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Study the possibility role of resveratrol and/or Gamma-Glutamyl Cysteine on Liver tissue damage by Azathioprine drug in Rats

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Drug-induced liver injury (DILI) is considered a serious complication that may lead to liver failure. Patients may have to discontinue therapy to prevent further hepatotoxic effects of some drugs. Azathioprine (AZA) is an immune suppression drug that effectively reduces immune reaction that has been used in some immune diseases like inflammatory bowel disease (IBD), acute lymphoblastic leukemia (ALL), and organ transplants. The current study attempted to the ameliorative effects of resveratrol (RSV) and gamma-glutamylcysteine (γ-GC) through antioxidative, anti-inflammation, and antiapoptotic against liver damage induced by Azathioprine. The period of the study was 28 days and included 50 male Wister Albino Rats were divided into five groups: G1 is the normal control group, G2 rats were fed AZA was administered orally (10mg/Kg body weight), G3 rats were fed resveratrol dissolved in dimethyl sulfoxide (DMSO) (8 mg/Kg body weight) were administered Intraperitoneally along with AZA administration, G4 rats were fed gamma-glutamylcysteine (100 mg/Kg body weight) were administered orally along with AZA administration and G5 rats were fed resveratrol dissolved in DMSO (8 mg/Kg body weight) were administered Intraperitoneally along with AZA administration and gamma-glutamylcysteine (100 mg/Kg body weight). The results show that AZA reduces antioxidant enzymes catalase (CAT) and superoxide dismutase (SOD). As a result, increased levels of oxidative stress reduce hepatic glutathione (GSH) and increase malondialdehyde (MDA). AZA-treated rats induce inflammation by an increase of tumor necrosis factor alpha (TNF-α). Administration of RSV or γ-GC or in combination restores antioxidant enzymes, improves oxidative balance represented in restoring the depletion of GSH, and reduced lipid oxidative damage as well as reduces inflammation. Thus, the current study can be on the ameliorative effects of RSV or γ-GC or in combination on liver injury in rats.

Keywords: Azathioprine, Drug-induced liver damage, Gamma- glutamylcysteine, Glutathione, Resveratrol.

INTRODUCTION

The liver plays a key role in multiple physiological processes, including Intra hepatic detoxification of xeno biotics and hormones e.g., Insulin-like growth factors, Angiotensinogen **(**Si-Tayeb et al. 2010). The liver is a metabolic organ that held fundamental pathways to produce energy and metabolism of carbohydrates, lipids, and proteins (Judge & Dodd, 2020), rather than the liver regulating blood clot factors, produces essential proteins e.g. albumin as well as production of bile acid, very-low-density lipoprotein (VLDL), cholesterol, store glucose as glycogen and lipid as triglycerides (TG) as well as other vitamins and mineral (Bizzaro et al. 2019; Huang et al. 2022; Ishikawa et al. 2021). Besides these roles, the liver is considered an immune organ since there are immune cells that are ready to eliminate the pathogen from the gastrointestinal tract (GI) (Hastings et al. 2020).

Drug-induced liver injury (DILI) is a condition of hepatic disorder that occurs due to drug abuse or other natural medicine such as herbs or other supplements. Inflammation, immune response as well as a high level of oxidative stress trigger Hepatic cell death by necrosis, apoptosis, and other types of cell death (Navarro et al. 2014).

Oxidative stress is a major mechanism in drug abused liver injury normally during liver metabolism and detoxification liver produce free radicals ROS and reactive nitrogen species (RNS) these free radicals establish an important normal physiological function but when their cellular concentration exceeds the normal level it is depleted cellular antioxidant and attacked cell membrane, form proteins adduct, DNA modification and trigger lipid peroxidation (Arauz et al. 2016; Frijhoff et al. 2015).

Azathioprine (AZA) (6-mercaptopurine) is a thiopurine form drug with corticosteroid-sparing properties. It has been discovered in the 1960s as an immune suppressive

drug for immune disorders such as leukemia (Björnsson et al. 2017), systemic lupus erythematosus and rheumatoid arthritis (RA), inflammatory bowel disease (IBD) (Axelrad et al. 2016), autoimmune hepatitis (AIH) organ transplant and has been used ever since. AZA can reduce inflammation effectively but its adverse drug reaction (ADR) has become an impediment and a reason to stop therapy (Sheiko et al. 2017).

