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# L-Ergothioneine protects hepatocytes against azathioprine-Induced toxicity via NIK/NF-KB signaling axis

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**Abstract**--This research study has major implications for the development of ESH as a safe and holistic hepatoprotectant and could be used as an adjuvant in the treatment of IBD to minimize DILI. We demonstrate that ESH regulates oxidative stress, NIK/NF-κB-mediated inflammatory axis, and improves the hepatocellular integrity against AZA-provoked deleterious events in the hepatocytes (Figure 5). However, further research is warranted to elucidate the efficacy, specificity, and safety of ESH in AZA-treated patients with normal genetic makeup and gene polymorphisms.

**Keywords**---Azathioprine-induced, L-Ergothioneine, Hepatocytes.

**Introduction**

Azathioprine (AZA), an antimetabolite, is a first-line immunosuppressive agent used in the treatment of various autoimmune diseases including inflammatory bowel disease (IBD) (van Liere et al. 2021). However, its therapeutic usage has been limited due to its dose-dependent adverse effects like hepatotoxicity, gastric intolerance, and bone marrow toxicity (Ana Casajús et al. 2022). Especially these adverse effects are more pronounced in thiopurine methyltransferase (TPMT) intermediate metabolizers (IMs) (Ana Casajús et al. 2022). Drug withdrawal is required in up to 9% of the IBD patients due to hepatotoxicity concerns (Labidi et al. 2020). Hence, it is immensely essential to gauge the dose of AZA as well as to co-treat the patients with an appropriate hepatoprotective drug to mitigate the noxious effects of AZA (van Liere et al. 2021). Numerous studies in human and

animal models underscored that treatment with antioxidants (e.g., allopurinol, gervital, N-acetyl-L-cysteine, etc.) is highly beneficial in the alleviation of hepatotoxic manifestations of AZA. (Menor et al. 2004; van Liere et al. 2021; Abdul-Hamid et al. 2022). In stark contrast, a recent report by Madrazo et al. (2021) indicated that allopurinol potentially enhances myelotoxicity (manifested as megaloblastic anemia and leukopenia). Hence, the hunt for more appropriate hepatoprotective agents is pushing the horizons of drug discovery in the context of cytoprotection against AZA-provoked toxicity.

Various deregulated molecular mechanisms are involved in the hepatotoxic effects of AZA. A comprehensive “OMICs”-based study revealed metabolic oxidative stress, inflammatory activation, aberrations in the bile acid/ fatty acid metabolism, and dysfunctional contractile proteins (Cho et al. 2014). Another seminal study demonstrated that inhibition of stress-activated protein kinases (c-Jun N-terminal kinase and p38 kinase) and amelioration of mitochondrial dysfunction negates the necrotic cell death process activated by AZA (Menor et al. 2004). Hence, targeting multiple pathophysiological mechanisms using a holistic and safe hepatoprotective agent is highly imperative to combat the hepatotoxic repercussions of AZA.

Ergothioneine (ESH) is a unique naturally occurring thiol-amino acid with an excellent safety profile in terms of no apparent cytotoxic, mutagenic, or carcinogenic effects (Marone et al. 2016). A study by Dare et al. showed that ESH proffers hepatoprotection through mitigation of oxidative stress, inflammatory activation, and dyslipidemia in a diabetic model. Fascinatingly, ESH has shown anti-inflammatory effects in ulcerative colitis via inhibition of TLR4/MyD88/NF- $\kappa$ B signaling axis (Pan et al. 2022). Based on this evidence, we surmised that ESH might be a suitable therapeutic agent which not only alleviates the hepatotoxic effects of AZA but also, complements its effect in the treatment of IBD.

