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L-Ergothioneine mitigates azathioprine-induced cytotoxicity in HepaRG cells via modulation of xenobiotic metabolizing enzymes and SIRT1/Nrf2 signaling

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***Abstract*---**Hepatotoxicity is one of the well-documented adverse effects of azathioprine (AZA), a linchpin in the immunosuppressant regimen in the treatment of various autoimmune diseases. In this study, L-Ergothioneine (ESH)—a unique thiol/thione amino acid—was investigated for its putative antioxidant and hepatocyte protective properties against AZA by using HepaRG liver cell model. The effect of ESH on AZA metabolizing enzymes (xanthine oxidase (XO) and glutathione S-transferase (GST) M1), reactive oxygen species (ROS) and glutathione (GSH) levels, mitochondrial parameters (ATP, succinate, and mitochondrial membrane potential) and upstream signaling parameters including SIRT1-mediated antioxidant genes (Nrf2, HO1, and γ-GCLC) were assessed. AZA exposure provoked XO and GST-M1-mediated ROS production and GSH depletion, mitochondrial dysfunction, and downregulation of SIRT1-Nrf2 axis and ultimately resulted in hepatocellular toxicity. ESH treatment effectively counteracted these aberrations and preserved the hepatocellular viability and functions. The present study demonstrated that ESH attenuates AZA-induced oxidative stress and mitochondrial dysfunction via suppression of AZA metabolizing enzymes and upregulation of SIRT1-Nrf2 signaling.

***Keywords*---**Azathioprine, L-Ergothioneine, mitochondrial dysfunction, oxidative stress, SIRT1/Nrf2.

1 Introduction

Azathioprine (AZA) is a linchpin immunosuppressant used for the treatment of various autoimmune diseases (e.g., inflammatory bowel disease (IBD), rheumatoid arthritis, etc.) and for the prevention of graft rejection in organ transplantation cases (Bhorade et al. 2011; Kakuta et al. 2018). However, patients treated with thiopurines (AZA or its primary metabolite, 6-mercaptopurine (6-MP) reported various adverse effects including hepatotoxicity, myelosuppression, nephrotoxicity, acute pancreatitis, skin cancer, etc. (de Abajo et al. 2004; de Jong et al. 2004; Jack et al. 2016). Clinical studies showed that 20-22% of patients withdrew AZA/6-MP due to adverse events (de Jong et al. 2004). AZA/6-MP treatment causes cholestatic hepatitis within three months of treatment initiation or dose escalation.

A population-based case-control study by Abajo et al. found that an alarmingly high incidence of drug-induced liver injury (DILI) was observed in the patients under AZA treatment (about 1 per 1000 patients) (de Abajo et al. 2004). Another study showed that AZA is associated with the highest risk of DILI (about 1 per 133 patients), although the actual number of patients was small (Björnsson et al. 2013). In this line, Siramolpiwata and Sakonlayac (2017) reported that liver toxicity—manifested as hepatocellular or cholestatic hepatitis or both—is a relatively infrequent but imperative repercussion of AZA. Even though post-withdrawal recovery from hepatoxicity was observed in all the patients, severe cholestasis can appear. According to the European Association for the Study of the Liver (EASL), various phenotypes of DILI include idiosyncratic DILI, nodular regenerative hyperplasia, and ductopenic (vanishing bile duct) syndrome are associated with AZA. Notably, idiosyncratic (unpredictable) DILI is a challenging entity for hepatologists, as it may precipitate grave complications like acute liver injury, for which effective treatment is still lacking. Hence, a high degree of cognizance about this condition and regular monitoring for cholestasis, transaminitis, and clinically significant rise in 6-methyl mercaptopurine (6-MMP) levels is quintessential in the patients undergoing AZA treatment.