Metabolism of AZA involves three competitive pathway that produces 6-thioguanine nucleotide (6-TGN), 6-methyl mercaptopurine (6-MMP), and 6 thiouric acid (6-TU) (Núñez et al. 2022). High levels of AZA metabolites may develop toxicity side effects e.g. myelotoxicity, Pancreatitis, hepatotoxicity, alopecia, and GI complications (Luber et al. 2019). 6-TGN is the active metabolite that integrates with the nucleic acid formation and induces apoptosis resulting in reducing the immune reaction as well as inhibiting activation of Ras-related C3 botulinum toxin substrate 1 (Rac1) resulting in T lymphocyte apoptosis (Gargallo-Puyuelo et al. 2021). Hepatotoxic side effect occurs from the accumulation of 6-MMP that elevates levels of hepatic enzymes and histopathologic features (Luber et al. 2019) and increase the level of ROS, depletion of antioxidant enzyme, and glutathione (GSH) (Stocco et al. 2014) depletion of ATP, mitochondrial dysfunction and necrotic cell death (Tapner et al. 2004).

Resveratrol (RSV) (3,5,4'trihydroxy-trans-toluylene) is a natural polyphenol naturally produced in plants as a result of the defense mechanism against

environmental stress, bacterial and fungal infection (Dong et al. 2016) RSV has anti-inflammatory, antioxidant effect (Izzo et al. 2021; Meng et al. 2021) and play a role in reducing the condition of a wide spectrum of disease including cancers, neurodegenerative disease, heart disease, liver disease and diabetes (Meng et al. 2020).

γ-glutamylcysteine (γ-GC) is a dipeptide that with glutathione synthase enzyme produces glutathione (Liu et al. 2021). γ-GC can serve as an antioxidant due to the ability of the thiol (-SH) side chain (Nakamura et al. 2012). γ-GC has anti-inflammatory and antioxidant properties (Salama et al. 2015; Yang et al. 2019). on the other hand, is a direct precursor of glutathione (Braidy et al. 2019).

The current study attempted to the ameliorative effects of resveratrol and gamma-glutamylcysteine through antioxidative, anti-inflammatory and antiapoptotic against liver tissue damage induced by Azathioprine and come up with positive results that can be indicators for resveratrol and/ or gamma-glutamylcysteine along with using immunosuppressive drugs.

MATERIALS AND METHODS

Chemicals

Azathioprine (AZA), 6-(1-Methyl-4-nitroimidazol-5-yl) thiopurine; C9H7N7O2S; ID IUPAC 6-(3-methyl-5-nitroimidazol-4-yl)sulfanyl-7*H*-purine tablets, provided from (Alnahdi pharmacy 5xidiz, Saudi arabia) as Imuran tablets. Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene; C14H12O3; ID IUPAC; 5-[(*Z*)-2-(4-hydroxyphenyl)ethenyl]benzene-1,3-diol) resveratrol 98% purity extract from Polygonum

cuspidatum (liftmode, Chicago, usa) and gamma glutamyl cysteine (γ-GC) provided from Biospecialties International Pty Ltd, Mayfield, NSW, Australia as a sodium salt (Glyteine®) with a minimum 95% purity γ-GC (90% reduced, 5% 6xidized). Other chemicals used in this study were obtained from Sigma-Aldrich (USA). Kits used for quantitative determination of different parameters were purchased from MyBioSource (San Diego, CA, USA), (Bio Basic, Canada).

Animals and treatment

Fifty Wistar Male Albino Rats weighing 170–220g were obtained from faculty of pharmacy, king Abdulaziz University, Jeddah, Saudi Arabia. The animals were maintained under standard conditions of humidity, temperature and light (12-h dark, 12- h light). They were provided with standard laboratory food pellets and water and libitum. Handling of rats was performed in accordance to the roles of King Abdulaziz University, Faculty of pharmacy. The animals were left for 7 days for adaptation.

Animals were classified into 5 groups, each of 10 rats as follows.

Group I: Control rats were treated orally with normal saline only.

Group II: Azathioprine animals in this group received AZA 10 mg/kg (Ajayi et al. 2018) for 28 successive days.

Group III: Rats were administered orally with AZA and intraperitoneal injection of resveratrol; 8ml/Kg body weight, (Ajayi et al. 2018; Ramalingam et al. 2019) daily for 28 days.

Group IV: Rats were administered orally with AZA along with oral administration of gamma-glutamylcysteine; 100mg/Kg body weight, (Ajayi et al. 2018; Liu et al. 2021) daily for 28 days.