## **2 Materials and Methods**

### *Cell culture and treatment*

After culturing the undifferentiated HepaRG cells (Biopredic International, Saint-Grégoire, France) according to the manufacturer's directions, seeding was done using William's E medium supplemented with 10% fetal bovine serum in 15% CO<sub>2</sub> at 37°C for two weeks. Differentiation of the HepaRG cells was then 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 (250  $\mu$ mol/L) AZA + ESH (ESH; 0.5  $\mu$ mol/L) or N-acetylcysteine (NAC; 0.5 mmol/L) groups.

### *Measurement of percentage DNA fragmentation*

Extraction of the DNA material from the hepatocytes was done as recommended by the method of Collins et al. (1997) by using a diphenylamine reagent in the investigation using agarose gel electrophoresis. Homogenized, centrifuged, and precipitated DNA fragments were obtained by addition of 1 ml Triton-X buffer

(pH;7.4), followed by dynamic vortexing to permit the external delivery of fragmented DNA. The released DNA was hydrolyzed with the help of 160  $\mu$ l of 5% trichloroacetic acid (TCA) and heating for a few minutes at 90°C. Colorimetric quantitation was done by employing diphenylamine (DPA)-based staining of the fragmented DNA using wave length 600 nm against blank reagent and the output was indicated as % fragmented DNA.

#### *Measurement of biochemical parameters*

8-hydroxy-2-deoxy guanosine (8-OHdG), an oxidative DNA injury biomarker was measured in the lysate obtained from HepaRG cells. The measurement was done by using ELISA technique using HRP-conjugated antibody for precise detection of both free 8-OHdG and DNA-incorporated 8-OHdG in the sample. Similarly, antibodies corresponding to the inflammatory mediators (TNF- $\alpha$  and IL-6) as well as the hepatic cell damage marker (FABP1) were used for the assays.

#### *Western blot analysis*

For the Western blot investigation, the HepaRG cells were homogenized in a radioimmunoprecipitation assay buffer using an electric homogenizer. For the separation of cellular protein contents, the cell homogenate was then allowed to centrifuge 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-SOD1, anti-SOD2, anti-NIK, and anti- NF- $\kappa$ B antibodies. Horseradish peroxidase-labeled with appropriate secondary antibody (Santa Cruz, CA, USA) was incubated for 1 hour at 37°C with the primary antibodies. Finally, the bands of protein expressions were quantified by using ECL technique.