Substantial pieces of evidence underscore that AZA-induced hepatotoxicity and treatment failure are the consequences of “skewed thiopurine metabolism”—leading to the formation of high levels of 6-MMP and defective conversion of AZA into its pharmacologically active derivatives, 6-thioguanine nucleotides (6-TGN) (van Asseldonk et al. 2012). Congruently, a recent meta-analysis report revealed that patients identified with a preferential 6-MMP generation pathway (“thiopurine shunters”) show diminished clinical outcomes despite dose escalation (Sousa et al. 2020). Besides, a positive correlation between liver toxicity with 6-MMP and a negative correlation with 6-TGN were confirmed. The hepatotoxicity of AZA involves two key mechanisms (Manuf et al. 2014): i) formation of 1-methyl-4-nitrothioimidazole, an inactive derivative of xanthine oxidase (XO)-mediated metabolism of AZA. ii) glutathione S-transferase (GST)-mediated reduction of the endogenous glutathione and formation of 6-thiouric acid (another inactive derivative of XO-mediated metabolism of AZA). Hence, co-treatment of the patients undergoing AZA therapy, with XO inhibitors and/or GSH precursors might attenuate AZA-associated hepatotoxicity and improve the clinical outcomes. In this line, a randomized controlled trial suggested that co-therapy of low-dose

azathioprine (LD-AZA) and allopurinol (AL; a xanthine oxidase inhibitor) has improved clinical remission in the ulcerative colitis patients (Kiszka-Kanowitz et al. 2022). However, treatment withdrawal due to adverse events was still reported in 30% of the patients who underwent LD-AZA/AL co-therapy. On the other side, N-acetylcysteine (NAC; a GSH precursor) substantially reduced the antiproliferative potential of AZA, although the cytotoxic profile of AZA remained unaltered. Lee et al., found that AZA provokes necrotic cell death of hepatocytes via GSH depletion-mediated oxidative stress and mitochondrial injury (Lee et al. 2001).

L-Ergothioneine (ESH)—a unique thiol/thione amino acid and a water-soluble dietary micronutrient found in fungi and bacteria—has potent antioxidant and cytoprotective properties (Paul et al. 2010; Williamson et al. 2020). ESH, with a well-documented safety, exhibits an unusual “adaptive” antioxidant property (i.e., active involvement only under high oxidative stress conditions) (Cheah et al. 2017). Fascinatingly, ESH depletion makes the mammalian cells more vulnerable to oxidative assault and mitochondrial injury leading to various diseases including inflammatory bowel syndrome (Lai et al. 2019). Interestingly, a recent study accentuated that ESH attenuates the hepatocellular oxidative stress via SIRT1/Nrf2 signaling (Dare et al. 2021). However, the effect of ESH and whether this signaling pathway is involved in AZA-induced hepatoxicity has not been investigated yet. Hence, we assumed ESH might be a holistic antioxidant and mitochondrial protectant suitable for the mitigation of AZA-induced hepatotoxicity plausibly via modulation of xenobiotic metabolizing enzymes and SIRT1/Nrf2 axis.

2 Materials and Methods

Cell culture and treatment

Undifferentiated HepaRG cells (Biopredic International, Saint-Grégoire, France) were cultured according to the manufacturer’s directions. Seeding was done using William’s E medium supplemented with 10% fetal bovine serum in 15% CO2 at 37◦C for two weeks. Then, differentiation of the cells was initiated using 2% DMSO in the William’s E medium (Petit et al. 2008). The cell viability was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay after the addition of AZA at various concentrations. For further studies, the HepaRG cells were segregated into control, AZA (250µmol/L) AZA + ESH (NAC; 0.5 mmol/) or N-acetylcysteine (NAC; 0.5 mmol/L) groups.

Measurement of oxidative stress and hepatocellular enzyme markers

Intracellular antioxidant content was measured by using the GSH assay kit (Abcam, Cambridge, UK). This assay is based on the enzymatic reaction in the presence of GSH and a chromophore, which undergoes reduction to form a stable product. The absorbance was measured at 450 nm using a microplate reader. The oxidant burden was measured by using the parameters: ROS (Abcam, Cambridge, UK) and MDA (Abcam, Cambridge, UK) assay kits. Intracellular ROS level was measured by using the oxidation conversion of 2,7-dichlorofluorescein diacetate (DCFDA) into a highly fluorescent compound 2,7-dichlorofluorescein (DCF). The

absorbance was measured at an excitation/emission wavelength of 485/535 nm using a microplate reader. The measurement of XO (Abcam, Cambridge, UK) and GSTM1 (Abcam, Cambridge, UK) was done by using ELISA assay kit as instructed by the manufacturer. For the measurement of AST (Abcam, Cambridge, UK) or ALT (Abcam, Cambridge, UK), conversion of a colorless probe to a colored one, by glutamate or pyruvate was measured at 450 nm or 570 nm respectively.

