Indexed, Abstracted and Cited: ISRA Journal Impact Factor, International Impact Factor Services (IIFS), Directory of Research Journals Indexing (DRJI), International Institute of Organized Research and Scientific Indexing Services

World Journal of Biology and Medical Sciences

Published by Society for Advancement of Science®

ISSN 2349-0063 (Online/Electronic)

Volume 1, Issue- 4, 72-80, October-December, 2014

WJBMS 1/3/32/2014

A Double Blind Peer Reviewed Journal www.sasjournals.com wjbmedsc@gmail.com wjbms.lko@gmail.com

RESEARCH PAPER

Received: 21/08/2014 Revised: 25/10/2014 Accepted: 25/10/2014

Zinc Sulphate in Azathioprine Induced Hepatotoxicity- An Experimental Study

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ABSTRACT

To investigate azathioprine (AZA) induced hepatotoxicity and possible protective role of Zinc sulphate in rabbit model. Experimental/Analytical study. Animal House, Isra University Hyderabad from July 2010 to November 2011. Eighty rabbits were studied at animal house of Isra University according to inclusion and exclusion criteria. Group 1. Controls (n=20) Rabbits received 0.9% isotonic saline orally Group 2. (n=20) Azathioprine 15 mg/kg. Group 3. (n=20) Azathioprine 15 mg/kg body + Zinc sulphate 1mg/kg orally & Group 4. (n=20) Azathioprine 15 mg/kg body + Zinc sulphate 2 mg/kg orally for four weeks. Blood samples were collected from tail veins. The animals were sacrificed, liver tissue, after fixation in 4% formaldehyde, was embedded in paraffin. Tissue sections of 5µ thickness were subjected to haematoxylin and eosin staining and were assessed by light microscopy. The data was analyzed on Statistix 8.1 using one-way analysis of variance and post hoc test. A p-value of ≤ 0.05 was taken statistically significant. The liver biochemical and histological findings showed significant differences among the controls, AZA and AZA+ Zinc sulphate (p=0.001).

Liver enzymes and histology was deranged significantly in AZA group compared to controls and AZA+ Zinc sulphate (p=0.01).The AZA+ Zinc sulphate showed low rise of liver enzymes and derangement in liver histology when compared to AZA group (p=0.01). The AZA group showed nodular regenerative hyperplasia, veno-occlusinve disease; peliosis hepatis, sinusoidal dilatation, cholestasis, hepatocyte necrosis and perisinusoidal fibrosis. Derangement of hepatocyte cords, hydropic changes with congestion of central venules and sinusoids were observed.Azathioprine induces hepatocellular injury and deranges liver biochemical parameters and Zinc sulphate may be used as an effective protector.
Key words: Azathioprine, Zinc sulphate, Liver injury and Sindh.

INTRODUCTION

Zinc (Zn) is an essential nutrient for all forms of life and its importance lies in the fact that many body functions are linked to zinc containing enzymes (**Fleet et al., 2000)**. Zn as a trace element has indispensible role in human health and diseases. It has been insufficiently recognized by a number of experts as an important public health issue, especially in developing countries. It is the most abundant intracellular metal ion found in cytosol, vesicles, organelles and in the nucleus **(King et al., 2003).** However, even a small deficiency is a disaster to human health, so as such the number of biological functions, health implications and pharmacological targets that are emerging for zinc has evoked further interest regarding its status in human health and nutrition **(King et al., 2003) and (Devi et al., 2014).**

Zinc is an integral part of several metallo-enzyme including superoxide dismutase which is present in the cytosol of various cells of body including liver cells. Low serum level of zinc is observed in patients with liver cirrhosis (**Chatterjea and Rana 2012**). Zinc is an efficacious agent in acute ethanol-induced liver injury (**Zhong et al., 2013**). Antagonism of redox-active, transition metal-catalyzed, site-specific reactions has led to the theory that zinc may be capable of reducing cellular injury that might have a component of site-specific oxidative damage. Zinc has been proven to be capable of reducing post ischemic injury to a variety of tissues and organs through mechanisms that may involve the antagonism of copper reactivity (**Powel, 2014)**. Hepatoprotective effect of zinc has been reported against chlorpyrifos induced liver injury. The zinc administered animals showed the preserved histology of liver (**Goel et al., 2005).**

Azathioprine (AZA) is widely used drug in clinical practice for prevention of rejection in renal transplantation and in treating various autoimmune diseases, including refractory severe rheumatoid arthritis, systemic lupus erythematous, psoriasis and inflammatory bowel disease (**Mruf et al., 2014)**. It is also used in treatment of multiple sclerosis, myasthenia gravis and malignancies. Despite these advantages, their therapeutic potential is limited by occasional adverse effects on the liver and bone marrow (**Moustafa and Badria, 2010)** recent studies recorded AZA-treated patients of inflammatory bowel disease admitted to hospital with fatigue, icterus, hepatosplenomegaly and ascites. The whole blood count revealed a pancytopenia, hyperbilirubinemia and elevated transaminases (**Moustafa and Badria, 2010)** and (**Trabelsi et al., 2013)**.

