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Hepatoprotective effect of vitamin A and E on diclofenac-induced hepatotoxicity in male Wistar albino rats

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ABSTRACT

This study was done to show the changes in the liver following diclofenac treatment and to study the hepatoprotective effects of Vitamin E and A in diclofenac treated rats. Rats were divided into four groups of six rats each. Group-1: Control rats (n= 6), Group-2: Rats (n= 6) treated with diclofenac at dose of 50 mg/kg IM for 7 days, Group-3: Rats (n= 6) treated with Vitamin A at dose of 400 IU/kg orally followed by diclofenac at 50 mg/kg IM 2 h later for 7 days, and Group 4: Rats (n= 6) treated with Vitamin E at dose of 200 IU/kg orally followed by diclofenac at 50 mg/kg IM 2 h later for 7 days. Later it was analysed with standard bio markers, and it was histologically interpreted. The results showed that there was an rapid increase in the levels of liver function test in diclofenac-treated group, which was significantly decreased after pre-treatment with vitamin E than vitamin A. The liver acinus showed centriacinar necrosis of hepatocytes after 7 days of diclofenac treatment, which was prevented by administration of Vitamin E and A. Drug-induced liver injury possesses a major clinical problem and has become a leading cause of acute liver failure and transplantation.

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INTRODUCTION

Diclofenac sodium belongs to NSAIDS, which are often used to relieve pain It is most commonly used to treat rheumatoid arthritis, musculoskeletal pain and nonspecific fever (Leon-Reyes *et al.*, 2008). Diclofenac is an antipyretic, analgesic and has anti-inflammatory properties which inhibit cyclooxygenase enzyme. The exact mechanism is not known, maybe it is because of decrease in the fatty acid entering the cell (Brater, 2002). NSAIDS gradually prevent prostaglandin synthesis by inhibiting cyclooxygenase and prostaglandin synthesis enzyme. Diclofenac sodium is commonly metabo-

lized in the liver by hydroxylation at 3,4 and 5^{th} position and conjugation with glucuronic acid to form unstable acyl glucuronide compound which is further oxidized by cytochrome(cyp2c8). These increased enzymatic actions lead to accumulation of more unstable compound which generates oxidative stress which causes tissue damage (Mitchell *et al.*, 1973). Oxidative stress is mediated by ROS generated in the liver. ROS include superoxide, peroxidase and hydroxyl radicles are highly unstable and most of ROS are converted to water molecule before they damage a cell. These damages are prevented by antioxidants such as superoxide dismutase, catalase, glutathione peroxidase and various vitamins such as vitamin E and A which act as a scavenger for this ROS (Adikwu and Brambaifa, 2012).

Catalase and GPX help to remove hydrogen peroxide, superoxidase dismutase helps to remove superoxide radicals (Jadhav *et al.*, 2010) GSH is one of the potent antioxidants present in the liver and helps to remove alkoxy radicals (Meister, 1984). These increased free radicals mediated by ROS can cause direct damage in hepatocytes leads to hepatotoxicity and indirectly by activating nuclear factors or by altering the protein level (Sies, 1986) (Bäuerle and Henkle, 1994; Zandi *et al.*, 1997). Vitamin E is a natural fat-soluble antioxidant and powerful scavenger for reactive oxygen species ROS and ameliorates the drug-induced oxidative stress. Vitamin A also had a hepatoprotective effect and its effects are reported in the previous literature, one among that is prevents the carbon tetrachloride, cadmium and gasoline vapour induced hepatic damage in rats (Hooser *et al.*, 1994; Bashandy and Alhazza, 2008; Uboh, 2009). The antioxidant effect of vitamin A and E is to scavenge the singlet oxygen and thereby, it reduces lipid peroxidation (Schafer *et al.*, 2002). The aim of the present study was to investigate the predominant hepatoprotective effect of vitamin A and E on diclofenac-induced oxidative stress in liver on male Wistar rats.

