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Research Article

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Hepatotoxic Effect of Intraperitoneal Administration of Amiodarone in Male Rabbits

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ABSTRACT

The purpose of this study was to investigate the hepatotoxic effect of intraperitoneal administration of amiodarone in male rabbits. Study was divided into 2 groups (control group and amiodarone group) and randomized control trial was used. Biochemical parameters such as ALT, AST, ALP, LDH, bilirubin, total protein, albumin, sodium, uric acid and haematological parameters were obtained before and after drug administration. Histopathological findings were also obtained. In this research we have used amiodarone in pure form and before use of amiodarone its identification and purity was determined. Significant results were obtained when compared to the control group. Based on the results it was concluded that amiodarone induced hepatotoxicity in male rabbits.

Keywords: Amiodarone; N-desethylamiodarone; Hyponatremia; Vasoreceptors; Aquaporins thrombocytes

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INTRODUCTION

Amiodarone hydrochloride ($C_{25}H_{30}Cll_2NO_3$, 2-Butylbenzofuran-3-yl)[4-[2(diethylamino)ethoxy]-3,5-diiodophenyl]) (Figure 1) is an anti-arrhythmic drug and it is classified as class III anti-arrhythmic agent in Vaugh Williams Classification. However it also shows the properties of class I, II and IV anti arrhythmic drugs.

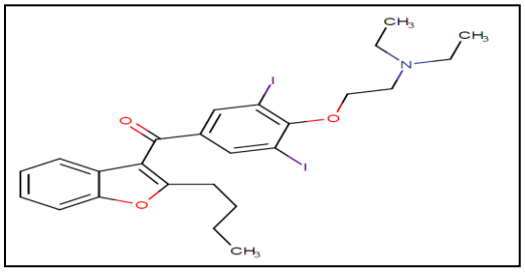

Figure 1: Chemical structure of amiodarone

Amiodarone has multi-dimensional actions as it also shows anti anginal properties and blocks α and β adrenergic receptors [1,2]. Amiodarone blocks both inward and outward currents. Amiodarone causes the prolongation of refractory period and also action potential. There will be the gene expression of K^+ channels by chronic administration of amiodarone. Amiodarone and its active metabolite both can antagonize the effect of triiodothyronine on the heart. ECG monitoring is required while amiodarone administration as it has numerous effects on various ECG intervals. Amiodarone administration causes 10% increase in PR and QT interval while there will be 15-20% decrease in sinus rate. However there will be appearance of U wave in ECG while abnormalities in T wave will be appeared [3].

Amiodarone undergoes variable and incomplete absorption. It shows about 22- 56% bioavailability with the slow extent of absorption. Amiodarone highly bounds to plasma protein (96.3 ± 0.6%) with 1.3-65.8 L/kg of volume of distribution (Vd). It biotransforms to various active and lipophilic potent metabolites by liver and N-desethylamiodarone is the most potent one. Elimination is through bile however negligible amount also eliminates through kidney. Elimination is through first order kinetics. Elimination half-life and body clearance is 3.2-52.6 days and 0.10-0.77 L/min respectively [4-6]. As Amiodarone has large Vd so loading dose must be given followed by maintenance dose. Guidelines for amiodarone dosage administration are given as follows in Table 1.

Table 1: Guidelines for amiodarone dosage administration (6)

Indication	Route of administration	Dosage	Adverse effects
Life threatening arrhythmia	IV	Bolus dose of 150 mg/10mins then 1 mg/min for 6 hrs then 0.5 mg/min for 18 hrs then further reduce IV dose or used oral dosing.	Bradycardia, Hypotension. Atrioventricular block
Atrial Fibrillation	Oral	10 g in divided doses of 600-800 mg/day then 200-400 mg/day.	GI upset, Bradycardia, Constipation, QT prolongation, torsaded de point.
Ventricular arrhythmia	Oral	10 g in divided doses of 800-1600 mg/day then 200-400 mg/day.	Bradycardia, Constipation, QT prolongation, torsaded de point

Amiodarone undergoes numerous adverse effects. Pulmonary toxicity is the first to occur which can occur with any dose of amiodarone. However other adverse effects include optic neuropathy, development of cataract symptomatic bradycardia, myocardial infarction, heart attack may also occur. Amiodarone causes bluish coloration of the skin and also deposits on eyes, skin, lungs and liver due to its lipophilic property. Amiodarone causes liver cirrhosis and hepatotoxicity also. Thyroid abnormalities, peripheral neuropathy, GIT disturbances and nausea also observed with amiodarone administration [7,8]. There will also be electrolyte disturbances with amiodarone [8]. Amiodarone falls under category D by FDA. It interacts and inhibits the metabolism of large number of drugs as it is inhibitor of cytochrome P450 [6]. In the present investigation, hepatotoxicity was investigated by amiodarone hydrochloride which is 2-Butyl-3-(3',5'-diiodo-4'α-diethylaminoethoxybenzoyl)-benzofuran compound.

MATERIALS AND METHODS

Experimental Animals

Male rabbits (1.5-2.5 kg weight) of local strain were used in the study. Rabbits were placed in iron cages at 25°C temperature with 24 hrs availability of fresh green fodder and water. Before the experiment, rabbits were acclimatized for period of one week. AEC/UCP/1016/4313 voucher number was obtained by the animal ethical committee of University College of Pharmacy, University Of Punjab, Lahore.