#### *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.

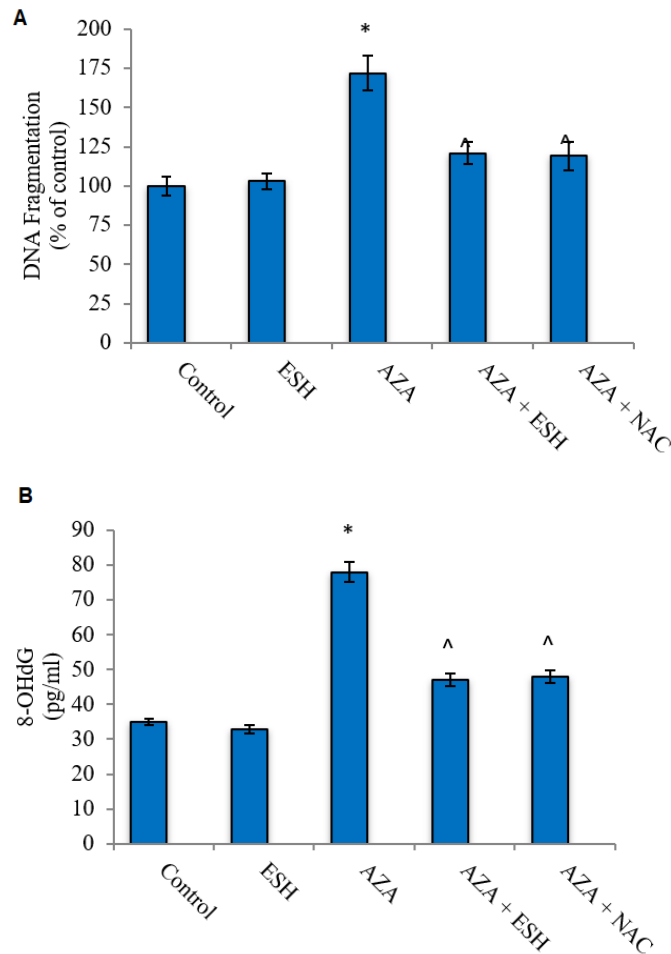
### **3 Results and Discussions**

#### *3.1. Results*

##### *Effect of ESH and AZA on the hepatocellular DNA*

AZA metabolism predisposes the hepatocytes to the oxidative stress load which in turn induces increased oxidative DNA damage. In our study, oxidative stress significantly (P<0.05) increased the percentage of DNA fragmentation in the AZA exposed cells, while ESH-treated cells effectively (P<0.05) countered the oxidative stress and associated DNA fragmentation (Figure 1). Besides, AZA-challenged cells produced significantly (P<0.05) more 8-OHdG entities, indicating a high amount of oxidative outburst in the hepatocytes. Nevertheless, ESH-treated cells

depicted normalcy and showed significantly ( $P < 0.05$ ) lower 8-OHdG levels, when compared to the AZA-challenged cells (Figure 1).



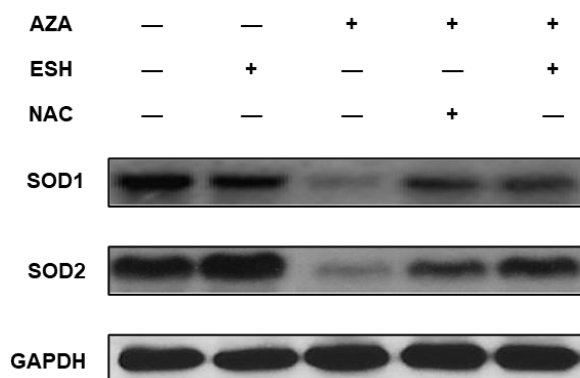
**Figure 1.** ESH treatment outcome in terms of DNA damage against AZA-provoked hepatocellular toxicity. A) DNA fragmentation (% of control) after various treatments. B) 8-OHdG levels in various treatments. \* $P < 0.05$  (AZA vs control); ^ $P < 0.05$  (AZA+ESH vs AZA); ^ $P < 0.05$  (AZA+NAC vs AZA).

#### *Effect of ESH and AZA on the antioxidant content*

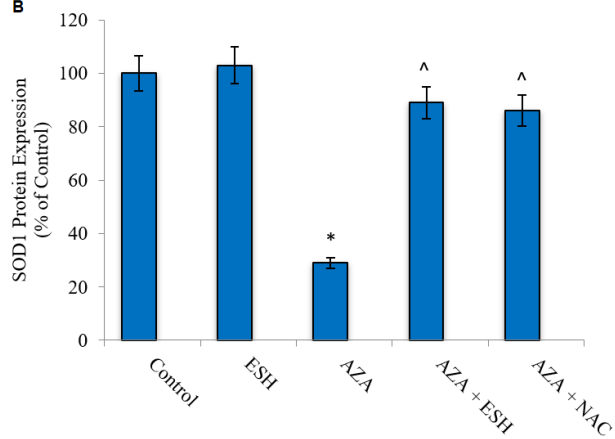
Two key antioxidant forms, SOD1 (cytosolic) and SOD2 (mitochondrial) counterparts were assessed in this study. We found that AZA-challenged hepatocytes significantly ( $P < 0.05$ ) suppressed the expression of both SODs. This underscores the failure of the cellular endogenous antioxidant defense mechanism at least in terms of the superoxide anion. On the contrary, ESH-treated cells showed significantly ( $P < 0.05$ ) replenished antioxidant status in terms

of SOD1 and SOD2, which is a clear signal of effective antioxidant defense support inside the hepatocyte (Figure 2).