Group V: Rats administered orally with AZA simultaneously with an intraperitoneal injection of resveratrol; 8ml/Kg body weight and gamma-glutamylcysteine; 100mg/Kg body weight for 28 days.

Resveratrol dissolved in dimethyl sulfoxide (DMSO) vehicle and administered intraperitoneally, γ-GC and azathioprine were administered orally with the use of a feeding tube, and supplies were prepared weekly.

Biochemical analysis

Liver tissue analysis

The liver tissues were quickly removed rinsed in ice-cold saline, and divided into two parts for homogenization and histopathological examination.

The concentration of SOD, GSH, and TNF-α, was estimated in liver tissue homogenate using a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) kit (Borgstahl et al. 1996; Kriegler et al.1988;

Pompella et al.2003) .CAT and MDA were determined in liver homogenate using a competitive enzyme-linked immunosorbent assay (ELISA) kit (Babusikova et al.2013; Ruiz-Larrea et al. 1994) all provided from (MyBioSource, Inc. P.O. Box 153308 San Diego, CA 92195-3308, USA) according to manufacturing instructions.

Histopathological studies

Liver tissues were examined to investigate the histopathological changes in various experimental groups. After collection of Liver specimens they were fixed in 4%

formaldehyde for 24 hours, then embedded into paraffin, sectioned for 5–6-µm thick, mounted on the microscope slides and stained with hematoxylin and eosin (H&E) (Bancroft and Gamble, 2008).

Statistical analysis

Data were statistically analyzed by comparing the values for different experimental groups with the values of individual normal ones. Results are expressed as mean ±SD. Significant differences among groups were analyzed using analysis of variance (ONE-WAY ANOVA) coupled with post-Hoc least significance difference (LSD). ANOVA at p≤0.05 was considered significant. The statistical analysis was performed by SPSS version 23.

RESULTS

Levels of liver homogenate antioxidant enzymes SOD, CAT. The influence of RSV and /or γ-GC on liver damage induced by AZA-treated rats are represented in (Figure 1). combination treatment of RSV and γ-GC along with AZA was the most advantageous in ameliorating levels of SOD comparing to other groups that were taking each treatment alone. Treatment of γ-GC with AZA also give liver homogenate level close to the combination G4 that was treated with γ-GC along with AZA were more beneficial compared to others.

Figure 2 shows the levels of liver homogenate oxidative stress markers GSH, and MDA. The effect of RSV and /or γ-GC on liver damage induced by AZA treated rats. combination treatments of RSV and γ-GC along with AZA in G5 as well as G4 th at treated with γ-GC along with AZA was the most advantageous in increasing the level of GSH. Treatment of G4 that take γ-GC with AZA shows the most beneficial group in decreasing the level of MDA compared to others.

Figure 3 shows the levels of liver homogenate inflammatory marker Tumor necrosis factor (TNF-α). The effect of RSV and /or γ-GC on liver damage induced by AZA treated rats. It's apparent from (Fig 3) that combination treatments of RSV and γ-GC along with AZA in G5 also treatment of γ-GC with AZA in G4 was the most advantageous in ameliorating levels of inflammatory marker TNF-α when compare to other groups.

(G3).

