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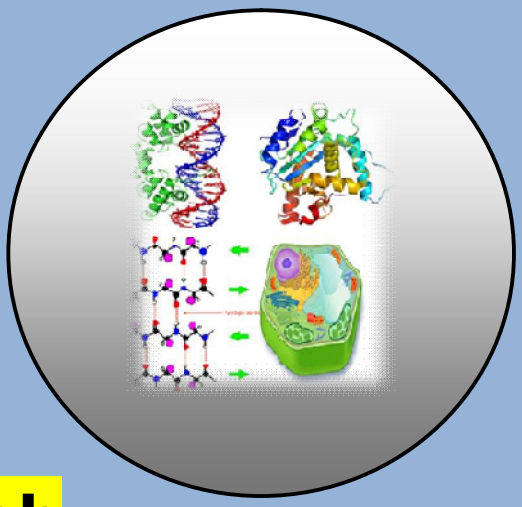

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RESEARCH PAPER

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Ascorbic Acid in Azathiopurine Induced Hepatotoxicity- An Experimental Study

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ABSTRACT

To investigate azathioprine (AZA) induced hepatotoxicity and possible protective role of ascorbic acid 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 + ascorbic acid 100 mg/kg orally for Group 4. (n=20) Rabbits received Azathioprine 15 mg/kg body + Ascorbic acid 200 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+Ascorbic acid (p=0.001). Liver enzymes and histology was deranged significantly in AZA group compared to controls and AZA+Ascorbic acid group (p=0.01).The AZA+Ascorbic acid 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 ascorbic acid may be used as an effective protector.
Key words: Azathioprine, Ascorbic Acid, Effective Protector, Hepatotoxicity, and Biochemical Parameters.

INTRODUCTION

Liver injury due to drugs is a possible obstacle of almost every medication prescribed, because the liver metabolizes approximately all therapeutic agents. Drug-induced liver disease accounts for about 10% to 50% of adult patients, present with elevated enzymes, specifically the patients over age 50 years. It may account for nearly 25% of patients with fulminant hepatic failure. (Watkins, 2006) (Teschke, 2008) though the most of drugs are metabolized without damaging the liver, but many lethal drug reactions may damage liver itself, and many cases are reported annually. Some agents produce metabolites which can cause damage to the liver in a regular dose-dependent manner. (Andrade, 2005) (Zimmerman, 2000) Approximately 11.7% of patients with drug-induced hepatocellular jaundice have chances of progressing to death or transplantation. (Bjornsson, 2006) For the handling and for avoidance of drug-induced hepatic damage it is necessary to recognize it as early possible to discontinue the drug, expeditiously. (Bjornsson, 2006) (Savvidou, 2007)

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.(Al Mruf, 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, 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, 2010) (Trabelsi, 2013)

L-Ascorbic acid (Vitamin C) is known for its antioxidant activity, protects against reactive oxygen species (ROS) which are generated through metabolism and also through exposure to toxins and carcinogens. (Halliwell, 2009) (Chaput, 2011) (Ristow, 2010) Vitamin C is a potent hydrophilic antioxidant which is able to scavenge a variety of free radicals and oxidative molecules such as hydroxyl radicals ($^{\cdot}OH$), superoxide anions ($O_2^{\cdot-}$), sulphydryl radicals, oxidized LDL, and others. There are evidences proving the L-ascorbic acid protects against oxidative stress, and thus may protect from various chronic diseases which originate primarily because of oxidative reactions. (Chaput, 2011) (Ristow, 2010) (Sahlin, 2010) (Yfanti, 2012)

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

MATERIAL 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) Rabbits received 0.9% isotonic saline orally on alternate day for four weeks and served as control group,

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

Group 3. (n=20) Rabbits received Azathioprine 15 mg/kg body + Ascorbic acid 100 mg/kg body orally for four weeks

Group 4. (n=20) Rabbits received Azathioprine 15 mg/kg body + Ascorbic acid at dose of 200 mg/kg orally for four weeks.