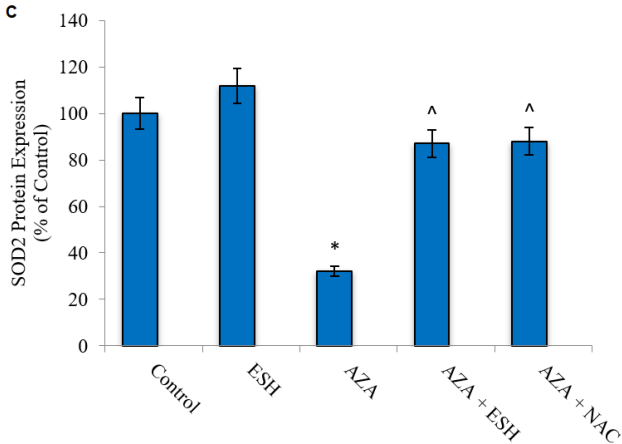
**A**



**B**



**C**

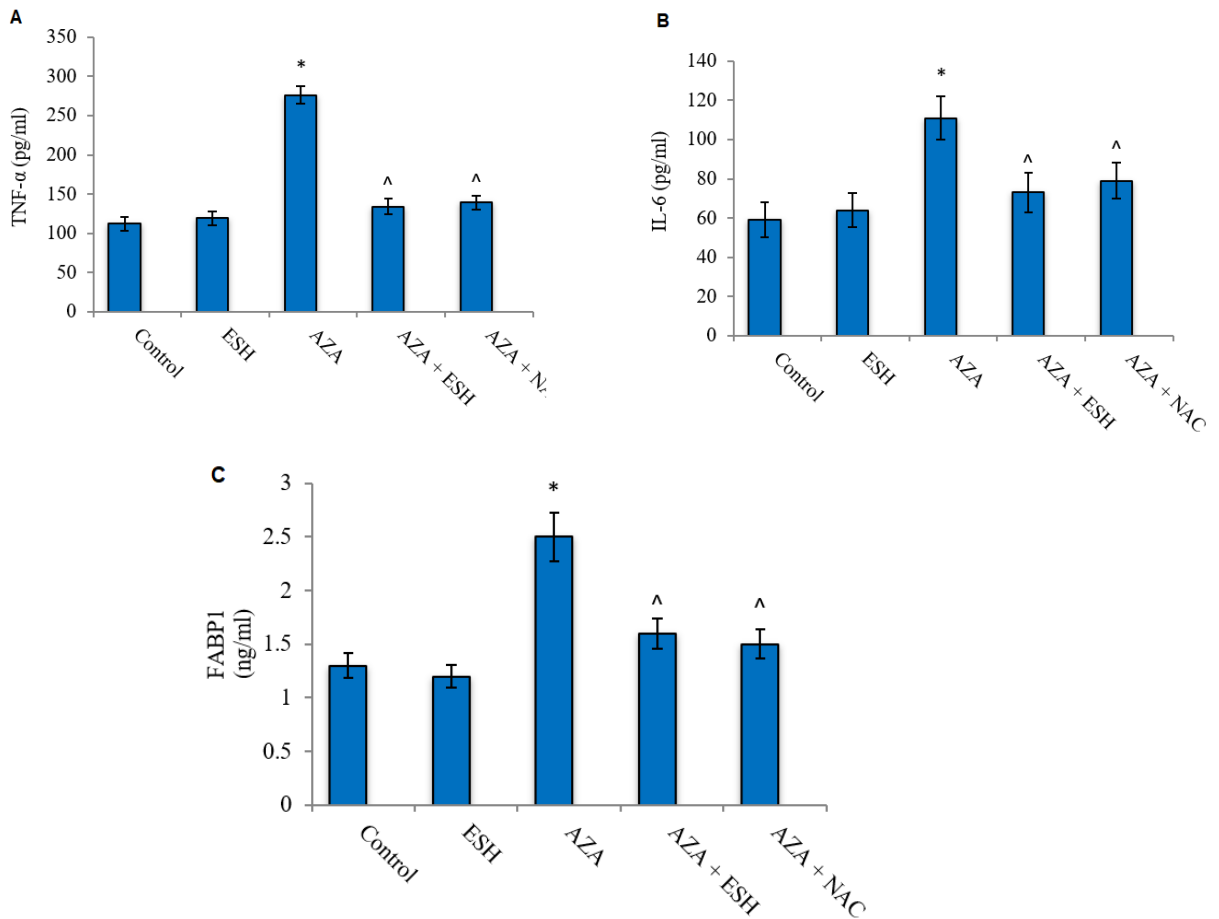


**Figure 2.** ESH treatment outcome in terms of the protein expression of antioxidants against AZA-provoked hepatocellular toxicity. A) Indicative western

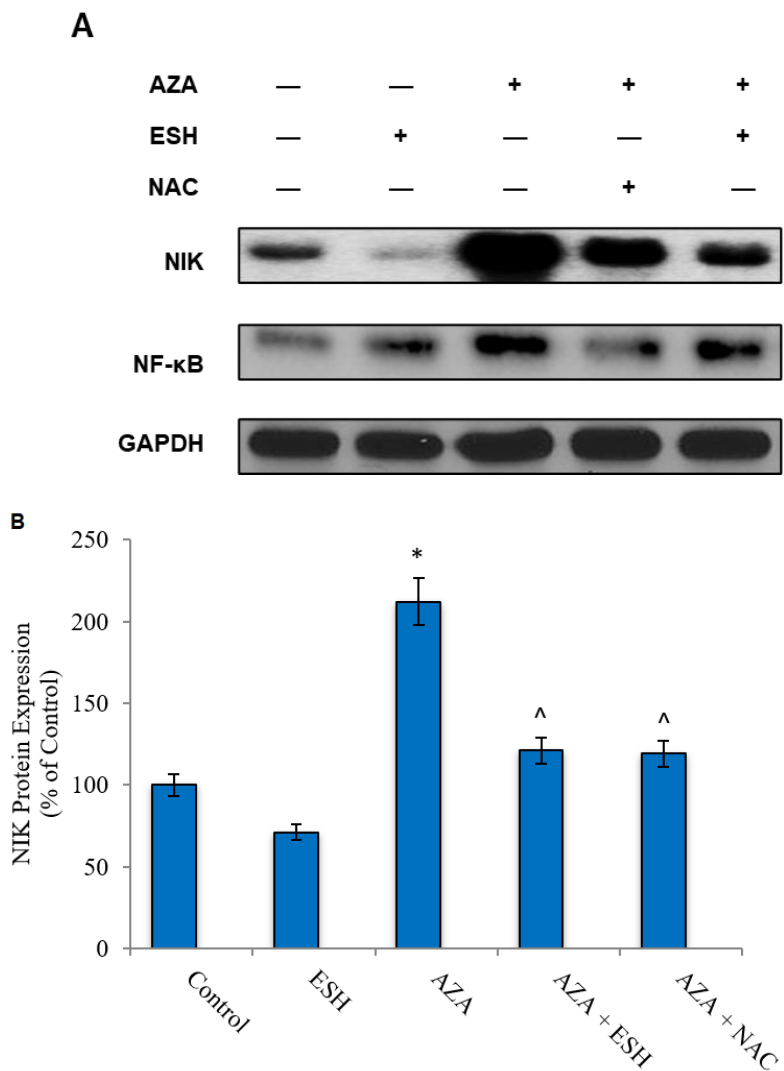
blot images of SOD1 and SOD2 expressions in various treatments. B-C) Relative SOD1 and SOD2 expression levels in each treatment. \* $P < 0.05$  (AZA vs control); ^ $P < 0.05$  (AZA+ESH vs AZA); ^ $P < 0.05$  (AZA+NAC vs AZA).