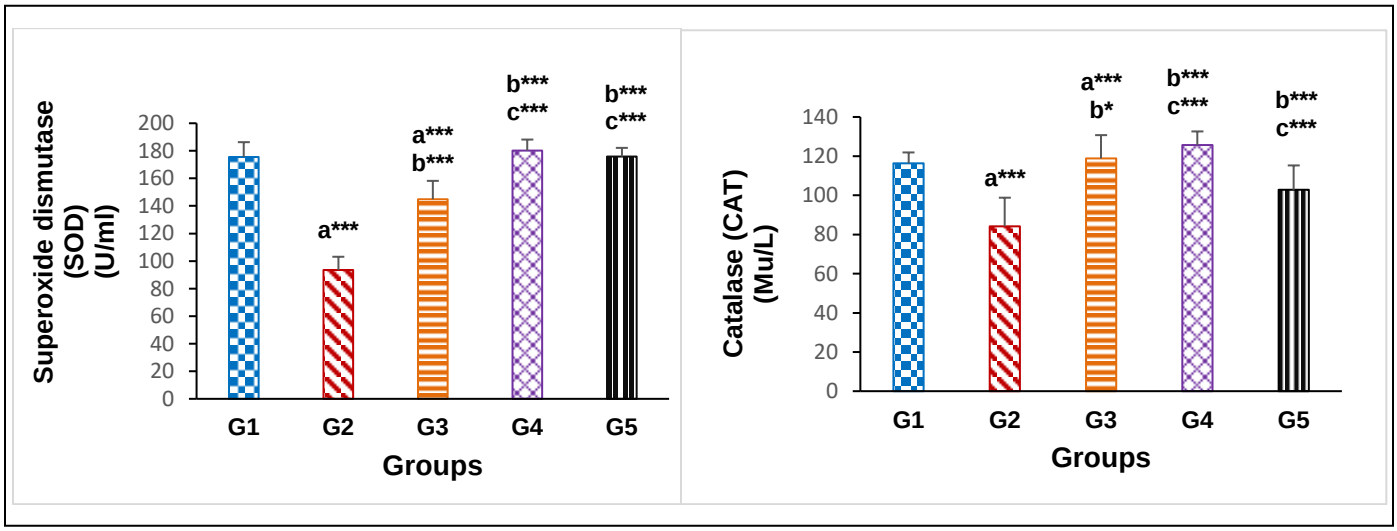

Figure1: Effects of resveratrol (RSV) and /or gamma glutamyl cysteine (γ-GC) on antioxidant enzymes in liver homogenate in azathioprine (AZA) intoxicated rats.

Values are expressed as mean ± SD of 10 rats. a*P≤0.001, compared with the control group (G1).b***P≤0.001, compared with the azathioprine (AZA) treated group (G2).b*P<0.05, compared with the azathioprine (AZA) treated group (G2).c***P ≤0.001, compared with the azathioprine + resveratrol group (AZA&RSV)**

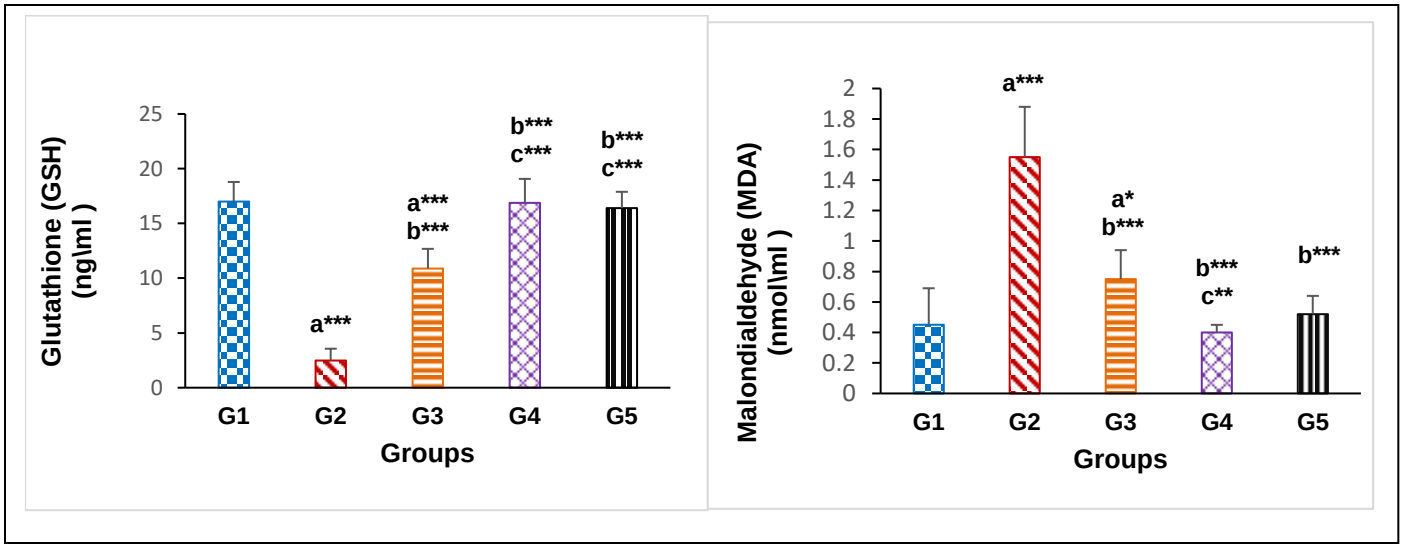

Figure2: Effects of resveratrol (RSV) and /or gamma glutamylcysteine (γ-GC) on oxidative stress markers in liver homogenate in azathioprine (AZA) intoxicated rats.

