Research Article

Ameliorative Effect of Vitamin E on Azithromycin Induced Biochemical and Histological Changes in Liver Tissue of Rats

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Abstract | The hepatotoxic potential of azithromycin and the ameliorative role of vitamin E in azithromycin induced toxicity were studied in adult Wistar rats. The study also assessed the effectiveness of pretreatment and posttreatment of Vitamin E in protecting against the injury caused. Azithromycin at 30mg/kg and Vitamin E at 50 IU/kg were administered orally for a period of fifteen days. Azithromycin at the administered dose did not affect the body weight gain or the relative liver weight in treated rats whereas it significantly (P<0.01) decreased the alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), superoxide dismutase (SOD) and catalase activities in liver. Pretreatment with vitamin E increased the levels of ALT, AST and SOD with no significant variations in the levels of ALP and catalase activities in the liver of rats. Significant (P<0.01) increase in the levels of AST and SOD in liver was also observed during posttreatment with vitamin E. The hematological parameters *viz*., total erythrocyte count, hemoglobin concentration, mean corpuscular volume, mean corpuscular hemoglobin, packed cell volume and differential leucocyte count did not show any significant variation among different groups while the total leukocyte count was significantly (P<0.01) increased in vitamin E alone and Vitamin E pre and posttreatment groups. Histopathology of liver of azithromycin treated group revealed cloudy swelling, periportal fatty change and bile duct hyperplasia. The results of this study revealed that pretreatment with vitamin E could mitigate the lesions induced by azithromycin more effectively than the posttreatment. Hence, vitamin E prophylaxis at 50 IU/kg may be considered beneficial for protection of liver against azithromycin induced damage.

Keywords | Azithromycin, Hepatotoxicity, ALT, AST, Catalase, SOD, Vitamin E

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INTRODUCTION

Azithromycin is a macrolide antibiotic recommended as first line therapy for prophylaxis and treatment of disseminated infection caused by *Mycobacterium avium intracellulare* in AIDS patients and for the treatment of pulmonary disease in non-HIV infected patients (Kovacs and Masur, 2000; Rao et al., 2014). Though it was considered as a safe drug with mild gastrointestinal symptoms and skin rashes (Leclercq, 2002), high doses employed for the treatment of *Mycobacterium avium complex* caused tinnitus, dizziness and reversible hearing loss (Wallace et al., 1994; Lin et al., 2010). Moreover, there are contradictory reports on lower and higher (FDA, 2013; Rao et al., 2014) risks of death associated with azithromycin therapy.

Hepatotoxicity and ototoxicity are the two well established adverse reactions to macrolide antibiotics. Azithromycin

with its unique pharmacokinetic properties besides being a member of this group could also cause hepatotoxicity following long term therapy. There were several clinical reports reiterating its potential for altering the biomarker enzymes during therapy which further resolved within a few days after cessation of the therapy (Hoepelman and Schneider, 1995; Chandrupatla et al., 2002; Suriawinata, and Min, 2002; Lockwood et al., 2010). Recent studies also have revealed its potential to cause hepatotoxicity in rats (Ebenezer and Ayokanmi, 2014; Singh et al., 2015). In addition, *in vitro* studies have also revealed its potential to alter the hepatocellular architecture (Gerbaux et al., 1996; Bambeke et al., 1998). The mechanisms for the azithromycin induced hepatotoxicity could be by membrane lipid peroxidation, free radical formation or mitochondrial impairment. Oxidative stress has been found to be an important mechanism in hepatotoxicity (Jaeschke et al., 2002; Amin and Hamza, 2005; Di Sario et al., 2007; Cheung and Sanyal, 2010; Jaeschke and Ramachandran, 2011)

Vitamin E is a group of biologically active tocopherol widely distributed in plant products. The naturally occurring active compound is d- α tocopherol. It is a strong phenolic antioxidant, donating hydroxyl group on its ring structure to free radicals, preventing lipid peroxidation thereby prolonging the biological life of polyunsaturated fatty acids in the cell membranes by slowing the formation of free radicals and hyper-peroxides (Phillips et al., 1982; Mukai et al., 2007; Traber and Atkinson, 2007). The protective role of Vitamin E in idarubicin induced myocardial toxicity (Kalender et al., 2002), heavy metal induced renal and testicular toxicity (Atef and Al-Attar, 2011) in dimethoate induced cerebral toxicity (Amara et al., 2011) and chlorpyrifos toxicity in Atlantic salmons (Olsvik et al., 2015) were already reported.