Measurement of mitochondrial function

Intracellular ATP content (Abcam, Cambridge, UK) was measured through phosphorylation of glycerol into a colored product using a microplate reader at 570 nm. Assessment of the mitochondrial membrane potential (ab113850) is based on the conversion of monomeric tetraethylbenzimidazolylcarbocyanine iodide (JC-1; emits green fluorescence at 530 nm) to J-aggregates (emits red fluorescence at 590 nm). Succinate level was measured at an absorbance of 450 nm using a microplate reader.

Western blot analysis

The Western blot analysis was performed by homogenizing the HepaRG cells in a radioimmunoprecipitation assay buffer using an electric homogenizer. The homogenate was centrifuged for 20 minutes at 16,000xg. The cellular protein contents were separated using the protein extraction kit (Cat# P0028; Beyotime Institute of Biotechnology). Proteins were separated on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis and incubated overnight at 4°C with the specific primary antibodies (Abcam, Cambridge, MA, USA): anti-SIRT1, anti-Nrf2, anti-HO-1, and anti-γ-GCLC antibodies. Horseradish peroxidase-labeled with appropriate secondary antibody (Santa Cruz, CA, USA) was incubated for 1 hour at 37°C with the primary antibodies and then protein expressions were quantified with the help of ECL.

Statistical analysis

Statistical data analysis was done using the software package, SPSS (V13.0; SPSS, Inc.) by one-way ANOVA by applying Tukey's posthoc test for comparing diverse groups. $P<0.05$ was the significance level considered.

3 Results and Discussions

3.1. Results
Effect of ESH on AZA-provoked oxidative stress and hepatocellular viability

Dysregulated cell viability is an eventful repercussion of oxidative stress-caused intracellular damage. ROS is the pandora's box of noxious entities formed due to the aberrations in a mitochondrial respiratory mechanism caused by provoked by AZA. Investigation of hepatocyte viability by using an MTT assay showed that AZA, at a concentration of 250µmol/L (but not at lower concentrations), significantly ($P<0.05$) reduced the cell viability at 24 h exposure as shown in Figure 1. Intracellular ROS levels include deleterious oxidant species such as superoxide anion and hydroxyl radical. We observed that exposure of the

hepatocytes to AZA elevated the ROS levels and increased lipid peroxidation (manifested by the formation of malondialdehyde [MDA]). Besides, a concomitant decrease in GSH was observed due to the metabolic reaction of AZA with GSH. However, pretreatment of the hepatocytes with ESH before the AZA challenge significantly ($P<0.05$) reduced the GSH depletion, ROS, and MDA levels, and upheld the cell viability against AZA-challenge (Figure 1).