Values are expressed as mean ± SD of 10 rats. a***P≤0.001, compared with the control group (G1). a*P<0.05, compared with the control group (G1). b***P≤0.001, compared with the azathioprine (AZA) treated group (G2). c***P ≤0.001, compared with the azathioprine + resveratrol group (AZA&RSV) (G3).c**P≤0.01, compared with the azathioprine + resveratrol group (AZA&RSV) (G3).

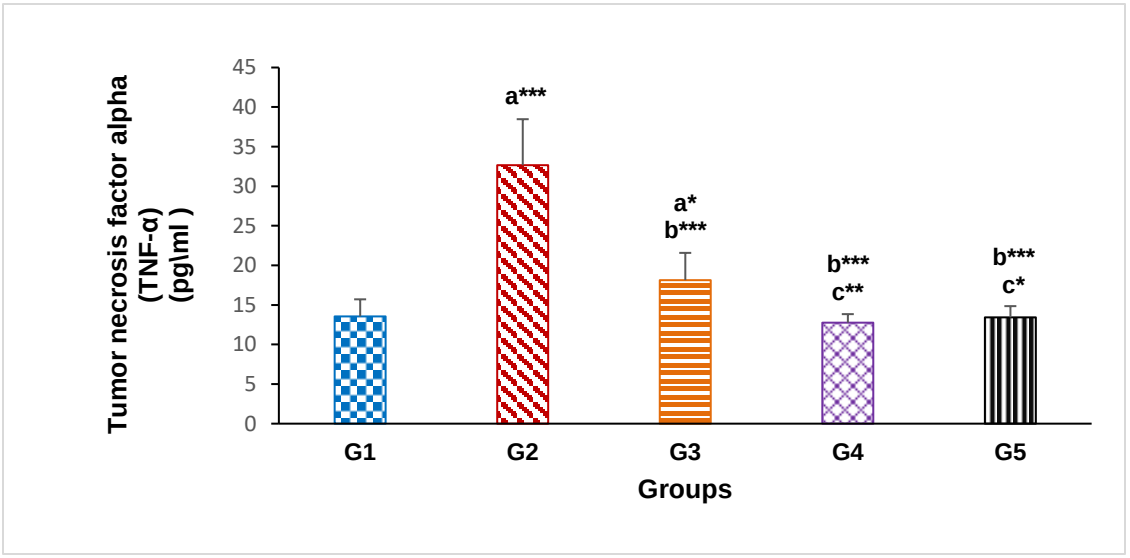

Figure3: Effects of resveratrol (Res) and /or gamma glutamyl cysteine (γ-GC) on liver homogenate inflammatory marker in azathioprine (AZA) intoxicated rats.

Values are expressed as mean ± SD of 10 rats. a***P≤0.001, compared with the control group (G1). a*P<0.05, compared with the control group (G1). b***P≤0.001, compared with the azathioprine (AZA) treated group (G2). c**P ≤0.01, compared with the azathioprine + resveratrol group (AZA&RSV) (G3). c*P<0.05, compared with the azathioprine + resveratrol group (AZA&RSV) (G3).

Histopathological observation of liver tissue

The histological sections of normal and AZA treated rat groups (Figure 4 a-f) were treated with H&E stain. (Figure 4-b and c) of rats treated with AZA showed focal necrosis of hepatocytes with loss of their outlines and karyolitic changes of their nuclei. Blood sinusoids looked dilated and showed aggregation of mononuclear inflammatory cells. (Figure 4-d) Liver sections of rats treated with AZA simultaneously with RSV only mild to moderate improvement was observed and still many hepatocytes showed necrotic changes. (Figure 4-e) In rats treated with AZA in concurrent with γ-GC Showing granuloma formation in the portal area & mild bile duct proliferation &grade 1 portal inflammation. In Liver sections of rats treated with AZA in concurrent with RSV combination with γ-GC (Figure 4-f) showed marked restoration of normal liver architecture with hepatocytes looked normal having active rounded nuclei. Blood sinusoids are thin and free of inflammatory cells showed that liver of control rat group (A) showed normal architecture where normal hepatocytes with rounded active nuclei are radiating from the central vein and separated by thin hepatic blood sinusoids, portal area also showed normal branches of hepatic artery, bile duct and portal vein. On the other hand Liver of AZA (B) and (C): showed focal necrosis of hepatocytes with loss of their outlines and karyolitic changes of their nuclei. Blood sinusoids looked dilated and showed aggregation of mononuclear inflammatory cells. In AZA+RSV (D): mild to moderate improvement was observed and still many hepatocytes showed necrotic changes. AZA+γ-GC (E) Showing granuloma formation in the portal area &mild bile duct proliferation &grade 1 portal inflammation However, liver of AZA+RSV+ γ-GC: showed marked restoration of normal liver architecture with hepatocytes looked normal having active rounded nuclei. Blood sinusoids are thin and free of inflammatory cells. (H&E, X20µm).