The present study was designed to observe effects of azathioprine on liver biochemical parameters and histology and possible protective role of zinc sulphate in rabbit model at animal house of Isra University.

MATERIALS AND METHODS

Eighty rabbits were studied at animal house of Isra University from July 2010 to November 2011. Rabbits 1000-15000 grams were included while female rabbits and weight more or less as mentioned above were excluded from the study. The Animals were housed in animal house at an optimal room temperature with 55-60% humidity and exposed to 12 hour light-dark cycles. The chaw like fresh alfalfa and clean water are provided freely.

Group 1. (n=20) 0.9% isotonic saline orally on alternate day for four weeks and served as control group,

Group 2. (n=20) Azathioprine 15 mg/kg orally for four weeks,

Group 3. (n=20) Azathioprine 15 mg/kg body + Zinc sulphate 1mg/kg body orally for four weeks,

Group 4. (n=20) Azathioprine 15 mg/kg body + Zinc sulphate 5 mg/kg orally for four weeks.

Azathioprine (Imuran 50 mg, Glaxo Smitth Kline Pharma) and Zinc sulphate (Zincate) were purchased from Pharmacy of Isra University Hospital. At the end of experimental period, blood samples were collected from tail veins. Sera were separated by centrifugation at 300xs for ten minutes. Serum samples were used to determine liver enzymes. The animals were sacrificed by over-dose of Ketamine and Xylazil as described by **Nayak et al., 2006** and liver was removed promptly for histological study. Liver enzyme assays were determined for alanine trans aminase (ALT), aspartate transaminase (AST), alkaline phophatase (ALP) and lactate dehyderogenase (LDH) using commercially available diagnostic kits.

Each sample of liver obtained was washed in normal saline and tissues were fixed in previously marked containers, containing 10% formaldehyde as preservative. The tissues were embedded in paraffin, cut into 5 um thick sections and stained with Hematoxylin-Eosin (H & E) and Masson`s trichrome staining for histological examination. The histological criteria included vacuolar degeneration, inflammatory cell infiltrate, congestion and necrosis. The histological parameters were graded as follows; 0 = no abnormal findings, + = mild injury, ++ = moderate injury and +++ = severe injury (**Murat-Bilgin et al., 2011)**.

The data was analyzed on *Statistix 8.1.* (USA). Kolmogrov-Smirnov and Levene`s test were used for normality and homogeneity. The continuous variables were analyzed by one-way analysis of variance and post hoc testing and presented as mean±S.D and range. Confidence interval was taken at 95%.

RESULTS

In present study, we observed major differences in liver enzyme assays among groups. The ALT, AST, ALP and LDH in serum of Rabbits treated with azathioprine (AZA) were found elevated compared to control group (p=0.0001) The AZA+Zinc sulphate revealed a significant reduction in the liver enzymes compared with the AZA groups alone (p=0.01) and control group (p=0.0001). The ascorbic acid when mixed with AZA showed significant reduction in the liver enzyme and improved liver histology. The finding showed significant hepatoprotection provided by the ascorbic acid against AZA injury. The liver enzyme assays among different groups are shown in table.1.

Different parameters of histological score of liver injury are shown in Table 2. The Liver sections from control group showed intact central portal venules and compact hepatocytes arrangement. Normal looking hepatocytes with prominent nucleus, nucleolus and well preserved cytoplasm

were seen in control group. (Figure 1). The AZA group showed nodular regenerative hyperplasia, veno-occlusinve disease; peliosis hepatis, sinusoidal dilatation, cholestasis, hepatocyte necrosis and perisinusoidal fibrosis have been noted. Derangement of hepatocyte cords, hydropic changes with congestion of central venules and sinusoids, and abundant inflammatory cell infiltration (Figure 2-6). Animals fed with 5mg/kg Zinc sulphate, liver tissue sections revealed least derangement of hepatocytes cords, hepatocyte damage and necrosis as compared to AZA and AZA+ Zinc sulphate 1mg/kg body weight. (Figure.4)

Table 1. Liver enzyme levels in controls, Azathioprine and Azathioprine combined with Zinc sulphate.