Azathioprine (Imuran 50 mg, Glaxo Smitth Kline Pharma) and Ascorbic acid (Ascorbic acid, Abbot) 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 transaminase (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. (Colli, 2008) 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 with control group (p=0.0001) The AZA+Ascorbic acid 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).

Table. 1. Liver enzyme levels in controls, Azathioprine and Ascorbic acid.

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+Ascrobic acid (100mg/kg) (n=20)	117.7±10.77	245.7 ±13.9	758.9±12.6	119.1±6.0
Azathioprine+Ascrobic acid (200mg/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 ascorbic acid.

	Sinusoidal dilation and Periportal inflammation	Steatosis Hepatis	Fibrosis	Peliosis Hepatis	Nodular regenerative hyperplasia	Veno-occlusive disease
Controls (n=20	0	0	0	0	0	0
Azathioprine (n=20)	++++	+++	++++	++++	++++	+++
Azathioprine + Ascrobic acid (100mg/kg) (n=20)	++++	++	+++	+++	+++	+++
Azathioprine+Ascrobic acid (200mg/kg) (n=20)	+++	++	++	++	++	+

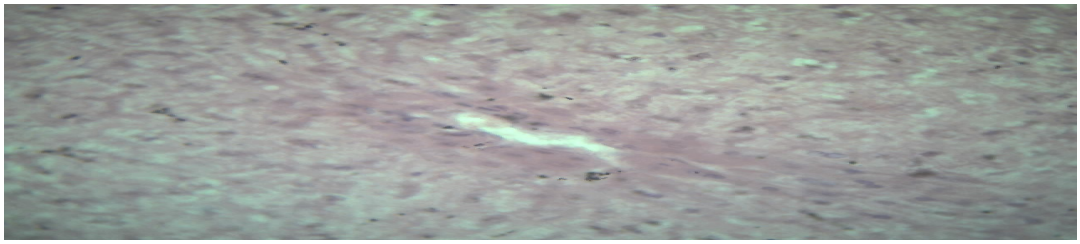

Figure 1. Liver slide of control group shows normal hepatocyte cords. Sinusoids with central venule are visible (H& E) (x40).

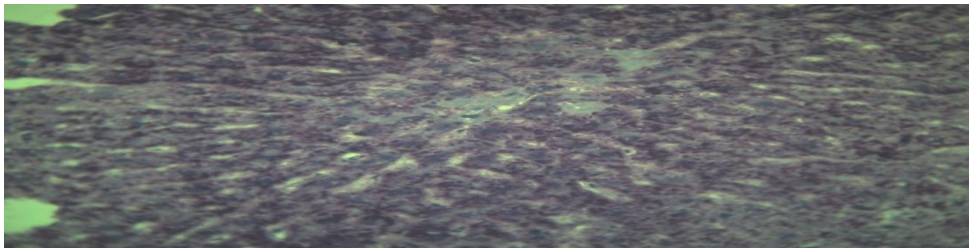

Figure 2. Liver tissue slide showing normal glycogen content on PAS staining. (x40).

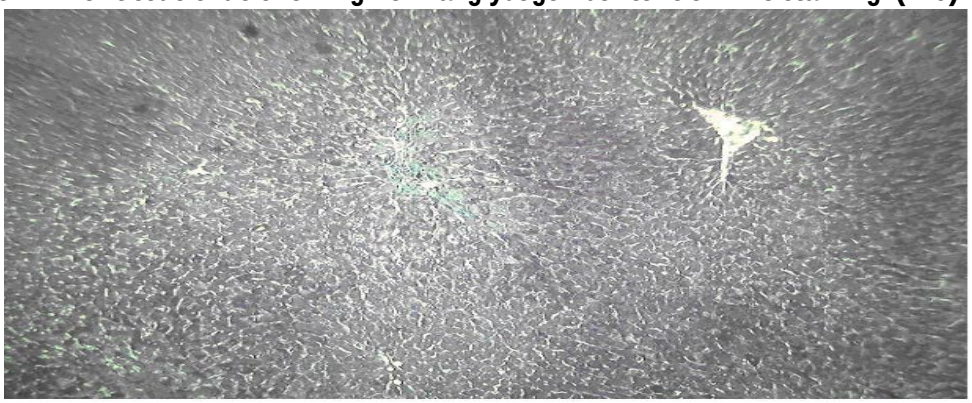

Figure 3. Liver tissue slide showing no fibrosis on methanemine staining normal glycogen content on PAS staining (x40).