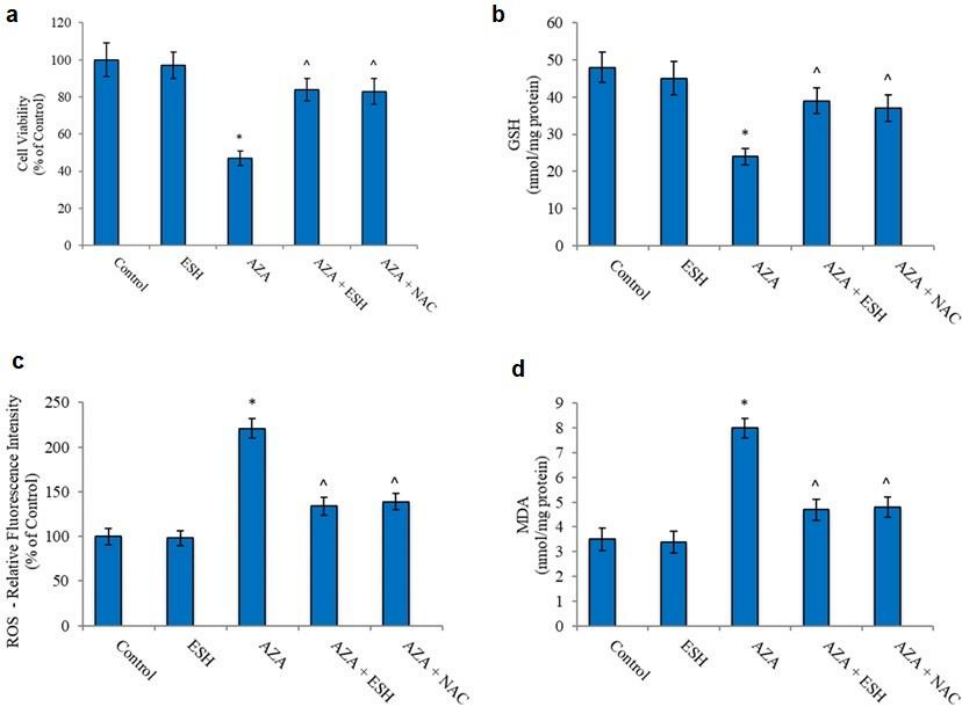

Figure 1. ESH treatment outcome in terms of the cell viability and oxidative stress parameters against AZA-provoked hepatocellular toxicity. A) Cell viability (% of control) after various treatments. B) GSH levels in various treatments. C) ROS levels in various treatments. D) MDA levels in various treatments. $^{*}P < 0.05$ (AZA vs control); $^{\wedge}P < 0.05$ (AZA+ESH vs AZA); $^{\wedge}P < 0.05$ (AZA+NAC vs AZA).

Effect of ESH and AZA on the xenobiotic metabolizing enzymes

Metabolism of AZA involves XO-mediated ROS production and GST-M1-mediated GSH depletion. Hence, suppression of the activities of these xenobiotic metabolizing enzymes alleviates the hepatocellular toxicity of AZA. In this study, we observed that exposure of the hepatocytes to AZA significantly ($P<0.05$) upregulated the expression of XO and GST-M1, while pre-treatment of the hepatocytes with ESH before the AZA challenge remarkably ($P<0.05$) downregulated the expression of XO and GST-M1 (Figure 2). This explains the reduced ROS and replenished GSH levels in the ESH treated hepatocytes.

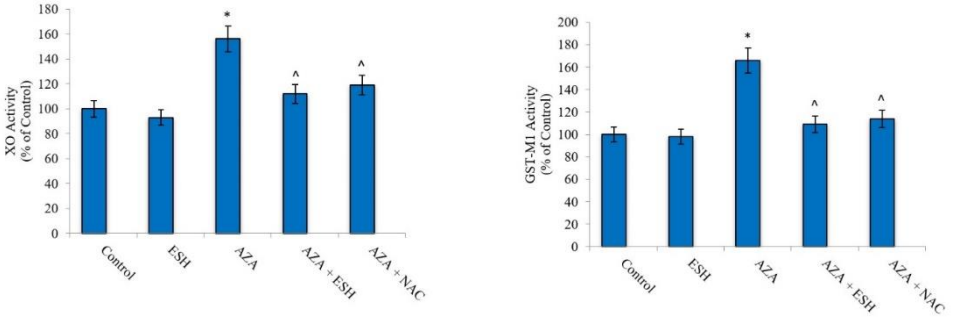

Figure 2. ESH treatment outcome in terms of AZA metabolizing enzymes. A) XO levels in various treatments. B) GST-M1 levels in various treatments. $^{*}P < 0.05$ (AZA vs control); $^{\wedge}P < 0.05$ (AZA+ESH vs AZA); $^{\wedge}P < 0.05$ (AZA+NAC vs AZA).

Effect of ESH on AZA-induced changes in hepatocyte membrane integrity

Elevated levels of transaminases (i.e., AST and ALT) are the pathognomonic markers of disrupted hepatocyte membrane integrity. AZA-induced oxidative stress has significantly (P<0.05) increased the plasma membrane permeability and led to a significant (P<0.05) increase in the release of AST and ALT (2.2-fold and 1.9-fold respectively) into the hepatocyte culture medium (Figure 3). On the contrary, ESH pretreatment preserved the hepatocellular membrane integrity and reduced the leakage of AST and ALT into the extracellular milieu.