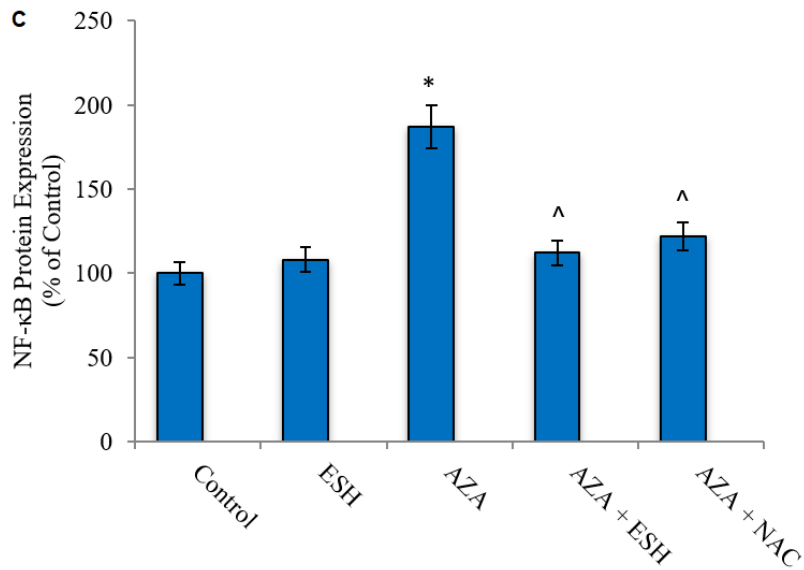
### Effect of ESH and AZA on the inflammatory status and hepatocellular integrity

Oxidative stress leads to inflammatory reactions, DNA damage, and cell death. In this study, AZA-induced oxidative-inflammatory stress activated the NIK/NF- $\kappa$ B signaling axis. This is manifested as significantly ( $P < 0.05$ ) upregulated protein levels of NIK and NF- $\kappa$ B, and significantly ( $P < 0.05$ ) elevated the levels of pro-inflammatory markers like TNF- $\alpha$  and IL-6 (Figure 3). However, these inflammatory deregulatory processes were effectively thwarted by the treatment with ESH. Furthermore, AZA-exposure significantly ( $P < 0.05$ ) increased the levels of fatty acid-binding protein 1 (FABP1) (Figure 4). It should be noted that FABP1 is a superior and effective alternative biomarker of hepatocellular membrane damage when compared to ALT. In contrast, we found that, plausibly, through the reduced oxidative-inflammatory stress in ESH-treated cells, the FABP1 level was maintained at near normalcy which is an obvious event of hepatocellular protection by ESH.



**Figure 3.** ESH treatment outcome in terms of pro-inflammatory molecules (TNF- $\alpha$  and IL-6) and cell membrane integrity (FABP1) against AZA-provoked hepatocellular toxicity. A-B) TNF- $\alpha$  and IL-6 levels in various treatments. C) FABP1 levels in various treatments. \* $P < 0.05$  (AZA vs control); ^ $P < 0.05$  (AZA+ESH vs AZA); ^ $P < 0.05$  (AZA+NAC vs AZA).





**Figure 4.** ESH treatment outcome in terms of the protein expression of the NIK/NF- $\kappa$ B signaling molecules and antioxidants against AZA-provoked hepatocellular toxicity. A) Indicative western blot images of NIK and NF- $\kappa$ B expressions in various treatments. B-C) Relative NIK and NF- $\kappa$ B expression levels in each treatment. \* $P < 0.05$  (AZA vs control); ^ $P < 0.05$  (AZA+ESH vs AZA); ^ $P < 0.05$  (AZA+NAC vs AZA).