Groups	ALT (IU/L)	AST (IU/L)	LDH (IU/L)	ALP (IU/L)
Controls (n=20)	47.5±3.3	92.5±15.61	723.5±45.8	96.9±7.88
Azathioprine (n=20)	182.7±10.9	473.7 ±13.9	2758.9±17.6	179.1±6.0
Azathioprine+ Zinc sulphate (1mg/kg) (n=20)	117.7±10.77	245.7 ±13.9	758.9±12.6	119.1±6.0
Azathioprine+ Zinc sulphate (2mg/kg) (n=20)	143.9±16.98	321.9±20.5	945.6±13.3	133.8±17.5

Table 2. Histology of liver injury of controls, azathioprine, and azathioprine combined with Zinc sulphate.

	Sinusoidal dilation and Periportal inflammation	Steatoheaptitis	Fibrosis	Peliosis Hepatis	Nodular regenerative hyperplasia	Veno-occlusive disease
Controls (n=20)	0	0	0	0	0	0
Azathioprine (n=20)	++++	+++	++++	++++	++++	+++
Azathioprine+ Zinc sulphate (1mg/kg) (n=20)	++++	++	+++	+++	+++	+++
Azathioprine+ Zinc sulphate (2mg/kg) (n=20)	+++	++	++	++	++	+

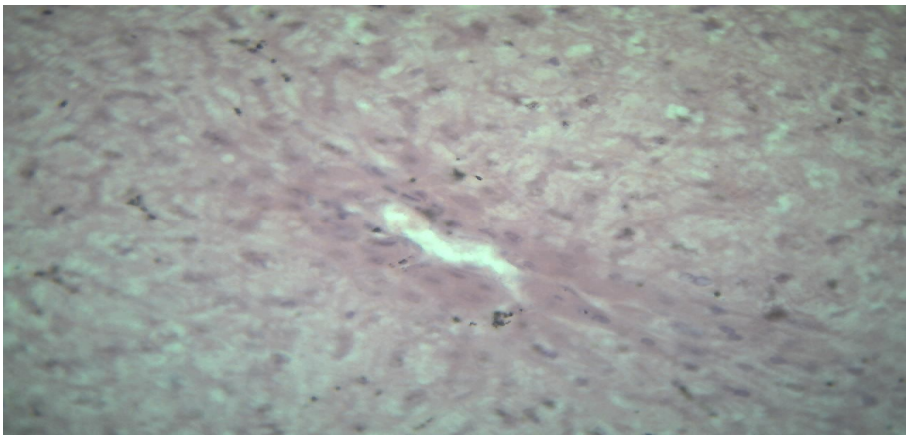

Figure 1. Liver slide of control group shows normal hepatocyte cords. Sinusoids with central venule are visible (H& E) (x40).

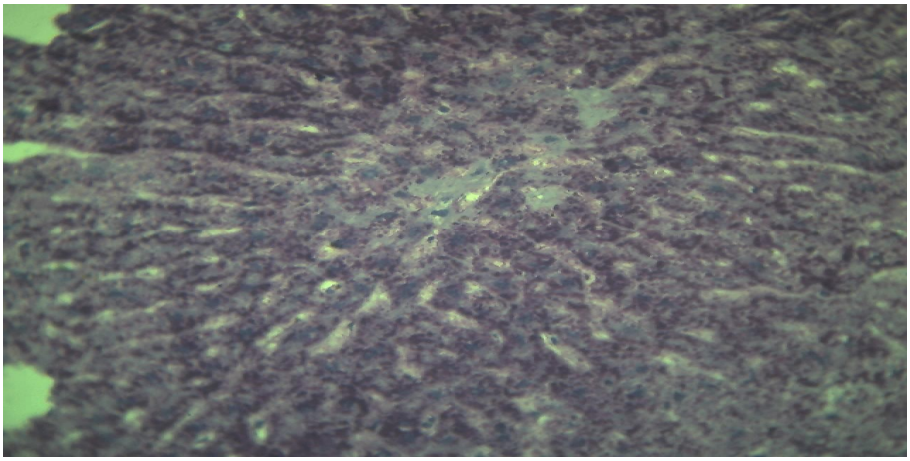

Figure 2. liver tissue slide of control group showing normal glycogen content on PAS staining. (x40)