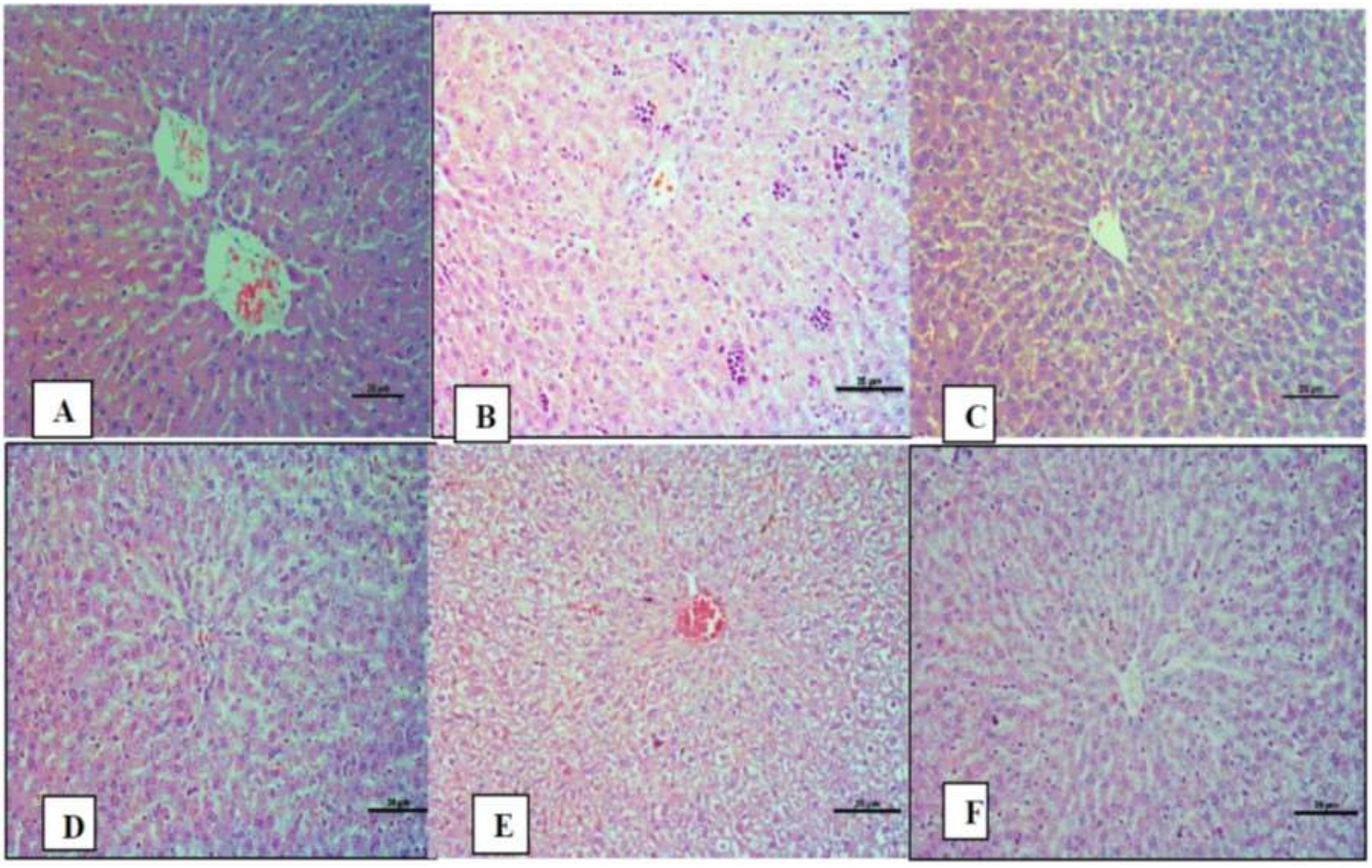

Figure 4: Light micrograph of rat liver sections of AZA treatment groups stained with H&E