## Discussion

Elevated oxidative stress coupled with an inflammatory outburst, orchestrated through GSH and xanthine oxidase-mediated reactions, is a pivotal pathological mechanism underlying AZA-provoked hepatocellular toxicity (Menor et al. 2004; Schaalán et al. 2018; van Liere et al. 2021; Abdul-Hamid et al. 2022). In our earlier study, it has been demonstrated that oxidative stress-mediated DNA fragmentation is one of the pivotal mechanisms underpinning hepatocyte cell death (Shanmugarajan et al. 2008). In this line, oxidative injury to the DNA of AZA-challenged hepatocytes was confirmed in our study through high-degree of DNA fragmentation and increased 8-hydroxy-2'-deoxyguanosine (8-OHdG), a biomarker of oxidative DNA assault in the cells. Another study led by Schaalán et al. (2018) has also demonstrated similar DNA fragmentation due to AZA challenge by using a testicular injury model. Further, the utility of 8-OHdG as an appropriate oxidized DNA biomarker is proven through its development as a point-of-care device for rapid and effective quantification of the oxidative DNA damage in cells (Martins et al. 2017). However, we observed that ESH effectively ameliorated these DNA abnormalities against AZA-provoked DNA injury. This outcome is in congruence with an earlier human study, wherein oral ESH administration effectively mitigated the oxidant overload through its antioxidant and anti-inflammatory properties (Cheah et al. 2017).

In our study, elevated oxidant burden in AZA-exposed hepatocytes diminished the intracellular antioxidant defense repertoire which is marked by an abnormal

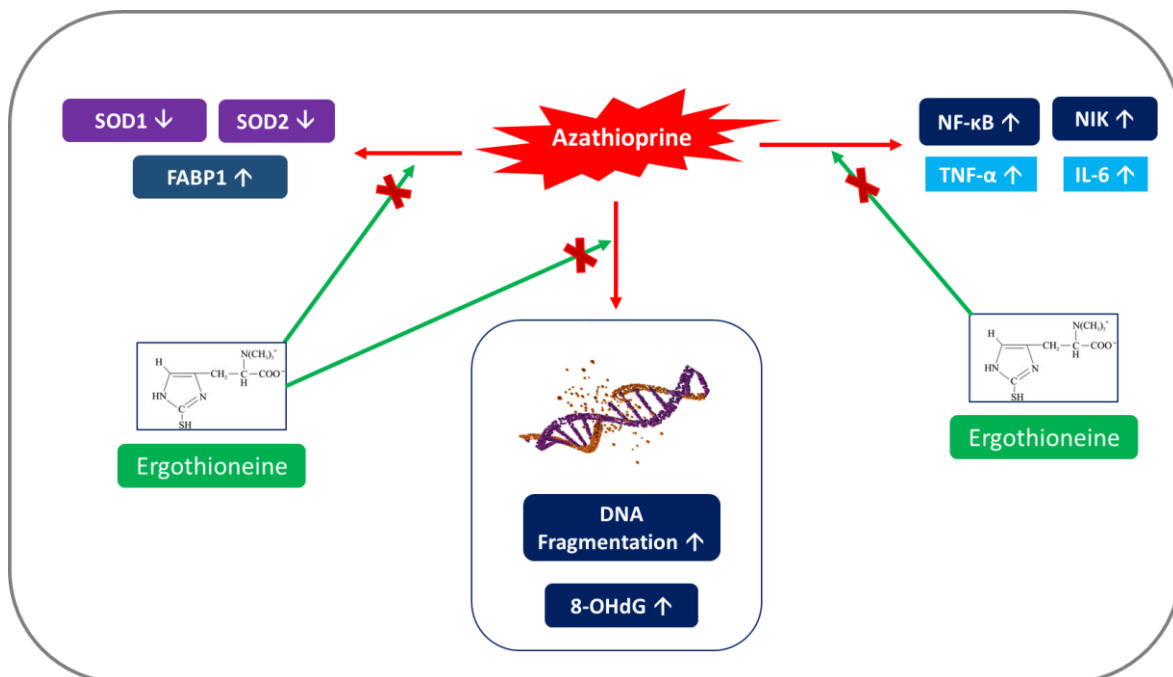
reduction in the levels of Cu,Zn-superoxide dismutase (SOD1), and manganese SOD (SOD2), two prominent forms of SOD combating oxidants to shield cytosol and mitochondria respectively. Polymorphisms in the SOD genes, toxin exposure, and drug-induced liver injury (DILI) are having robust causal associations with the altered levels of SOD (Sukkasem et al. 2020; Nikam et al. 2018; Kim et al. 2015; Liu et al. 2015). These studies conducted in the *in vitro* and *in vivo* preclinical and clinical models advocate the pivotal role of SOD in neutralizing the oxidant activity of superoxide anion in distinct subcellular compartments. Interestingly, we observed that both SOD1 and SOD2 were upregulated in the ESH-treated hepatocytes against AZA. The SOD upregulating effect of ESH has been document already (Mishra et al. 2018).