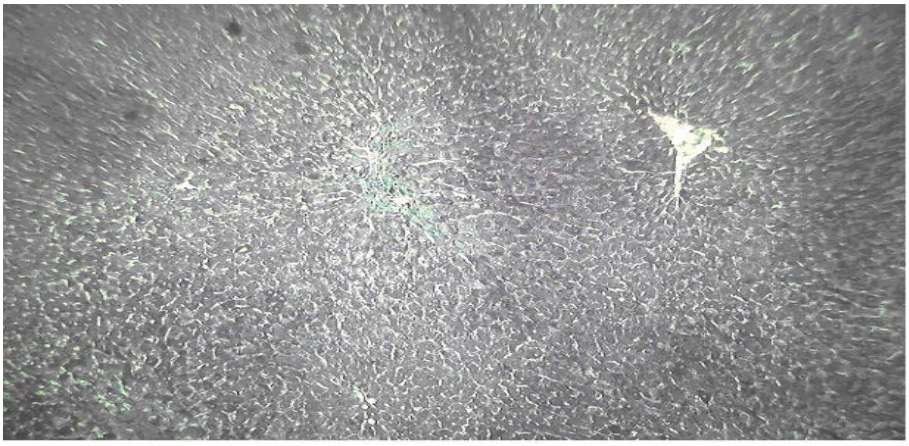

Figure 3. liver tissue slide showing no fibrosis on methanemine staining normal glycogen content on PAS staining. (x40)

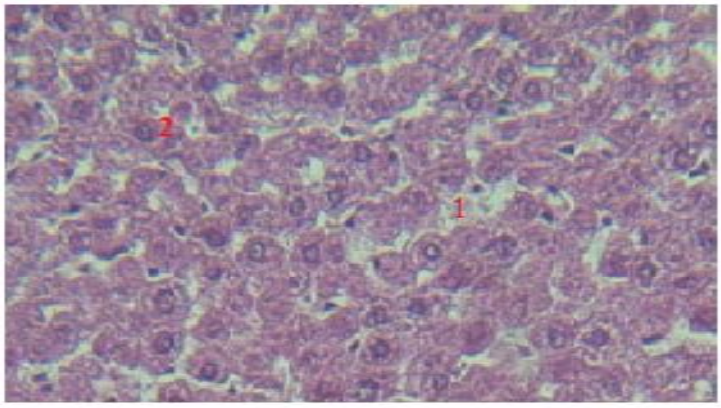

Figure 4. liver tissue slide showing Peliosis hepatis in azathioprine group (H& E) (x40)

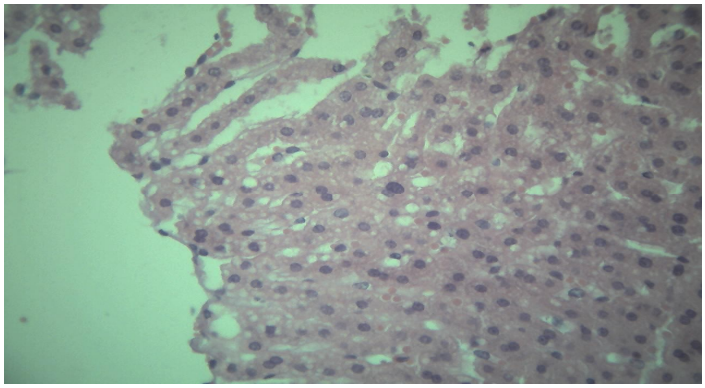

Figure 5. liver tissue slide showing near normal histology of liver in zinc sulphate (1mg/kg) group (H& E) (x40)

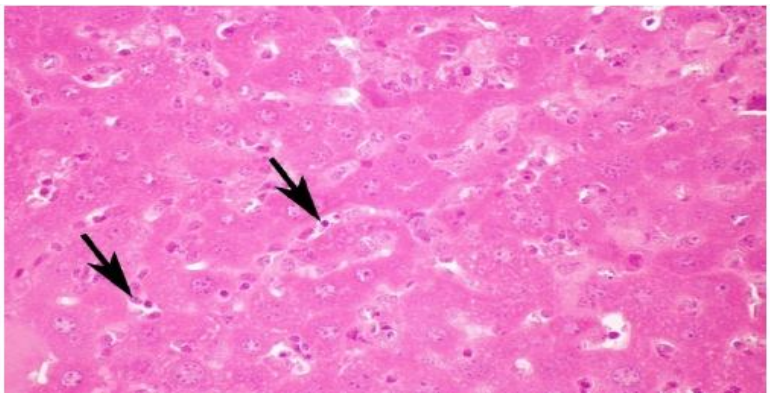

Figure 6. liver tissue slide showing near normal histology of liver in zinc sulphate (5mg/kg) group; however areas of cellular injury are visible (H& E) (x40)