Thus, the present study was conducted to evaluate the hepatotoxic potential of azithromycin and the possible protective effect of vitamin E, since it has not been investigated.

MATERIALS AND METHODS

Animals

Fifty adult Wistar rats of either sex weighing 150 to 200 g were procured from the Small Animal Breeding Station, College of Veterinary and Animal Sciences, Mannuthy. The animals were housed in a room with controlled temperature (21-22°C), light (12 hour light dark cycle) and humidity (50 ± 10%). The animals were allowed free access to standard laboratory food and water. The tests were performed between 9 AM and 1 PM. The experiments were performed according to the guiding principles in the use of animals in toxicology and approved by the Institutional Animal Ethics Committee.

Chemicals

Azithromycin technical grade was gifted by M/s INTAS Pharmaceuticals Limited, Ahmedabad, India with purity more than 98%. All chemicals and reagents used in this study were of analytical grade and procured from M/s Merck India Ltd., Bangalore, India.

Experimental Design

The rats were randomly divided into five groups each comprising of ten animals. Group I served as normal control and kept on sterile distilled water orally for 15 days. Group II received azithromycin at the rate of 30 mg/kg orally for 15 days. Group III received vitamin E at 50 IU/kg for 15 days as a pretreatment followed by azithromycin orally (dosage and duration were as in group II). Group IV received azithromycin as in group II followed by vitamin E at 50 IU/kg for 15 days. Group V were treated with vitamin E at 50 IU/kg orally for 15 days. All the dosings were done at 9.30 AM.

The body weights of animals were recorded before, and on the last day of experiment. At the end of experiment, all the animals were sacrificed by humane method. Fifty per cent of animals in each group were sacrificed by cervical dislocation and specimens were collected for biochemical and haematological examinations. Liver samples were collected in ice cold normal saline for tissue biochemical studies. The tissues were then blotted, removed fascia, minced and homogenized with ice cold normal saline in a glass homogenizer with motor driven teflon coated pestle (Remi Model no. C-24). The homogenate was centrifuged at 10^4g in an automatic high speed refrigerated centrifuge (Eppendorf 5810R) for 30 minutes and the supernatant was stored at -20°C until biochemical analysis. The biomarkers assessed were alanine aminotransferase (ALT), aspartate aminotransferase (AST) (Yatazidis, 1960), alkaline phosphatase (ALP) (Kind and King, 1954), superoxide dismutase (SOD) (Mimami and Yoshikawa, 1979) and catalase (CAT) (Cohen et al., 1970).

Blood samples were collected at the time of sacrifice into sterile vials containing disodium salt of ethylene diamine tetra acetic acid (EDTA sodium) at the rate of 1 mg/mL for the estimation of haematological parameters like total leucocyte count (TLC), total erythrocyte count (TEC), haemoglobin concentration (Hb), packed cell volume (PCV), differential leucocyte count (DLC), mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH).

Remaining animals of each group were sacrificed by anesthetic over dosage using ether and liver samples were collected in 10 per cent formol saline for histopathological examination. The specimens were processed by standard procedure (Smith and Burton, 1977).

Table 1: Effect of Vitamin E on azithromycin induced changes in liver tissue biomarker enzymes of rats

Group	Drug Treatment	ALT (µg pyruvate/mg of protein/60 min)	AST (µg pyruvate/mg of protein/60 min)	ALP (µg of phenol/ mg protein/15 min)	SOD (units/ mg of protein)	CAT (units/ assay mixture)
I	Control	108.3±6.11^{d}	136.68 ± 12.18^{b}	0.0046±0.00067^{b}	12.17±1.02^{d}	431.98±12.57^{b}
II	Azithromycin at 30 mg/kg	52.84±4.34^{a}	92.55±7.48^{a}	0.00035±0.0000082^{a}	0.86±0.09^{a}	301.06±19.66^{a}
III	Vit E 50 IU/kg pretreated group	82.27±4.93^{bc}	112.62±3.78^{ab}	0.00033±0.000010^{a}	7.84±0.65^{c}	314.42±23.41^{a}
IV	Vit E 50 IU/kg posttreated group	65.98±5.18^{ab}	105.92±2.49^{ab}	0.0014±0.00084^{a}	4.39±0.45^{b}	324.63±6.7^{a}
V	Vit E at 50 IU/kg	95.83±2.36^{cd}	109.93±5.67^{ab}	0.00047±0.000054^{a}	8.3±0.63^{c}	317.43±23.99^{a}

Values are mean ± SE; **n**=6 in each group; Values (means) bearing the same superscript do not differ significantly at P<0.01.