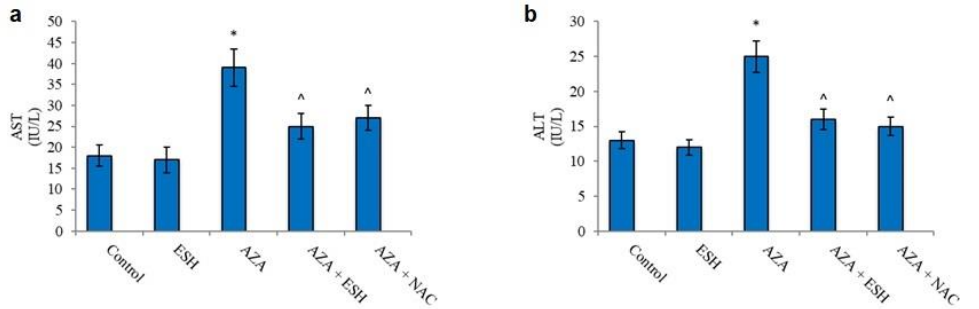

Figure 3. ESH treatment outcome in terms of the cell membrane integrity parameters against AZA-provoked hepatocellular toxicity. A) AST levels in various treatments. B) ALT levels in various treatments. $^{*}P < 0.05$ (AZA vs control); $^{\wedge}P < 0.05$ (AZA+ESH vs AZA); $^{\wedge}P < 0.05$ (AZA+NAC vs AZA).

Effect of ESH on the mitochondrial injury provoked by AZA

AZA provokes mitogen-activated protein kinase (MAPK)-mediated necrotic death of hepatocyte due to oxidative mitochondrial dysfunction—manifested as ATP depletion-mediated deregulation of cellular bioenergetics, disruption of intracellular ion homeostasis, and opening of mitochondrial membrane permeability pore (Lee ey al. 2001; Menor et al. 2004). In our study, we observed that AZA exposure significantly (P<0.05) decreased the ATP level (to 57% vs. control) and Δψm (54% reduction vs. control). Besides, AZA-induced mitochondrial dysfunction is associated with a significant (P<0.05) elevation (≈

2.4-fold) of succinate (Figure 4). Nevertheless, ESH pretreatment prevented these deregulatory changes caused by AZA exposure and preserved the mitochondrial integrity.

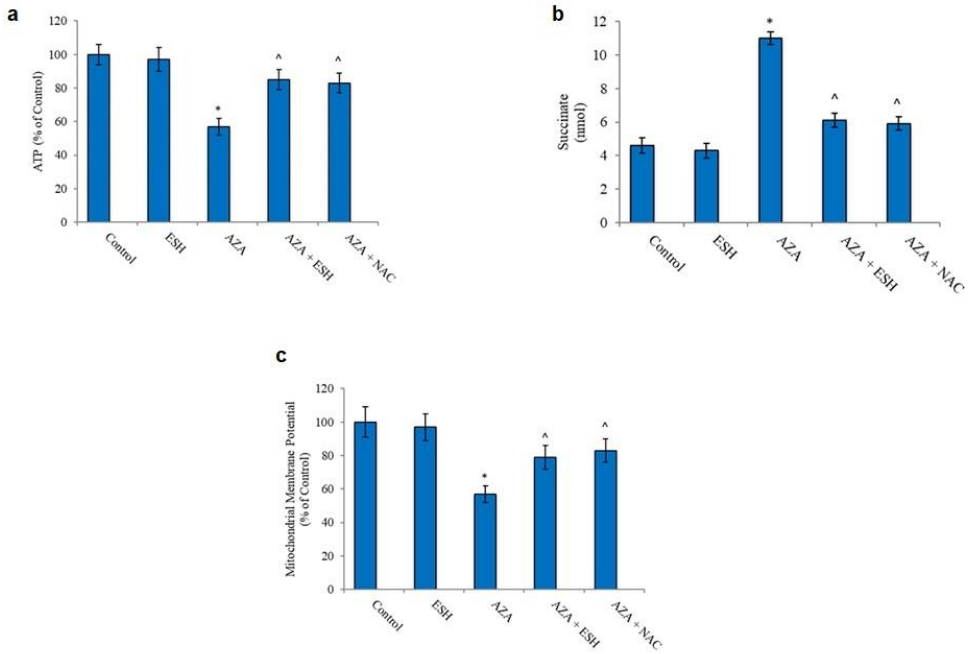

Figure 4. ESH treatment outcome in terms of the mitochondrial function parameters against AZA-provoked hepatocellular toxicity. A) ATP levels in various treatments. B) Succinate levels in various treatments. C) MMP in various treatments. $^{*}P < 0.05$ (AZA vs control); $^{\wedge}P < 0.05$ (AZA+ESH vs AZA); $^{\wedge}P < 0.05$ (AZA+NAC vs AZA).

