to some conclusions. Three weeks of treatment caused no notable alteration in BGL, total bilirubin and AST, although there was a trend of higher values in total bilirubin in treated animals compared to control group. Biochemical markers of hepatotoxicity, AST and ALT were significantly elevated in experimental group. This is in accordance to previous studies that demonstrated how isoniazid has inhibiting property on ALT enzyme assay which can produce false negative test for hepatotoxicity. Also, there is evidence suggesting that ALT is not a potent biochemical marker for toxic liver injury in rats, due to its short half-life in blood and low intrahepatic activity [28,29].

To our knowledge, there are no studies presenting detailed qualitative histological analysis of liver damage induced by isoniazid and rifampicin. Applying quantitative histological analysis in further investigations can provide more accurate results and help to elucidate underlying morphological changes in liver tissue.

**Conclusion**

Observed morphological changes of liver tissue in isoniazid-rifampicin treated group of animals were mild, while biochemical assay did not show notable alterations.

**Declaration of interest**

The authors declare no conflict of interest for this study.

**References**


