## Therapeutic Effect of Resveratrol and Gamma-GlutamylCysteine in Azathioprine Drug-Induced Hepatotoxicity

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#### Abstract

The purpose of the current literature is to examine the protective properties of resveratrol (RSV) and gamma-glutamylcysteine ( $\gamma$ -GC) against hepatic injury induced by Azathioprine (AZA) that may have a favorable impact on using resveratrol and/ or gamma-glutamylcysteine with immunosuppressant drugs therapy. The study duration was 4 weeks; fifty Male Wister Albino Rats were classified into 5 groups: control group: and rats were orally treated with normal saline. AZA group, Rats were taken AZA orally at 10 mg/kg. RSV group, rats had oral administration of AZA along with IP injection of RSV; 8ml/Kg, GC group, rats had oral administration of AZA and  $\gamma$ -GC; 100mg/Kg after 2 h post-injection for 4 weeks.

The results indicate that AZA treatment increases alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels, decreases antioxidant enzymes catalase (CAT) and superoxide dismutase (SOD) and depleted hepatic microRNA-122 (miR-122) referred to hepatic injury. Administration of RSV/ $\gamma$ -GC separately or together modulates levels of liver enzymes and protective hepatic tissue.  $\gamma$ -GC reduced the degree of histological hepatic damage in rats. The results reveal that co-administration of RSV and/or  $\gamma$ -GC can reduce immunosuppressant drug hepatotoxicity.

Keywords: Azathioprine, Drug-induced liver injury (DILI), Gamma- glutamylcysteine, Resveratrol

#### INTRODUCTION

The liver is a metabolic organ that works in many ways and performs different actions. The liver plays a vital role in circulation and executes multiple jobs in metabolism, immune system, excretion, secretion, and vascular. It is the major organ of metabolism for different macromolecules carbohydrates, lipids, proteins, also detoxifying drugs chemicals, and carcinogens [1].

Drug-induced liver injury is hepatotoxicity due to drugs and their metabolites, supplements, or herbal medicine. Other factors that might help in the disease development can be environmental, immunological, or inherited. It is a critical cause of adverse drug reaction (ADR) known as hepatic injury insulted by drugs and a major cause of acute liver failure (ALF) [2].

Azathioprine (AZA) is a prodrug of 6-mercaptopurine (6-MP) and they are forms of thiopurine that have been used in immune diseases like acute lymphoblastic leukemia (ALL), inflammatory bowel diseases, and organ transplant. AZA is metabolized to 6-thioguanine nucleotide (6-TGN), 6-methyl mercaptopurine (6-MMP), and 6-thiourea acid (6-TU) [3].

Active metabolite 6-TGN targets leukocytes, since it is a purine analog it will bind to ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) instead of guanine and adenosine nucleotides leading to alteration in DNA structure, motivating cell apoptosis and stopping the synthesis of protein and nucleotides [4]. 6-thioguanine triphosphate (6-TGTP) specially incorporated into ras-related C3 botulinum toxin substrate 1 (Rac1) is a GTP binding protein and vav signaling protein and block the activation of their genes such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ b) and Signal transducer and activator of transcription 3 (STAT3) resulting in stopping the production

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This is an open-access article distributed under the terms of the Creative Commons Attribution-Non Commercial-Share Alike 4.0 License, which allows others to remix, tweak, and build upon the work non commercially, as long as the author is credited and the new creations are licensed under the identical terms.

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AZA treatment increases reactive oxygen species (ROS) levels, leading to oxidative stress, mitochondrial dysfunction, ATP depletion, and necrotic cell death [7]. Adverse drug reaction of AZA occurs in two ways it can be dose-dependent that happens in long duration months or years and may cause myelosuppression, hepatotoxicity, and lymphomas second type of dose-independent can cause pancreatitis. High concentration of 6-TGN the active metabolite highly related to myelotoxicity [8, 9]. Accumulation of 6-MMP in hepatocytes may be responsible for the hepatotoxic side effect of the drug. 6-TU is the inactive metabolite secreted in urine [5].

Resveratrol (RSV) is stilbene phytoalexin synthesized in plants due to ecological stress, sunlight, and pathogens like microbes and fungi [10]. RSV stimulates molecular signals like nuclear factor erythroid 2–related factor 2 (Nrf2), SIRT1, mitogen-activated protein kinases (MAPK), and protein kinase B (Akt) moreover, it increases antioxidant enzymes and reduces inflammation [11].