Effect of ESH and AZA on SIRT1-mediated antioxidant signaling mechanism

SIRT1 exhibits a multi-faceted role in the regulation of cellular stress adaption and longevity. Activation of the SIRT1/Nrf2 antioxidant signaling pathway stabilizes the hepatocytes against oxidative assault. AZA exposure predisposed the hepatocytes to oxidative injury and downregulated the protein expression of SIRT1 (43% reduction vs. control) and also Nrf2 (37% reduction vs. control) and their downstream effectors: HO-1 (50% reduction vs. control) and γ-GCLC (26% reduction vs. control). Interestingly, ESH pretreatment effectively thwarted these devastating effects of AZA (Figure 5).

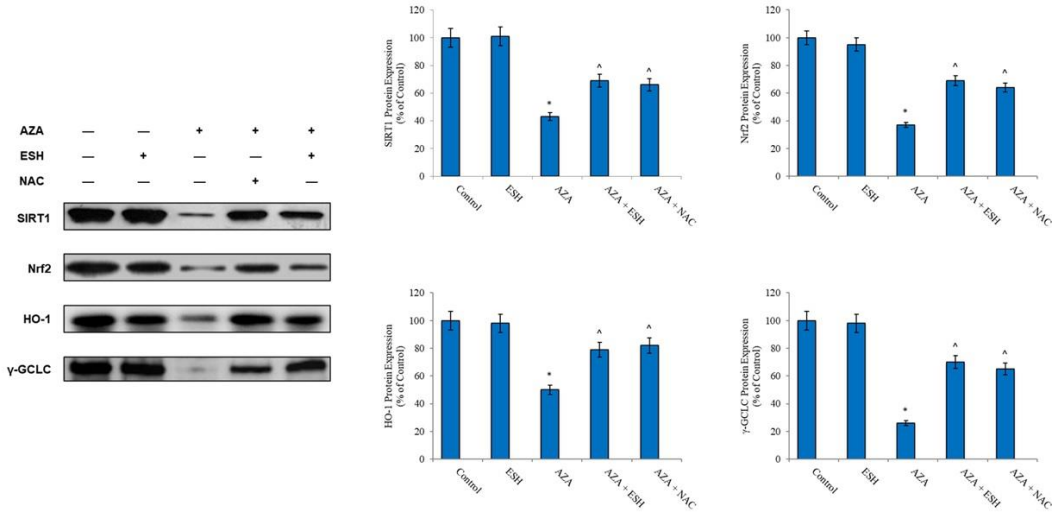

Figure 5. ESH treatment outcome in terms of the protein expression of the SIRT1-Nrf2 signaling pathway proteins against AZA-provoked hepatocellular toxicity. A) Indicative western blot images of SIRT1, Nrf2, HO-1 and γ-GCLC expressions in various treatments. B) Relative SIRT1, Nrf2, HO-1 and γ-GCLC expression levels in each treatment. $^{*}P < 0.05$ (AZA vs control); $^{\wedge}P < 0.05$ (AZA+ESH vs AZA); $^{\wedge}P < 0.05$ (AZA+NAC vs AZA).