MATERIALS AND METHODS

Experimental animals

Twenty-four male albino Wistar rats (weighing 180-200g) were used in this study. The animals were housed individually in cages and maintained under standard laboratory conditions (temperature 252°C) 12 hours light and dark cycle with free access to a standard commercial diet and water ad libitum throughout the experimental period. The rats were acclimatized to laboratory conditions for 7 days before commencement of the experiment. Experimental method

Rats were divided into four groups

1. Group 1
2. Group 2
3. Group 3
4. Group 4

The animals were given over anesthesia with an intramuscular injection of ketamine hydrochloride 50 mg/kg and sacrificed by cervical dislocation. The blood samples were taken by retro-orbital vein puncture, for biochemical analysis and following parameters like AST, ALT, ALP, bilirubin, total protein. The liver was dissected and fixed in 10% formalin solution for 24 hrs and processed through the paraffin embedding technique. Paraffin blocks were cut by rotary microtome into 5 microns, thin sections which were stained with hematoxylin and eosin for histopathological studies. The dissected liver was washed with ice-cold saline and 10% homogenate prepared in phosphate buffer (ph 7.0). The portion of liver homogenate was centrifuged at 3000 rpm for 15 min at 4 °C, and the supernatant was used for the estimation of TBARS.

Biochemical analysis

Serum alanine aminotransferase (ALT) and serum aspartate aminotransferase (AST), alkaline phosphate and total protein were determined calorimetrically according to the method (Reitman and Frankel, 1957; Kind and King, 1954).

Antioxidant analysis

For the assessment of malondialdehyde (MDA) and glutathione (GSH), the liver tissue was kept frozen at 70°C. After washing with ice water, the tissues were weighed and homogenized in 9 volumes saline 09% and kept at 70°C. Lipid peroxidation and GSH was measured by the spectrophotometric method (Placer *et al.*, 1966; Sedlak and Lindsay, 1968).

Histopathological Changes

The histopathology of liver sections was visualized using a light microscope, and its images were photographed. Changes observed in the slides were described in the order, Figure 1 control group shows the normal central vein, hepatocytes, and hepatic sinusoids.Figure 2 Diclofenac (50 mg/kg im) treated rats showing distorted central veins, oedematous enlargement of cytoplasm, nuclear degeneration, and centrilobular necrosis of hepatocytes. Figure 3. Vitamin A pre-treated 400IU/kg orally showing the normal arrangement of the central vein, hepatocyte,

and hepatic sinusoids with minimal degenerated hepatocytes. Figure 4Vitamin E pre-treated 200 IU/kg orally showing the normal arrangement of the central vein, hepatocytes, and hepatic sin usoids which is seen like the control groupFigure 4.

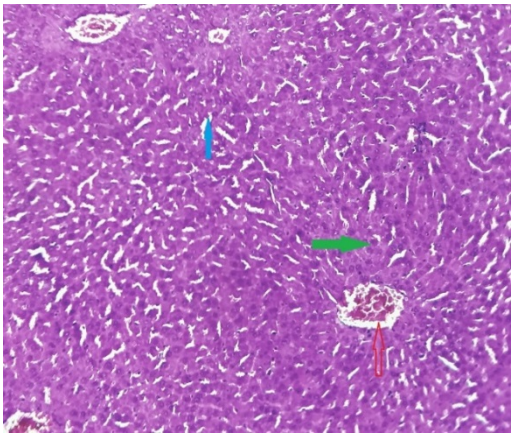

Figure 1: Control rats showing normal central vein(Red arrow) and hepatocytes (Green arrow) and hepatic sinusoids (Blue arrow)

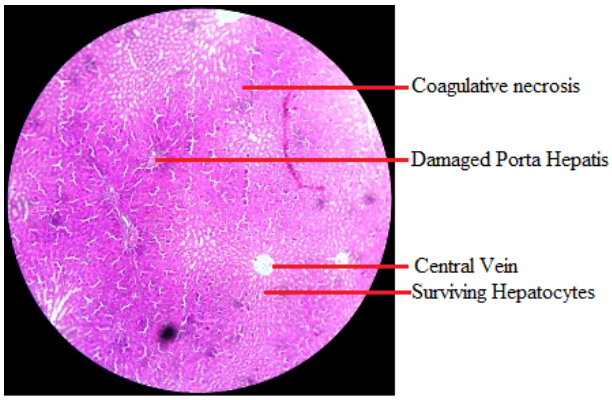

Figure 2: Diclofenactreated rats shows coagulative necrosis, Damaged Porta hepatis and distortedcentral vein