Statistical Analysis

The data were expressed as mean ± standard error (SEM), n = 6. The differences between groups were calculated by one way analysis of variance (ANOVA) coupled with Duncan's multiple range test. A difference with P <0.01 was considered as significant.

RESULTS

No mortality was observed in any of the experimental groups during the period of observation. Azithromycin did not affect the weight gain compared to vitamin E pre and posttreatment. Similarly, vitamin E did not interfere with the weight gain when compared to other treatments. Also, there was no significant difference in the relative organ weight of liver of different groups.

The mean and standard error values of liver ALT, AST, ALP, SOD and CAT activities of various groups are depicted in Table 1.

The liver ALT activity was significantly decreased in azithromycin treated animals compared to the control and vitamin E treated group. On the other hand, pretreatment with Vitamin E significantly (P<0.01) increased the enzyme activity compared to azithromycin treated group. However, the enzyme activity in the posttreatment group did not differ significantly with that of azithromycin treated group.

Likewise, the AST activity was significantly (P<0.01) decreased in the liver of rats treated with azithromycin at 30 mg/kg compared to control. Vitamin E, when given alone did not significantly affect the liver enzyme activity. However, pre and posttreatment with vitamin E in azithromycin treated rats caused an increase in the enzyme activity to the level comparable to that of vitamin E alone and control group.

ALP activity was significantly (P<0.01) decreased in liver in azithromycin and vitamin E treated groups compared to control. Pretreatment as well as posttreatment with vitamin E did not alter the enzyme status in azithromycin treated rats.

The SOD activity in the liver tissue of azithromycin treated group differed significantly (P<0.01) from control and vitamin E alone treated groups. Though the enzyme activity significantly increased in both pretreated and posttreated groups compared to azithromycin treated group, the activity in pretreated group was comparable to vitamin E alone treated group.

The CAT activity was significantly (P<0.01) decreased in the liver of azithromycin treated rats compared to control group. However, no significant effect on CAT activity was observed following pre and posttreatment with vitamin E.

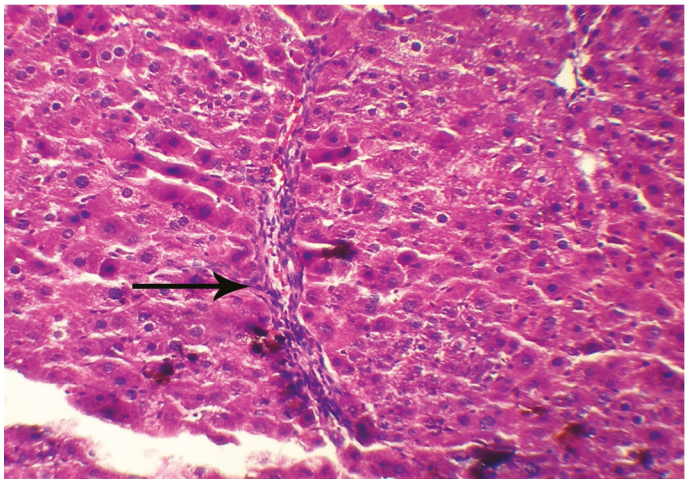

Figure 1: Liver of azithromycin treated animals showing cloudy swelling, extensive fatty changes on the periportal area and bile duct hyperplasia

The hematological parameters *viz.*, TEC, Hb, MCV, MCH, PCV and DLC did not show any significant variation among different groups (Table 2). The increase in the TLC was significant in vitamin E alone treated group compared to control. Both pre and posttreatment with vitamin E also significantly increased the TLC in azithromycin treated rats.