3.2 Discussion

Azathioprine has been reported to cause four patterns of hepatotoxicity including transient increase in serum aminotransferase enzymes (AST and ALT), idiosyncratic, cholestatic hepatitis, sinusoidal obstruction syndrome, and nodular regenerative hyperplasia (Björnsson et al. 2017). Earlier studies from our lab and other institutes underscored that AZA-provoked oxidative stress plays a pivotal role in the inflammatory trigger, mitochondrial, and nuclear dysfunctions—ultimately culminating in necrotic hepatocyte death (Lee et al. 2001; Shanmugarajan et al. 2009; Matsuo et al. 2014). Induction of oxidative stress by AZA metabolism entails two key pathways: glutathione S-transferase- (GST-) catalyzed depletion of reduced glutathione (GSH) and xanthine oxidase- (XO-) catalyzed generation of reactive oxygen species (ROS) (Matsuo et al. 2014; Maruf et al. 2014; Shanmugarajan et al. 2009). Diminished endogenous GSH and increased oxidant levels perturb the hepatocellular redox homeostasis and trigger the pathological signaling mechanisms leading to liver cell death.

Xenobiotic metabolizing enzymes (XO and GST-M1) involved in the biotransformation of AZA are responsible for the ROS production and GSH depletion. Treatment with ESH effectively reduced intracellular ROS production and replenished GSH levels in the ESH treated hepatocytes. HepaRG cells serve as a unique liver cell model due to the presence of a repertoire of metabolizing enzymes that almost match the metabolic functions of human hepatocytes. In this study, we observed that AZA-treatment reduced the hepatocellular GSH level and amplified the level of malondialdehyde (MDA), a biomarker of lipid peroxidation. However, ESH treatment improved the antioxidant status

(manifested as increased GSH level) and mitigated the oxidant stress (manifested as increased ROS level and decreased MDA level). This outcome is in harmony with other studies which showed that ESH upregulated GSH (Hseu et al. 2020) and also, ESH exhibits potent antioxidant capacity, which is superior to the effects of the classic antioxidant entities such as GSH, Trolox, and uric acid (Franzoni et al. 2006). In addition to the altered redox balance, AZA exposure hampered the viability of hepatocytes, and release the intra-hepatocellular enzymes (AST and ALT) into the cell culture medium. However, we observed that ESH preserved the membrane integrity of hepatocytes and reduced the leakage of these enzymes. These roles of ESH might be orchestrated through its adaptive antioxidant and cytoprotective properties (Matsuo et al. 2014).

Mitochondria are the pivotal cellular organelles that generate intracellular ROS during cellular respiration. But, exposure of hepatocytes to AZA provokes aberrant production of mitochondrial ROS and increased mitochondrial membrane permeability (Lee et al. 2001). The cytoplasmic shuttling of these excessive, noxious entities leads to hepatocellular injury and cell death. In our study, we observed that AZA-challenge has altered the mitochondrial membrane potential ($\Delta\psi m$) in the hepatocytes and has led to ATP depletion and release of succinate in the culture medium of hepatocytes. Disrupted tricarboxylic acid (TCA) cycle due to ROS accumulation and increased mitochondrial membrane permeability might be the reason underpinning the rise in succinate in the extracellular milieu. Nonetheless, ESH treatment restored $\Delta\psi m$, countered the ATP depletion, and mitigated the release of succinate. This indicates that ESH preserves mitochondrial integrity of the hepatocytes and offers cytoprotection against AZA. In congruence, Paul and Snyder showed that ESH is more localized in the mitochondrial region and offers substantial defense against mitochondrial oxidative stress (Paul et al.2010).

Ample studies emphasized that nuclear factor-erythroid factor 2-related factor 2 (Nrf2) is a central factor in the regulation of redox homeostasis during exogenous oxidative stress including AZA-induced hepatocellular toxicity (Schaalan et al. 2018). Consistence with this study, our observation also indicated that AZA downregulated the expression of Nrf2, while ESH treatment prevented the decrease in Nrf2 protein expression. Plausibly, the cytoprotective effect of ESH is mediated through the activation and nuclear shuttling of cytoplasmic Nrf2 and the subsequent induction of antioxidant entities including heme oxygenase-1 (HO-1) and gamma-glutamyl-cysteine ligase catalytic subunit (γ-GCLC) (Hseu et al. 2015; Hseu et al. 2020). HO-1 is a pivotal antioxidant and cytoprotective enzyme, while γ-GCLC is a key enzyme in the endogenous production of GSH. Akai et al reported that knockdown of γ-GCLC causes hepatic GSH depletion and increases the vulnerability of hepatocytes to drug-induced toxicity (Akai et al. 2007). In this line, our study showed that protein expressions of HO-1 and γ-GCLC in the AZA-challenged hepatocytes were downregulated, while ESH treated hepatocytes upheld the expression of these proteins.