DISCUSSION

In most cases, hepatotoxicity is an unpredictable side effect of AZA, whose molecular and pathogenic mechanisms remain unknown (**Ehmsen et al., 2008)**. A variety of histopathologic findings have been observed in AZA-induced hepatotoxicity. Nodular regenerative

hyperplasia, veno-occlusinve disease, Peliosis hepatis, sinusoidal dilatation, and perisinusoidal fibrosis have been reported.[13-15] Cholestasis, with or without associated hepatocyte necrosis, has also been reported for these thiopurine drugs in clinical studies.[14,15] The findings of present study are highly consistent with previous studies as above. The histological findings are shown in figure 1-6. The disturbed liver biochemical parameters as are shown in table 1.

The AZA increased alanine transaminases, aspartate transaminases, lactate dehydrogenase and alkaline phosphatase. The findings are consistent with previous studies **(Ehmsen et al., 2008)**, **(Farrell, 2004) and Ardeshiri et al., 2012).** The AZA toxicity appears to be dose related, and sometime idiosyncratic reactions are also involved. Increase in AST and ALP in AZA-treated rats explains the leakage of these enzyme into circulation which suggests hepatocellular damage, this occurs because of the damage of vascular membrane resulting in impaired liver enzyme levels (Figure 2). The observed intensity of damage in tissues in AZA treated Rabbits is shown in (Figure 2 to 6) comparing to control group (Figure 1), it appears that the damage due to AZA intake is highly justified as apoptosis was observed. Portal fibrosis and inflammation of the blood vessels around portal triad can be hydropic liver cells and even because cell death is induced **(Ehmsen et al., 2008)**, **(Farrell, 2004) and Ardeshiri et al., 2012)**. The veins were widely dilated and the cytoplasm showed degeneration. According to previous reports about AZA **(Ehmsen et al., 2008)**, **(Farrell, 2004) and Ardeshiri et al., 2012)** seemingly obvious explanations could be that AZA selectively inhibit synthesis of purine nucleotides, which are required for DNA synthesis.

Comparing the findings with zinc sulphate at 1 and 5mg/kg body weight, some astonishing findings have been noted in present study on rabbit model. The protective effects of zinc sulphate were pronounced at 5 mg/kg body weight, this shows protective role of zinc sulphate against AZA induced hepatotoxicity.

Zinc exerts anti-oxidant activity, as zinc stabilises cytosolic Zinc/Cu superoxide dismutase which catalyses superoxide removal by virtue of zinc–histidyl-Cu triad acting as a proton donor during the oxidation cycle. It also inhibits the enzyme NADPH oxidases which catalyze the production of superoxide O2- from O2. Cytotoxic cytokines TNF- α, IL-1β and IL-8 which generate free radicals are also inhibited by Zinc. The production of cysteine-rich metallothionein, an excellent scavenger of hydroxyl (OH-) radical is also induced by zinc (**Prasad, 2002).**

Zinc supplementation protects against toxin-induced liver damage and is used as a therapy for hepatic encephalopathy in patient's refractory to standard treatment **(Grungreiff et al., 2000 and Grungreiffand Reinhold, 2010).** Zinc supplementation has proved to decrease hepatic encephalopathy and blood ammonia levels **(Grungreiff et al., 2000)**. Supplementation of zinc in chronic Hepatitis-C-Virus infected patients has been shown to reduce gastrointestinal disturbances, weight loss, hair loss and mild anemia **(Grungreiff et al., 2000, Grungreiff and Reinhold, 2010, Grungreiff and Reinhold, 2010, Samir and Zine 2013 and Sidhu et al., 2004)**. The findings of present experimental study are highly consistent with most of previous studies mentioned in literature **(Samir and Zine 2013 and Sidhu et al., 2004, Bolkent et al., 2006, De Boer et al., 2008, Ardeshiri et al., 2012 and Naqvi et al., 2010)**. The present study proved that the AZA induced liver damage may be minimized by zinc sulphate, which may be used in

patients taking AZA for indications which need drug for long time periods.

ACKNOWLEDGEMENTS

Authors are thankful to Isra University for grant and support in conducting the research.

CONCULSION

The present study concludes that Azathioprine induces hepatocellular injury and zinc sulphate may be used as an effective protector against azathioprine induced liver damages. However, further studies are recommended to elucidate the underlying protective mechanism of zinc sulphate.

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