Table 2: Effect of Vitamin E on azithromycin induced changes in hematology in rats

Drug treatment	TEC (millions per mm³)	Hb(g%)	MCV(fl)	MCH (pg)	PCV(%)	TLC (thousands/mm³)
Control	6.9±0.06[a]	13.63±0.08[a]	60.21±1.64[a]	1.98±0.02[a]	41.5±0.85[a]	6291.67±27.42[a]
Azithromycin at 30 mg/kg	6.85±0.05[a]	13.50±0.09[a]	59.91±1.29[a]	1.97±0.01[a]	41.0±0.73[a]	6733.33±180.33[ab]
Vit E at 50 IU/kg pretreated group	6.86±0.03[a]	13.43±0.15[a]	60.47±1.12[a]	1.96±0.03[a]	41.5±0.76[a]	7208.33±58.33[b]
Vit E at 50 IU/kg posttreated group	6.92±0.04[a]	13.50±0.15[a]	58.98±0.61[a]	1.95±0.02[a]	40.83±0.48[a]	7150.00±27.42[b]
Vit E at 50 IU/kg	6.95±0.06[a]	13.8±0.05[a]	60.23±1.01[a]	1.98±0.02[a]	41.5± 0.70[a]	7200.00±64.55[b]

Values are mean ± SE, n=6 in each group; Means bearing the same superscript do not differ significantly at P<0.01.

The liver of azithromycin treated animals showed cloudy swelling, extensive fatty changes on the periportal area and bile duct hyperplasia (Figure 1), severity of which were seen decreased in the liver of Vitamin E pretreated animals (Figure 2). Pretreatment with vitamin E reduced the severity of lesions as evidenced by the fatty changes whereas the post-treatment with the drug did not make significant effect.

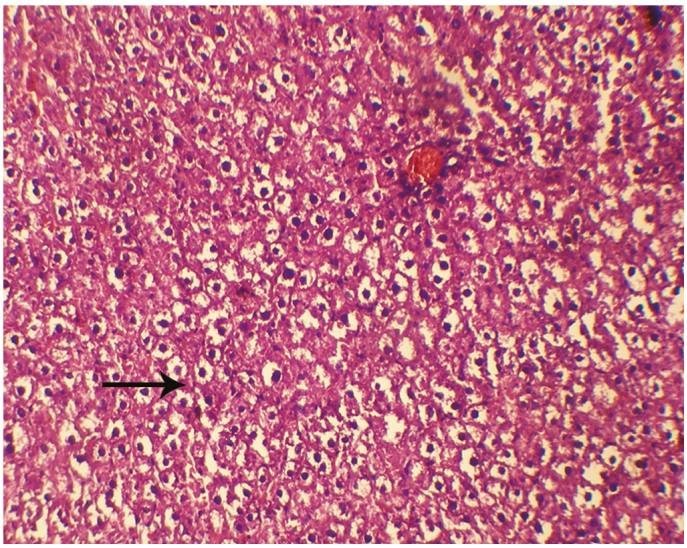

Figure 2: Liver of group III animals showing (pretreated with vitamin E before administered with azithromycin @ 30mg/kg) decreased severity of liver lesions

DISCUSSION

Determination of the activity of hepatic enzymes was one of the most important tools in the study of hepatotoxicity. In the present study, it was observed that treatment with azithromycin reduced the activities of tissue ALT, AST, ALP, SOD and CAT compared to the control. The histopathological studies further confirmed the findings wherein it was noted that treatment with azithromycin induced damages in the liver. Azithromycin, in comparison to the older generation macrolides had higher bioavailability, considerably prolonged serum half-lives and higher tissue concentration following oral administration. Azithromycin's long half-life resulted from its extensive uptake and slow release from the tissues rather than delayed metabolism. Thus the tissue concentrations were generally 10-100 times than those achieved in serum (Giguere et al., 2006). Therefore, in the present study it is expected that high tissue retention might be one of the explanations for the tissue injury induced by azithromycin as evidenced by histopathology.