A recent study revealed that positive modulation of SIRT1—one of the epigenetic determinants of longevity with a well-established role in hepatoprotection—induces Nrf2-mediated antioxidant genes (Farghali et al. 2019; Wang et al. 2020; Higgins et al. 2022). In consistence with this evidence, ESH treatment

upregulated SIRT1 as well as SIRT1-mediated expression of Nrf2 and its downstream targets (HO-1 and γ-GCLC) against AZA-induced hepatocellular toxicity.

4 Conclusion

To summarize, our study throws light on the plausible mechanisms (Figure 6) underlying the beneficial effects of ESH in an in vitro model of AZA-induced hepatocellular toxicity. Accordingly, ESH exhibits its hepatocellular protective effects through its antioxidant, mitochondrial stabilization, and SIRT1-upregulation mechanisms. Based on the triage theory, Ames proposed that ESH might be regarded as a "longevity vitamin" due to its multifaceted role against oxidative stress and in healthy aging (Ames et al. 2018). This proposal emphasizes that ESH might have both cytoprotective and longevity-enhancing properties. However, further, in vivo preclinical and clinical studies are highly warranted to consider the use of ESH to uphold human health and manage various diseases.

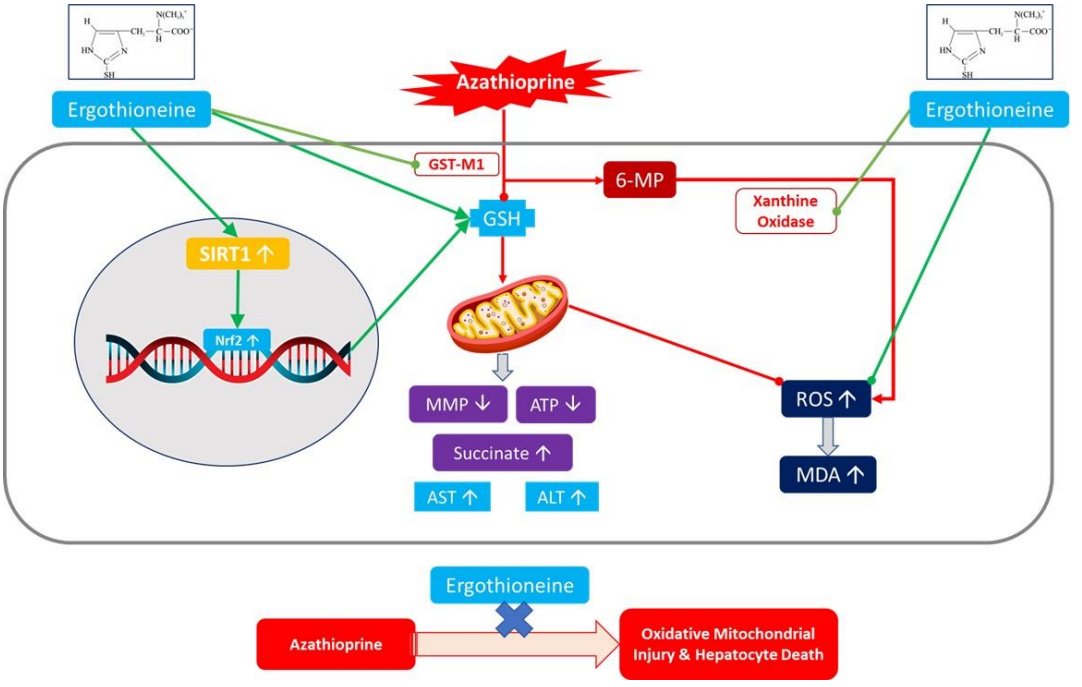

Figure 6. AZA causes GSH depletion during its metabolic conversion into 6-MP and other metabolites. ESH exhibits its hepatocellular protective effects through its antioxidant (manifested as mitigated GSH depletion), mitochondrial stabilization (ATP, succinate, and mitochondrial membrane potential enhancement), and SIRT1-mediated antioxidant gene (Nrf2, HO1, and γ-GCLC) upregulation effects.

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Disclosure statement
The authors report no declarations of interest.

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