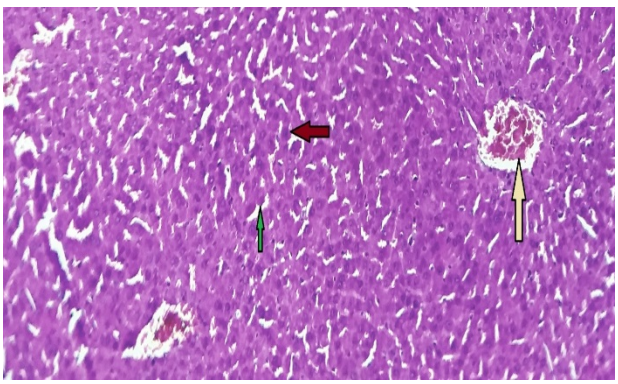

Figure 3: Vitamin Etreated rat showing normal central vein (Red arrow), hepatocyte (Blue arrow)

Statistical analysis

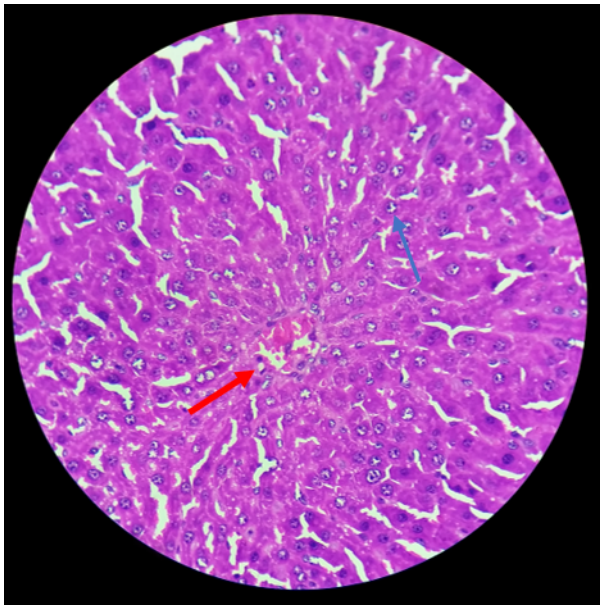

Figure 4: Vitamin A treated rat showing normalcentral vein (Red arrow), hepatocyte (Blue arrow)

Results were statistically analysed and Normally distributed data were analysed with a parametric test using one-way analysis of variance (ANOVA) and data are represented as mean and standard deviation. Kruskal Wallis test were performed for non-parametric test and the data are represented as median (interquartile range). Calculations are done using computerized SPSS software version 16. A notable change was accepted at $p<0.001$.

RESULT

All the values are expressed as median (interquartile range) (Kruskal Wallis test). $P<0.05$ significant difference with control.

The results obtained from this study are shown in Table 1 and Table 2. It was observed that there was a significant increase ($p<0.001$) in serum liver enzymes AST, ALT, ALP and total bilirubin in diclofenac administrated group of rats when compared to control with the value of {41.50(40-42), 54(52.75-56.25), 80(76.50-81.25) and 0.8(0.7-0.9)} respectively whereas there was a significant decrease($p<0.001$) in total protein levels when compared to control with value of (9.20(9.09-9.30). In group 3 pre-administration of vitamin A followed by diclofenac showed a significant ($p<0.001$) reduction in the values of serum AST, ALT, ALP and total bilirubin (63.50(59.50-66.25), 63.50(62-63.50), 96.50(92-98.25) and 0.9(0.7-0.9)) when compared to diclofenac treated groups which is almost nearer to control group and also there was a significant increase ($p<0.001$) in total pro-

Table 1: Changes in serum liver enzymes in rats treated with diclofenac and protective groups

Parameters	Group 1 (Control)	Group 2 (diclo treated)	Group 3 (vitamin A + diclo)	Group 4 (vitamin E+ diclo)	Pvalues
AST (IU/L)	41.50(40-42)	102.5(99.75-105.75)	63.50(59.50-66.25)	52(49.50-55.50)	‹0.001
ALT (IU/L)	54(52.75-56.25)	132(122.25-136.25)	63.50(62-63.50)	59.50(57.25-60.50)	‹0.001
ALP (IU/L)	80(76.50-81.25)	164(158.75-166.75)	96.50(92-98.25)	85(83-88.75)	‹0.001
Totalprotein(g/dl)	9.20(9.09-9.30)	6.02(5.88-6.17)	8.07(7.93-8.26)	9(8.88-9.02)	‹0.001
Total bilirubin (μ mol/L)	0.8(0.7-0.9)	2.05(1.99-2.20)	0.9(0.7-0.9)	0.85(0.8-0.9)	‹0.001