Gamma-glutamylcysteine ( $\gamma$ -GC) is a dipeptide antioxidant consisting of glutamic acid and cysteine residue,  $\gamma$ -GC eliminates ROS by two mechanisms first, in the presence of GSH synthetase (GS) is converted into glutathione (GSH), GSH is an antioxidant tripeptide that performs an essential role in cellular detoxification, ROS, and RNS elimination and maintains normal cellular and organelles function [12]. Due to the thiol group (SH)  $\gamma$ -GC maintain intracellular thiol homeostasis and works as a cofactor for glutathione peroxidase 1 (GPX1) is an antioxidant enzyme which has crucial mechanism in the elimination of hydrogen peroxides (H<sub>2</sub>O<sub>2</sub>) as water, and a cofactor for GPX4 that reduce H<sub>2</sub>O<sub>2</sub> and lipid hydroperoxides (LOOHs) [13, 14].

## MATERIALS AND METHODS

Azathioprine (AZA) was obtained from (Alnahdi pharmacy, Saudi Arabia) as Imuran tablets. Resveratrol extract from Polygonum cuspidatum (Liftmode, Chicago, USA) and gamma-glutamylcysteine ( $\gamma$ -GC) obtained from Biospecialties International Pty Ltd, Mayfield, NSW, Australia in the form of sodium salt (Glyteine®) (90% reduced, 5% oxidized). All used chemicals were provided by Sigma-Aldrich (USA). Kits that were used in the determination of parameters were provided by MyBioSource (San Diego, CA, USA), (Bio Basic, Canada), (and TaKaRa Inc., Japan).

Fifty male Wistar albino rats weighing 180–230 g were provided by the Faculty of Pharmacy, King Abdulaziz University, Jeddah, Saudi Arabia. Rats were housed under standard conditions. They were given standard pellets and treated according to the instructions of the Faculty of Pharmacy, King Abdulaziz University. Rats were acclimated for 1 week.

Rats were divided into five groups: control group: and rats were orally treated with normal saline. AZA group, Rats were taken AZA orally at 10 mg/kg [15] for 4 weeks. RSV group, rats had oral administration of AZA along with IP injection of RSV; 8ml/Kg for 4 weeks [15, 16].  $\gamma$ -GC group, rats had oral administration of AZA and  $\gamma$ -GC; 100mg/Kg [15, 17] for 4 weeks. Combination group rats had oral administration of AZA with IP injection of RSV; 8ml/Kg; and  $\gamma$ -GC 100mg/Kg after 2 h post-injection for 4 weeks

Resveratrol dissolved in Dimethyl sulfoxide (DMSO) vehicle for intraperitoneal injection, supplies prepared weekly.

## Specimen Collection

After finishing the study period, the day after blood samples were taken by eye puncture of the anesthetized rats in plain tubes to separate serum, centrifuged at 2000 g-15 min and preserved at -20 °C for determination of biochemical investigation, rats were euthanized by exsanguination under pentobarbital sodium anesthesia 65 mg/kg, IP for collecting liver tissue samples.

### **Biochemical Analysis**

ALT and AST were determined in blood serum by the use of a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) kit [18, 19]. SOD was determined in liver tissue homogenate by the use of a double antibody sandwich ELISA kit [20]. CAT was determined in liver homogenate using a competitive ELISA kit [21]. All were obtained from (MyBioSource, Inc. P.O. Box 153308 San Diego, CA 92195-3308, USA).

# Measuring miR-122 Expression by Quantitative PCR (qPCR)

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis was done to measure the expression of MiR-122 (as a target gene), compared with Beta 2 Microglobulin (B2M) (as a reference gene) using SYBR Premix Ex Taq II (TaKaRa Inc., Japan) by Rotor-Gene 6000 qPCR machine (Qiagen, Germany). The specific primers for MiR-122 & B2M (HKGs) Primer sequences are represented in (**Table 1**).

All samples were run in duplicate. Relative expression of miR-122 with b2m as control was expressed using the comparative CT method [22].

Table 1. RT-PCR of Primer sequences		
Target gene		Primer sequence
MiR-122	Forward	5'-TTGAATTCTAACACCTTCGTGGCTACAGAG-'3
	Reverse	5'-TTAGATCTCATTTATCGAGGGAAGGATTG-'3
B2M	Forward	5'-CTTCAGCAAGGACTGGTC-3'
	Reverse	5'-TCTCGATCCCAGTAGACG-3

#### Histopathological Examination

After tissue collection part of the liver was treated with formaldehyde for 24 hours then dehydrated with ethyl, clarified with xylene, and stick in paraffin. Paraffin blocks were cut using a microtone at 4-5 micrometers, and then fixed on glass slides and stained with Massons Trichrome (MT) [23].

#### Statistical Analysis

Statistical analysis of the data was performed using SPSS and the values of the various experimental groups were compared with the values of the individual normal groups. Results are expressed as mean  $\pm$  SD. Combine analysis of variance (one-way ANOVA) with post hoc least significant difference (LSD) to express significant differences between groups. ANOVA with  $p \leq 0.05$  was considered significant.