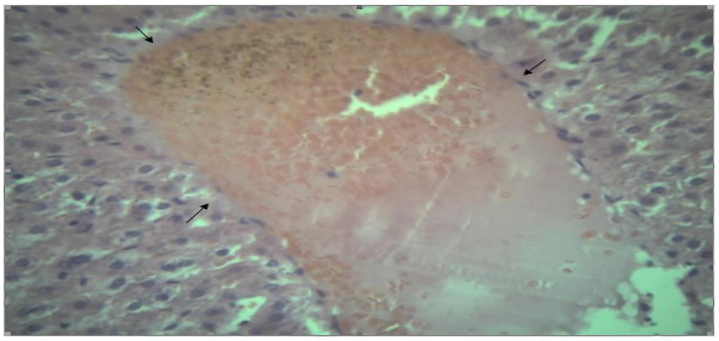

Figure 4. Liver tissue slide showing Peliosis hepatis in azathioprine group (H& E) (x40).

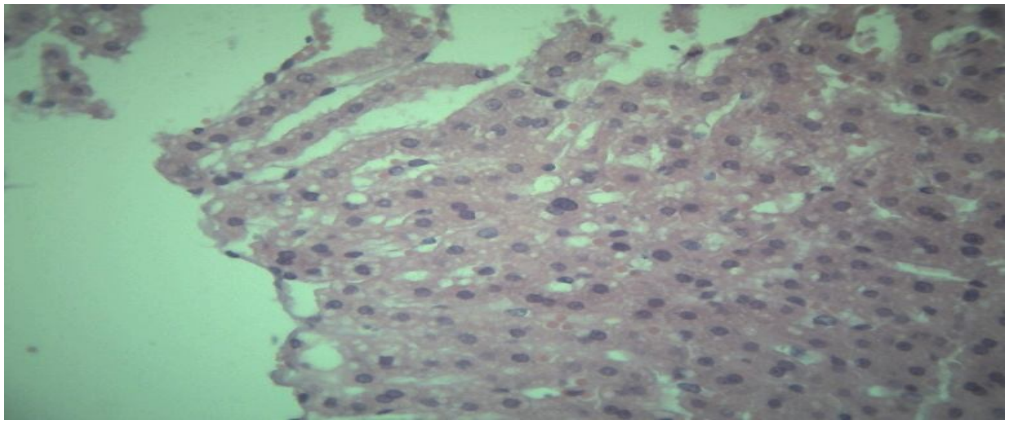

Figure 5. Liver tissue slide showing near normal histology of liver in ascorbic acid group (100mg/kg) (H& E) (x40).

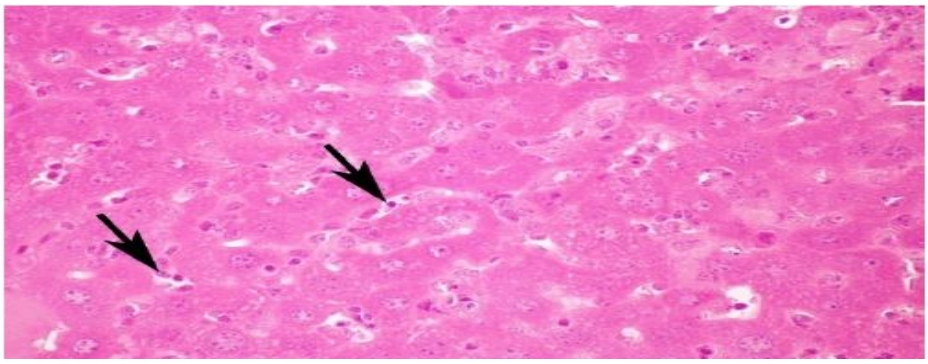

Figure 6. Liver tissue slide showing near normal histology of liver in ascorbic acid group, however areas of cellular injury are visible (200mg/kg) (H& E) (x40).