It was already stated by Hoe and Wilkinson (1973) that in the event of any cell wall damage, the permeability of cell membrane either increased or the cell wall ruptured resulting in diffusion of the enzyme into the blood stream causing increased serum enzyme activity. Once the cell death happened, the source of enzyme declined. Therefore, any change in the enzyme activity actually reflected the status of the liver. In the current study, the liver ALT values were significantly decreased in azithromycin treated group, indicating the hepatotoxic potential of the antibiotic at 30 mg/kg body weight. Our findings are further supported by Hoepelman and Schneider (1995) wherein transient abnormalities in the level of hepatic enzymes were observed in 1 per cent of azithromycin treated patients. Similarly, in another study, higher serum ALT values were recorded in a 33 year old patient following azithromycin treatment (Suriawinata and Min, 2002). Further, recent studies have revealed that azithromycin when given alone (Ebenezer and Ayokanmi, 2014) or in combination with paracetamol (Singh et al., 2015) have significantly produced liver toxicity by increasing the serum level of hepatic enzymes (ALT, AST and ALP) and oxidative parameters in liver of rats. It is remarkable to observe that even though vitamin E did not have significant effect on the enzyme activity, it provided a prophylactic effect in azithromycin treated animals by increasing the enzyme level up to the level of activity as in group treated with only vitamin E. On the contrary, vitamin E posttreatment did not significantly affect the enzyme activity. This was reinforced by the fact that the presence of hepatic cellular levels of α-tocopherol during toxic insult alone provided protection (Fariss et al., 1993). The hepatic cells were unable to maintain the cellular tocopherol levels as they were lost once the cell death ensued (Fariss et al., 1993). AST was a nonspecific enzyme that

reflected injury to extrahepatic tissue. In most instances, the activity was greater than ALT activity.

In the rat, ALP was found in the liver, kidney and intestine. It exerted a role in down regulation of secretory activities of the intrahepatic biliary epithelium. Since the major routes of excretion of azithromycin were bile and the intestinal tract (Giguere et al., 2006), the decrease in the enzyme activity by azithromycin in fact could have reflected the pathological alteration of the bile duct function. Usually, the normal level of serum ALP in rat was reported exceptionally high, independent of growth and unusually susceptible to variations in the diet. Further, in a comparative sub-chronic study in rats for clinical chemistry and liver histopathology, it was observed that decreases in tissue ALP activity were not remarkable (Travalos et al., 1996).

The decreased SOD levels in the liver tissue of azithromycin treated group might be attributed to the scavenging of the free radicals generated during metabolism by SOD. The rats pretreated with succinate esters of tocopherol were completely protected from CCl_4 mediated liver damage (Fariss et al., 1993). The super oxide anion was more likely to be transformed to hydrogen peroxide by superoxide dismutase because of its higher cellular concentration (Fariss et al., 1993).

Unlike vitamin E, azithromycin did not significantly increase the total leukocyte count compared to control. The increased count was also appreciably significant in vitamin E pre and posttreated rats. Vitamin E was reported to mitigate leucopenia caused by cancer chemotherapy in rats (Branda et al., 2006). However, in contrast to our findings, an increase in neutrophil and leukocyte counts were also reported in azithromycin treated patients (Hoepelman and Schneider, 1995).

It was surprising to note that the severity of azithromycin induced changes in the liver was considerably seen reduced in the liver of animals pretreated with Vitamin E. The potential of Vitamin E to ameliorate the damages induced was further supported by the reports that vitamin E could reduce the progressing degrees of fibrosis induced by thioacetamide (Borai et al., 2005), cardiotoxicity induced by idarubicin (Kalender et al., 2002) and reduce the morphological changes like Kupffer cell hyperplasia, portal triaditis, necrosis and lymphocytic infiltration in the lipopolysaccharide administered rats (Bharrhan et al., 2010). The results of this study revealed that pretreatment with vitamin E could mitigate the lesions induced by azithromycin more effectively than the posttreatment. Hence, vitamin E prophylaxis at 50 IU/kg may be considered beneficial for protection of liver against azithromycin induced damage.

CONCLUSION

The results of the study revealed that azithromycin at the rate of 30 mg/kg body weight orally produced hepatotoxicity as evidenced by the changes in the biomarkers as well as histopathology of liver. Vitamin E, which is a proven antioxidant when given as a pretreatment can reduce the severity of the toxicity. Thus, vitamin E prophylaxis is recommended when azithromycin is prescribed for long term therapies of disseminated infection caused by *Mycobacterium avium intracellulare* in AIDS patients and for the treatment of pulmonary disease in non-HIV infected patients.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this research paper.

AUTHORS CONTRIBUTION

Praseena, Sujith and Suresh Narayanan Nair carried out the experiment trial, drafted and revised the manuscript. Leena Chandrasekhar and Ajith Jacob George participated in histopathological studies. Sanis Juliet, Gopakumar and Koshy John conceived the study, and participated in its design and coordination. Reghu Ravindran reviewed and revised the manuscript. All authors read and approved the final manuscript.

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