DISCUSSION

AZA is [6-(1-methyl-4-nitro-5-imidazolyl) and its metabolites 6-mercaptopurine (6-MP) a thiopurine prodrug that represents corticosteroid-sparing features effect and reduces remission of the different immune disorders including acute lymphoblastic leukemia (ALL), and inflammatory bowel disease (IBD) (Sousa et al. 2020). AZA has been reported to have a role in the development of liver damage (Wong et al. 2017). Accumulation of 6-MMP metabolites causes hepatotoxicity and drug withdrawal (Núñez et al. 2022). Several clinical and experimental studies have indicated that AZA treatment is associated with the development of hepatotoxicity (Al-Judaibi et al 2017; Munnig-Schmidt et al 2018). Furthermore, AZA treatment increases ROS level and induce oxidative stress, mitochondrial dysfunction, ATP depletion, and necrosis (Misdaq et al. 2015).

Antioxidant enzymes CAT and SOD function to reduce oxidative stress they eliminate excuses amount of free radicals thus preventing oxidative cellular damage. On the other hand, evidence documented that AZA depletes the antioxidant enzymes along with oxidative damage a study indicates that administration of AZA reduces antioxidant properties via diminished SOD and CAT concentration (Amin & Hamza, 2005).

The liver has its mechanism of defense against oxidative stress with antioxidant agents that control levels of ROS and RNS, GSH, glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT) (Ntamo et al. 2021). SOD and CAT are important antioxidant enzymes where they eliminate free radical and maintain normal oxidant balance, they express high activity in liver (Goyal & Basak, 2010). SOD is found in three forms in cytosol CuZn-SOD, mitochondria Mn-SOD and extracellular

SOD (He et al. 2017; Miao & Clair, 2009). CAT located mainly in peroxisomes decomposes hydrogen peroxide into oxygen and water (Scibior & Czeczot, 2006).

The mechanisms of AZA-induced hepatic injury have been addressed by previous investigations. Some studies have reported that AZA can induce hepatic injury by the action of their active metabolites during drug detoxification, metabolites can directly attack cell membrane. accumulation of 6-MMP inactive metabolite of AZA in hepatic tissue trigger the formation of free radicals by the effect of its XO in AZA metabolism also produce ROS during the process, all these mechanisms trigger GSH cellular concentration promote increasing levels of oxidative stress, these factors cause modifications of DNA, proteins, and lipid (Maruf et al.2014; Stocco et al.2014). It has been found that AZA induces mitochondrial dysfunction, ATP depletion, and cell death due to necrosis, not apoptosis (Lee & Farrell, 2001).

Combining administration of RSV and γ-GC has been indicated to increase antioxidant enzymes CAT and SOD compared to a decrease there amount in AZA-induced hepatic injury. RSV plays a key role in activating cellular defense mechanisms to protect the hepatocytes from harmful consequences caused by oxidative stress (Dalaklioglu et al. 2013). Output agrees that RSV increases hepatic antioxidant enzyme CAT and SOD thus triggering oxidant recovery In thioacetamide-induced hepatic injury and alcoholic liver injury models (Hussein et al. 2017; Peiyuan et al. 2017).

AZA elevates the level of ROS, depletes cellular GSH, triggers mitochondrial injury and necrotic cell death (Tapner et al. 2004), and promotes liver damage by increasing

oxidative damage and lipid peroxidation. Reducing GSH increases lipid peroxidase 4-HNE and MDA (Ayala et al. 2014).

GSH is an important thiol-peptide that plays a key role in cellular physiological function and maintains oxidant cellular integrity, eliminating exclusive mitochondrial ROS while transformed to oxidized glutathione disulfide (GSSG) form in the process (Chen et al. 2013), on the other hand, serve as a cofactor for other antioxidant enzymes like glutathione peroxidase(GPx) that metabolize hydrogen peroxide (H2O2) and lipid peroxides (Arthur, 2000; Abdali et al. 2019) at increasing levels of free radicals Nrf2 play a central role in GSH level thus trigger expression of antioxidant mediators, including GCL, to enhance GSH levels (Han et al. 2006).

MDA is one of the aldehydes end products of lipid peroxidation and has been used as a marker for lipid oxidative damage (Maurya et al. 2021). Increasing levels of MDA are linked to a lot of diseases in order of oxidative stress including liver diseases, MDA causes modifications in DNA and proteins (Ayala et al. 2014).