## **RESULTS AND DISCUSSION**

**Figure 1** represents serum levels of liver function enzymes ALT and AST and the effect of AZA-intoxicated rats. combined administration of RSV and  $\gamma$ -GC syncing with AZA gives the best results in improving levels of ALT and AST when comparing with each compound separately. Administration of  $\gamma$ -GC and AZA also gives liver serum enzymes level close to the combination group.





**Figure 1.** Impact of RSV and /or  $\gamma$ -GC on liver enzymes serum in AZA-treated rats. Values are expressed as mean  $\pm$  SD of 10 rats. <sup>a\*\*\*</sup>P $\leq$ 0.001, compared with the control group (G1).<sup>a\*\*</sup>P $\leq$ 0.01, compared with the control group (G2).<sup>c\*</sup>P  $\leq$ 0.001, compared with AZA treated group (G2).<sup>c\*</sup>P  $\leq$ 0.05, compared with AZA&RSV (G3).<sup>c\*\*</sup>P $\leq$ 0.01, compared with AZA&RSV (G3).

**Figure 2** displays the levels of liver homogenate antioxidant enzymes SOD and CAT. The effect of RSV and /or  $\gamma$ -GC on hepatic injury indices in AZA-treated rats. Combination administration of RSV and  $\gamma$ -GC syncing with AZA gives the best results in improving levels of SOD when comparing each compound separately. Administration of  $\gamma$ -GC and AZA gives liver antioxidant levels close to the combination group. For CAT antioxidants level in the  $\gamma$ -GC group that were treated with AZA+ $\gamma$ -GC were most advantageous compared to others.





**Figure 2.** Impact of RSV and /or  $\gamma$ -GC on antioxidant enzymes in liver homogenate in AZA-treated rats. Values are expressed as mean  $\pm$  SD of 10 rats. <sup>a\*\*\*</sup>P $\leq$ 0.001, compared with the control group (G1).<sup>a\*</sup>P<0.05, compared with the AZAtreated group (G2).<sup>c\*\*\*</sup>P $\leq$ 0.001, compared with AZA&RSV group (G3).<sup>c\*\*</sup>P $\leq$ 0.01, compared with AZA&RSV group (G3).

**Figure 3** displays the levels of liver homogenate genetic marker MicroRNA-122 (miR-122). The effect of RSV and /or  $\gamma$ -GC on hepatic injury indices in AZA-treated rats. Combination administration of RSV and  $\gamma$ -GC syncing with AZA gives the best result in improving levels of miR-122 when comparing with each compound separately, it's almost the same as the control group.



**Figure 3.** Impact of RSV and /or  $\gamma$ -GC on liver homogenate genetic marker in AZA-treated rats. Values are expressed as mean  $\pm$  SD of 10 rats. <sup>a\*\*\*</sup>P $\leq$ 0.001, compared with the control group (G1).<sup>a\*\*</sup>P $\leq$ 0.01, compared with the control group (G1).<sup>b\*\*\*</sup>P $\leq$ 0.001, compared with AZA treated group (G2).<sup>c\*\*</sup>P  $\leq$ 0.01, compared with AZA&RSV group (G3).<sup>d\*</sup>P<0.05, compared with AZA&QC group (G4).

#### Histopathological Examination of the Liver

#### Masson's Trichrome Stain of the Liver

Micrograph of liver sections stained with Masson's trichrome (Figure 4). The stained blue color represents collagen distributions in liver tissue: the control group 1 shows normal hepatic lobules showing a central vein with cords of hepatocytes separated by blood sinusoids and a normal degree of collagen fibers in the central vein and portal tract area. However, rats treated with the AZA group 2 shows massive intralobular deposition of collagen fiber in both Porto-portal and Porto-central bridging fibrosis apparently as intense blue-stained collagen fibers content in the tissue. Rats' liver sections that consumed AZA along with the RSV group 3 showed nearly normal blue-stained collagen fibers content in the tissue, while rats that consumed AZA along with  $\gamma$ -GC group 4 liver show a minimal degree of collagen fibers surrounding the central vein. Rats' liver sections that consumed AZA along with RSV and  $\gamma$ -GC group 5 shows moderate collagen fibers surrounding the central vein.



G2 (AZA)



G3 (AZA+RSV)



G4 (AZA+y-GC)



G5 (AZA+RSV+Y-GC)

**Figure 4.** Light micrograph of rat liver sections stained with Masson's Trichrome (MT).

AZA has an immunosuppressive effect and has been used in immune disease and organ transplants. 6-MMP accumulation during AZA treatment may lead to hepatotoxicity thus therapy is discontinuous. AZA metabolism increases free radicals and ROS that lead to mitochondrial dysfunction, ER stress, ATP depletion, and necrotic cell death [3].