ALP : Alkaline phosphatase, ALT: Alanine aminotransferase, AST: Aspartateaminotransferase

Table 2: Antioxidant enzyme level in rats treated with diclofenac and protective groups

Parameters	Group 1 (Control)	Group 2 (diclo treated)	Group 3 (vitamin A + diclo)	Group 4 (vitamin E+ diclo)	Pvalues
TBARS (nmol/MDA/g)	4.66(4.47-4.82)	7.45(7.30-7.57)	5.04(4.83-5.30)	4.83(4.5-5)	‹0.001
SOD (U/mg protein)	2.20(0.14)	1.06(0.16)	1.26(0.85)	1.80(0.17)	‹0.001
GSH (μmol/g)	16.26(0.31)	12.81(0.79)	14.99(0.04)	15.80(0.24)	‹0.001
GPX (U/mg protein)	18.18(0.90)	14.43(0.37)	16.25(0.15)	17.60(0.33)	‹0.001
CAT (U/mg protein)	4.02(4.01-4.16)	2.82(2.56-2.91)	3.92(3.64-4.0)	3.92(3.64-4.0)	‹0.001

The values are expressed in Mean (SD) for theparameter of SOD, GSH and GPX (ANOVA). TBARS and CAT values are expressed inmedian (interquartile range) (KruskalWallis test)

tein level (8.07(7.93-8.26)). In group 4 with pre-administration of vitamin E with diclofenac showed a significant decrease (p<0.001) in serum liver enzymes in AST, ALT, ALP and total bilirubin (52(49.50-55.50), 59.50(57.25-60.50), 85(83-88.75) and 0.85(0.8-0.9)) which is similar to control group and significant (p<0.001) raise in total protein levels 9(8.88-9.02).

Table 2 shows changes in the antioxidant parameters after the diclofenac administration there was a significant reduction (p<0.001) in the SOD, GSH, GPX and CAT when compared to the control group with the values of (2.20(0.14), 16.26(0.31), 18.18(0.90) and 4.02(4.01-4.16)) respectively, however, there was a significant increased TBARS values seen in diclofenac group Table 2. After the pre-administration of vitamin, A reversed these values by resorted the antioxidant levels by showing a significant (p<0.001) increase on SOD, GSH, GPX and CAT levels (4.02(4.01-4.16), 14.99(0.04), 16.25(0.15) and 3.92(3.64-4.0)) respectively when compared to diclofenac-treated group and a reduced TBARS level shown in Table 2. Similar effects were seen in pre-administration with vitamin E shows a significant (p<0.001) increase in SOD, GSH, GPX and CAT (1.80(0.17), 15.80(0.24), 17.60(0.33) and 3.92(3.64-4.0)) respectively when compared to diclofenac-treated group and also significant (p<0.001) reduce values of TBARS.

The results of the present study indicated that there was a significant (p<0.001) decrease in the serum liver enzymes levels, and total bilirubin levels, as well as significant (p<0.001) increase in total protein levels in both the vitamin-treated groups however when compared between the vitamin-treated groups it clearly shows vitamin E administration shows more significant (p<0.001) than vitamin A treated group. This result suggested that the hepatoprotective activity of vitamin E tends to the more than that of vitamin A.

DISCUSSION

The liver is a major organ for the metabolism of various compounds; hepatotoxicity is usually caused by several factors such as hepatitis virus or certain chemical toxins such as CCl4 administration, alcohol intake or certain drugs such as NSAIDS leads to liver damage. In the present study, the hepatotoxic effects of diclofenac were studied by the administration in rats for 7 days intramuscularly, and the results revealed that a significant increase in the serum biochemical enzymes like ALT, AST and ALP in relation to the normal control group and also it causes alterations in antioxidant and lipid peroxidation levels. However, pre-treatment with vitamin A and E ameliorates the toxic effects of diclofenac on hepatocytes. Alterations in the biomarkers of liver enzymes like AST, ALT and ALP on the administration of diclofenac is an indication of hepatic oxidative damage (Uboh *et al.*, 2005) (Uboh *et al.*, 2007; El-Demerdash *et al.*, 2004). Increased AST and ALT level in human and animals have been previously reported for diclofenac (Fry and Seeff, 1995) (Baravalia *et al.*, 2011a). In previous literature, it was reported that administration of diclofenac leads to hepatotoxicity and even liver failure (Boelsterli, 2003). The toxicity is mainly due to the formation of reactive metabolites produced by the compounds responsible for the metabolism of diclofenac in the liver by CYP2C9, CYCP3A4 and UGT2B7 (z yan, 2005; Daly *et al.*, 2007). This reactive species interact with the hepatocytes to cause lipid peroxidation and thereby exhibiting their toxic effects.