Animals fed with 200mg/kg ascorbic acid, liver tissue sections revealed least derangement of hepatocytes cords, hepatocyte damage and necrosis as compared to AZA and AZA+Ascorbic acid 100mg/kg body weight (Figure.4).

DISCUSSION

Azathioprine (AZA), prodrug of 6-mercaptopurine, is widely used as an immunosuppressant for several diseases such as inflammatory bowel disease (IBD) and autoimmune diseases and following transplantation to avoid organ rejection.(Colli, 2008) (Etchevers, 2008) (Petit, 2008) (Schumann, 2008) In most cases, hepatotoxicity is an unpredictable side effect of AZA, whose molecular and pathogenic mechanisms remain unknown.(Ehmsen, 2008) One previous study reported that 3.5% of inflammatory bowel diseases patients developed hepatitis as a consequence of AZA treatment.(De Boer, 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. (Tong, 2000) (Chattopadhyay, 2008) (Shukla 2009) (Nanji, 2001) (Zheng, 2008) (Bergmeyer, 1978) (Kesiova 2006) Cholestasis, with or without associated hepatocyte necrosis, has also been reported for these thiopurine drugs in clinical studies. (Zheng, 2008) (Kesiova 2006)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. (Ardeshiri, 2012) (César, 2004) 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 the damage is due to AZA intake is highly justified as apoptosis was observed in. Portal fibrosis and inflammation of the blood vessels around portal triad can be hydropic liver cells and even because cell death is induced.(Farrell, 2004) The veins were widely dilated and the cytoplasm showed degeneration. According to previous reports about AZA (César, 2004), seemingly obvious explanations could be that AZA selectively inhibit synthesis of purine nucleotides, which are required for DNA synthesis. It has been suggested that, in rat hepatocyte treated with AZA, ROS production could damage membranes and macromolecules at this level, although there are no convincing results supporting this hypothesis. Another potential source of ROS that could initiate oxidative stress may be related to production some metabolites as 6 mercaptopurine that is toxin and ROS may be formed during their metabolism. (Ardeshiri, 2012) (César, 2004). In present study AZA induced changes were severe. The findings of AZA induced hepatotoxicity noted were as; sinusoidal distortion, Periportal inflammation, Peliosis hepatis (Figure 4), regenerative nodular hyperplasia and veno-occlusive changes were evident. Comparing the findings with ascorbic acid at 100 and 200mg/kg body weight, some astonishing findings have been noted in present study on rabbit model. The protective effects of ascorbic acid were pronounced at 200 mg/kg body weight, this shows protective role of ascorbic acid against AZA induced hepatotoxicity. Portal fibrosis and inflammation of the blood vessels around portal triad can be hydropic liver cells and even cause cell death is induced. The veins were widely dilated and the cytoplasm showed some degeneration. According to previous reports about AZA seemingly obvious explanations could be that AZA selectively inhibit synthesis of purine nucleotides, which are required for DNA synthesis. (Ardeshiri, 2012) (César, 2004). It has been suggested that, in hepatocyte treated with AZA, ROS production could damage membranes and macromolecules at this level, (Ardeshiri, 2012) (César, 2004) although there are no convincing results supporting this hypothesis. Another potential source of ROS that could initiate oxidative stress may be related to production some metabolites such as 6 mercaptopurine. According to previous reports about AZA, seemingly obvious explanation could be that AZA selectively inhibit synthesis of

purine nucleotides, which are required for DNA synthesis. (Ardeshiri, 2012) (César, 2004) The present study proved that the AZA induced liver damage may be minimized by ascorbic acid, which may be used in patients taking AZA for indications which need drug for long time periods.

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CONCULSION

The present study concludes that Azathioprine induces hepatocellular injury and ascorbic acid may be used as an effective protector against azathioprine induced liver damages. However, further studies are warranted.

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