ALT and AST are liver enzymes that are found in higher amounts in liver tissues, released to blood due to liver damage, and are considered markers of hepatic injury [24] transaminases reflect hepatic injury, and the degree of injury correlates with their levels [25, 26]. The current study showed that oral consumption of AZA to male rats cause a significant increase in liver injury markers, ALT, and AST. Other reports revealed that treatment with AZA can cause an increase in hepatic transaminases [27]. Oral consumption of AZA-intoxicated rats in combination with RSV and /or  $\gamma$ -GC had a significant reduction in the levels of hepatic AST and ALT versus the AZA-intoxicated group.  $\gamma$ -GC was the potential therapy for modulating the levels of liver function indices close to normal levels. The advantageous hepatoprotective effects of both compounds may be due to their action in reducing oxidative damage and inflammation of AZA hepatic injury. Abdu and Al-Bogami [28] reported that ingestion of RSV into DMN-injected rats significantly reduced serum ALT and AST. Also, the potential prophylactic effect of RSV by decreasing the impact of liver transaminases level against liver injury has been proved by clinical studies [29-31].

 $\gamma$ -GC function modulates hepatic enzyme concentration as a hepatoprotective marker in liver injury, studies show the decreased concentration of ALT and AST after administration of  $\gamma$ -GC that has been increased in hepatic injury rat models [32, 33].

Free radicals ROS and RNS normally produced during hepatic metabolism and play a role in cellular function and signaling but an increasing amount of them cause cellular oxidative stress. The Liver has an antioxidant defense system to eliminate free radicals including CAT and SOD [34]. Superoxide dismutase (SOD) is found in three forms cytoplasm (CU/ZnSOD), mitochondrial (Mn/SOD), and Extracellular (CU/ZnSOD), SOD works to get rid of superoxide onion as a form of hydrogen peroxide [35]. CAT converts hydrogen peroxide to  $H_2O$  [36].

Depletion of cellular GSH and continuous generation of ROS by XO cause depletion of antioxidant enzymes SOD, and CAT oxidative damage to hepatocytes [37].

RSV and  $\gamma$ -GC therapy along with AZA administration restore levels of SOD and CAT compared to the group treated with AZA only depleted their concentration thus increasing oxidative damage in the liver. Studies agree that RSV and  $\gamma$ -GC reduce ROS by increasing SOD and CAT in vivo models [17, 38].

Mir-122 is a small segment of noncoding RNA consisting of 22 nucleotides, it regulates gene expression by binding to complementary bases on targeting RNA and terminating transcription. miR-122 is the most abundant type of miRNA in hepatocytes representing 70% of total miRNAs and plays a major role in different biological functions of the liver including enzymes, cholesterol regulation, iron homeostasis, and cell proliferation. A high concentration of miR-122 correlates with liver injury [39]. Different studies *in vivo* agree that miR-122 is liver specific biomarker [40, 41].

In our current study as a result of hepatic injury AZA hepatic miR-122 concentration is decreased. Mostly referred to that miR-122 are released from hepatocyte in hepatic toxicity at first it represents a high level but then reduced when it is depleted due to constant hepatic injury. Like our results in

thioacetamide (TAA) induced liver injury, miR-122 was significantly decreased in TAA intoxicated rats' group [42].

Supplementation with RSV and/or  $\gamma$ -GC with AZAintoxicated rats increases the concentration of hepatic miR-122 compared to the decreased concentration in the group that consumes AZA only. Similar to our results in RSV plus AZA group, a study on rat hepatoma cell line represents a decreased concentration of miR-122 after treatment with RSV miR-122 shows an increased level [43].

Under histopathological examination Treatment with RSV and/or  $\gamma$ -GC showed remarkable improvement in collagen deposition as well as moderate collagen fiber ratio around the central vein compared to intralobular collagen deposition in AZA treated group, RSV protective effect on liver histology also documented in concanavalin-A- (ConA-) hepatotoxicity mice model [44].  $\gamma$ -GC reduced the degree of histological liver injury in mice sepsis model [45].

## CONCLUSION

From the present study, we conclude that AZA-induced liver damage through oxidative damage, inflammation, and necrotic cell death. RSV and  $\gamma$ -GC exhibit antioxidant and anti-inflammation properties, which modulate liver enzymes level, restore cellular antioxidant enzymes as well reducing histopathological damage.

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#### CONFLICT OF INTEREST: None

#### FINANCIAL SUPPORT: None

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