Therefore, increase in the liver enzymes in this present study may be due to the accumulation of these reactive metabolised produced by metabolism of diclofenac in the liver is likely to enhance the formation of lipid peroxidation which affects the biomembranes and cause leakage on cellular components (Linden *et al.*, 2008). The present study showed a significant increase in serum AST, ALP, ALT and total bilirubin in a diclofenac-treated group, whereas total protein level was significantly reduced when compared to the control group. These results are highly correlated with (Thanagari *et al.*, 2012; El-Maddawy and El-Ashmawy, 2013). Bilirubin is the excretory product from the catabolism of heme, which is normally conjugated by the liver and excreted through bile, increased serum bilirubin is an indicator of liver impairment our results are also correlated with (Orinya *et al.*, 2017). It has been reported that increase in the total bilirubin level is an indication of either excessive production of bilirubin in hemolytic anemia, potential damage by chemical toxins, decreased hepatocellular excretion and impaired intrahepatic and extrahepatic bile flow (Lee, 1995).

This observation supports our present study that administration of diclofenac sodium produced a significant increase in the activities of serum ALT, AST, ALP and Total bilirubin and also reduction in the total serum protein reported on this study indicated that the hepatotoxic effects of diclofenac may result in chronic liver damage. Our findings showed a significant reduction in the level of GSH and CAT on diclofenac treated rats. These results were highly correlated with the statement given by (Khan and Ahmed, 2009) and (DeLeve *et al.*, 1996). Oxidative damage in the cell occurs either due to decreased antioxidant capacity in the cell, or increased production of ROS exceeds the antioxidant capacity of the cell (Sies and Stahl, 1995).

The levels of GSH and CAT are the main determinants of the antioxidant defence mechanism of the cell. There are few studies reported the involvement of oxidative stress during diclofenac mediated tissue toxicity (Baravalia *et al.*, 2011b). Thus, the reduced production of GSH and CAT is due to the inhibition of its metabolising enzymes in liver of diclofenac intoxicated rats may reduce the tissue capacity to protect itself from the diclofenac-induced oxidative tissue damage (Hickey *et al.*, 2001). Few authors stated that depletion in antioxidant levels indicate an increase in free radicals and thereby cellular damage is increased (Lee *et al.*, 2003)our results also supported by showed reduced antioxidant levels in diclofenac treated animals.

Our histological findings are also supports the biochemical values and showed significant changes in liver tissues in diclofenac treated rats. On microscopic examination shows that severe periacinar necrosis, moderate hydropic degeneration, fatty changes, and degenerating hepatocytes similar changes were also noted in the administration of diclofenac 50 mg/kg/bw by Nasir et al. in our study, pre-treatment was done with Vitamin A, and Vitamin E reversed these changes. Both biochemical and histological results show significant hepatoprotective effects on diclofenac treated rats. There were a merely changes in biochemical values almost within the normal limits; however, there were no significant changes noted in Vitamin A groups when compared to control. Histological evidence shows almost normal hepatocytes with the absence of necrosis on both pre-administration of Vitamin A and Vitamin E. From these results, it is evidenced that pre-administration of Vitamin A and Vitamin E ameliorates the effect of diclofenac on liver cells and thereby it protects the cellular damage and hepato-

toxicity from metabolites produces NSAIDs free radicals.

CONCLUSION

To conclude, the liver function markers (ALP, AST, ALT, and BR), serum electrolytes were found to be altered in diclofenac-induced hepatotoxicity rats. Antioxidants (SOD, CAT, GPX, GST, and glutathione reductase) and histopathological changes were also found to be altered due to diclofenac-induced toxicity. Treatment with the effective dose of Vitamin E and A significantly improved biochemical profiles as well as the histopathological changes caused by diclofenac. The present study shows that protective role of Vitamin E is predominant than vitamin A in diclofenac-induced hepatotoxicity. Hence, it is concluded from the present findings that Vitamin E hepatotoxicity exhibit role through the restoration of serum biochemical profiles and histopathological changes as well as antioxidant enzymes in the liver tissue of diclofenac-induced hepatotoxicity in male rats.

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