NF- $\kappa$ B-inducing kinase (NIK), a serine/threonine-based mitogen-activated protein kinase kinase kinase 14, is a kinase is the regulatory hub of non-canonical NF- $\kappa$ B signaling activation in various pathological conditions (Kucharzewska et al. 2019; Zhong et al. 2021). Stimulation of NIK activates NF- $\kappa$ B signaling via activation of inhibitor of  $\kappa$ B (IkB) kinase- $\alpha$  (IKK $\alpha$ ). In harmony with this evidence, we observed that AZA-challenged hepatic cells aberrantly expressed increased levels of NIK and NF- $\kappa$ B. Activation of NF- $\kappa$ B is an established target that kicks off the inflammatory cascade thereby increasing the levels of TNF- $\alpha$  and IL-6. We observed that activation of NIK/NF- $\kappa$ B signaling axis and increased levels of pro-inflammatory markers like TNF- $\alpha$  and IL-6 were effectively countered by the anti-inflammatory action of ESH. The positive effects of anti-inflammatory and antioxidant activities against AZA-induced deleterious reactions has been previously demonstrated by other researchers using green tea polyphenols and taurine-chloramine (El-Beshbishy et al. 2011; Schaalán et al. 2018).

Fatty acid-binding protein 1 (FABP1), a 14-kDa protein, has been shown to be a superior biomarker over alanine aminotransferase (ALT) in drug-induced liver injury (Mikus et al. 2017). We found that this meaningful liver injury biomarker (FABP1) level was abnormally elevated in the culture fluid due to AZA-induced hepatocellular membrane damage and extracellular leakage of FABP1. However, maintenance of oxidant-antioxidant balance and regulation of NIK/NF- $\kappa$ B-mediated inflammatory axis by ESH preserved the hepatocellular integrity and reduced the leakage of FABP1.

## **Conclusion**

This research study has major implications for the development of ESH as a safe and holistic hepatoprotectant and could be used as an adjuvant in the treatment of IBD to minimize DILI. We demonstrate that ESH regulates oxidative stress, NIK/NF- $\kappa$ B-mediated inflammatory axis, and improves the hepatocellular integrity against AZA-provoked deleterious events in the hepatocytes (Figure 5). However, further research is warranted to elucidate the efficacy, specificity, and safety of ESH in AZA-treated patients with normal genetic makeup and gene polymorphisms.



**Figure 5.** AZA causes oxidative DNA damage (manifested as increased DNA fragmentation and 8-OHdG levels), and diminished antioxidant status, especially SOD1 and SOD2. Furthermore, activation of NIK/NF-κB signaling axis results in increased inflammation, marked by pro-inflammatory molecules (TNF-α and IL-6). Oxidative-inflammatory changes in the AZA-treated cells ensue in leaky membrane and release of FABP1 in the extracellular milieu — ultimately leading to hepatocellular death. On the other side, ESH treatment effectively ameliorates these abnormalities and preserved the hepatocellular integrity.

### Acknowledgements

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### Disclosure statement

The authors report no declarations of interest.

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