Our results indicate that combining administration of RSV and γ-GC have been indicate to increase GSH and decrease MDA comparing to the increase in AZA induced hepatic injury. On methotrexate-induced liver injury rat model RSV enhance GSH, as well as decrease lipid peroxidation, appears on reducing MDA thus prevent hepatic oxidative stress (Tunali-Akbay et al. 2010). Antioxidant properties of RSV has positive impact on reducing lipid peroxidation since they there connected. Ethanol-fed rats correlate with a high concentration of lipid peroxides, RSV shows decreasing levels of MDA in different organs including the liver which documents RSV on lower lipid peroxidation (Ara et al. 2005). γ-GC exerts an antioxidant role by reducing cellular oxidative stress due to the -SH group, γ-GC can work independently rather than affect GSH as an antioxidant (Nakamura et al. 2012). γ-GC has a direct effect on the increasing level of hepatic GSH and declines lipid peroxidation that presented in decreasing levels of MDA on iron overload which induces hepatic damage in rats' model (Salama et al. 2015).

AZA 50 mg/kg i.p administration in male rats presents increased levels of inflammatory marker TNF-α and diminishes reduced glutathione, CAT, and accumulation of cellular oxidized glutathione while enhancing antioxidant

enzymes and glutathione reducing inflammation (El-Beshbishy et al. 2011). oral administration of AZA in mice increases the expression of proinflammatory cytokines such as TNF-α and IL-1β (Matsuo et al. 2014).

TNF-α is a pro-inflammatory cytokine mainly produced by macrophages and increases disease (Parameswaran & Patial, 2010) TNF-α is a key factor that contributes to the triggering of an inflammatory cascade involving the induction of cytokines after liver injury (Chu, 2012). Ethanol Sensitizes hepatocytes showing increased levels of TNF- α due to necrosis caused as a result of mitochondrial glutathione pool depletion and increase ROS (Colell et al. 1998).

Supplement of RSV and /or γ-GC along with AZA reduce TNF-α compared to the AZA hepatotoxicity group, this result presents the potency of anti-inflammatory impact in both RSV and γ-GC. Presumably the anti-inflammatory effect of RSV by inhibiting NF-kB activation results in reduced TNF- α and IL-6 and reduce ROS production (Di Pascoli et al. 2013; Faghihzadeh et al. 2014; Sadeghi et al. 2017).

Sepsis-induced mice model either by injection of lipopolysaccharide (LPS) or cecal ligation and puncture (CLP) mice show increased levels of TNF-α and IL-1β, after oral administration of γ - GC (1200 mg/kg) in 30 min post-injection, attenuates serum levels of TNF-α and IL-1β and reduced mortality rate of LPS and CLP from 80% to 40% and 90% to 40% respectfully (Yang et al. 2019).

Continues supplementation of RSV and/or γ-GC had protective effect to AZA intoxicated rats liver against the AZA-induced hepatic histomorphology damage as referred by more or less normal liver architecture with normal hepatocytes. The hepatoprotective effects of both RSV and/or γ-GC on liver histological deterioration have been documented by other investigations. It has been illustrated that administration of RSV could significantly decrease the incidences of liver histological lesions induced by Concanavalin-A-induced acute liver injury in mice (Zhou et al. 2015; Sulimani et al. 2021), suggest that RSV exhibits potential hepato-prophylactic effect against liver hepatotoxicity. Recent studies have also indicate that treatment of rats with γ-GC, effectively protected the liver against histopathological liver lesions induced by cadmium in the Wistar rats model (Salama et al. 2019).

CONCLUSION

The present study demonstrates that the use of AZA has the potential to cause liver side complications which can progress to liver tissue damage through inducing oxidative stress, inflammation, and necrosis.

The ameliorative mechanisms of RSV and γ-GC against AZA-induced hepatotoxicity were through their antioxidant and anti-inflammatory impact, different investigations indicate that increasing antioxidant liver enzymes, modulate other oxidation markers, as well as increases intracellular GSH of RSV and γ-GC, reduce the AZA hepatic toxicity.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

Huda S Almutairi performed the experiment, tissue collection, sample, and data analysis. Jehad M Yousef also did data analysis. Jehad M Yousef and Manal A Tashkandi wrote experimental design, wrote the manuscript, and reviewed it.

All authors read and approved the final version.

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