Chemicals

Amiodarone pure material was purchased from the sigma Aldrich. Liquid paraffin, ethanol, formalin and methanol were purchased from sigma Aldrich. Kits of Cresent Diagnostics, Saudi Arabia was used for estimation of ALT, AST, ALP, Bilirubin, Total protein, Albumin and uric acid. Kits of Human, Germany and Global *in vitro* LLP, UK were used for LDH and sodium estimation respectively. Acetonitrile and methanol HPLC grade are from J.T Baker (NJ, USA). Glacial acetic acid and ammonia are from Merck (Darmstadt, Germany). All the chemicals and reagents used in the experiment were of analytical grade.

Apparatus

HPLC system (Shimadzu Model: LC-2010CHT) with pump, auto sampler, column oven, and L-2490 UV detector was employed. The novochrom Elite software was employed. C18 column (5 µm, 150 mm length, 4.6 mm inner diameter) was used in this study and it is from Waters Corporation (Milford, Massachusetts, USA). The column is kept at room temperature.

Shimadzu UV-VS 2550 Spectrophotometer, Human Humalyzer, Human Hemacount machine, Centrifuge machine BHG and weighing balance Setra BL-410S was used in the study. Amiodarone Hydrochloride contains NL T 98.5% and NMT101.0% of $C_{25}H_{29}I_2NO_3 \cdot HCl$, calculated on the dried basis.

Procedure
Buffer: Dissolve 6.80 g of monobasic potassium phosphate in 900 mL of water, and add 1.0 mL of triethylamine. Adjust with phosphoric acid to a pH of 6.00 ± 0.05, and dilute with water to 1000 ml.
Diluent: Acetonitrile and water (1:1).
Mobile phase: Acetonitrile and Buffer (1:1).
Standard stock solution: 0.5 mg/mL of USP Amiodarone Hydrochloride RS in methanol.
Standard solution: 0.1 mg/mL USP Amiodarone Hydrochlo-ride RS in diluent from standard stock solution.
Sample stock solution: 0.5 mg/mL of Amiodarone Hydro-chloride in methanol.
Sample solution: 0.1 mg/mL of Amiodarone Hydrochloride in diluent from sample stock solution.

Chromatographic System
Mode: LC
Detector: UV 240 nm
Column: C18 column (5 µm, 150 mm length, 4.6 mm inner diameter)
Flow rate: 1.5 mL/min
Injection size: 10 µL

Analysis
Samples: Standard solution and sample solution. Calculate the percentage of $C_{25}H_{29}I_2NO_3 \cdot HCl$ in the portion of amiodarone hydrochloride taken.

Preparation of Amiodarone Solution and Toxicity Induction
200 mg amiodarone solution was freshly prepared (by using 1 portion of high grade ethanol and 3 portions of distilled water) and injected intraperitoneally thrice a day to male rabbits and toxicity was induced [9].

Experimental Design and Treatment Protocol
Study was divided into two groups; control and amiodarone group.
Group I:
Control group: 2 ml 25% hydroalcoholic solution was administered intraperitoneally three times a day.
Group II:
Amiodarone group: 200 mg amiodarone solution was freshly prepared and administered intraperitoneally three times a day for three days.
Before administration of drug, blood sample was obtained from marginal ear vein and all biochemical, haematological and histopathological parameters were note down to estimate the health of rabbits and only healthy rabbits were selected for study and sample was obtained after 24, 48 and 72 hours of amiodarone administration.

Histopathological Analysis
Rabbit's liver were removed carefully and fixed, processed and hardened in 38% formalin, ethanol and xylene respectively. Paraffin was used to embed liver tissues. Blocks were then prepared for microtoming. Gelatin was used to fix the slices which were then dried in the oven at 58°C for 10-12 hrs. Hematoxylin and eosin stain was used to stain the slides. Slides were examined after covering and sealing with cover slip and wax respectively.

Statistical Analysis
Values were expressed as mean ±SE which were compared by applying student T test using SPSS Statistics 13.0. P value was then calculated from student T-table [10]. $P<0.05$ was considered statistically significant.

RESULTS

Assay of Amiodarone
On the HPLC assay basis which is according to USP monogram we find that material which we are going to use for our experiment is pure and its assay is under USP limit (Figures 2 and 3).

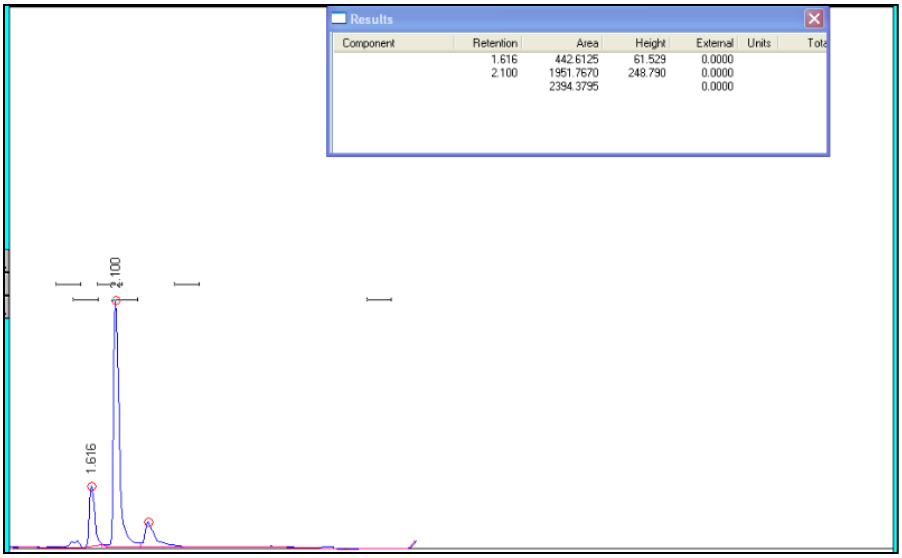

Figure 2: Assay of reference standard

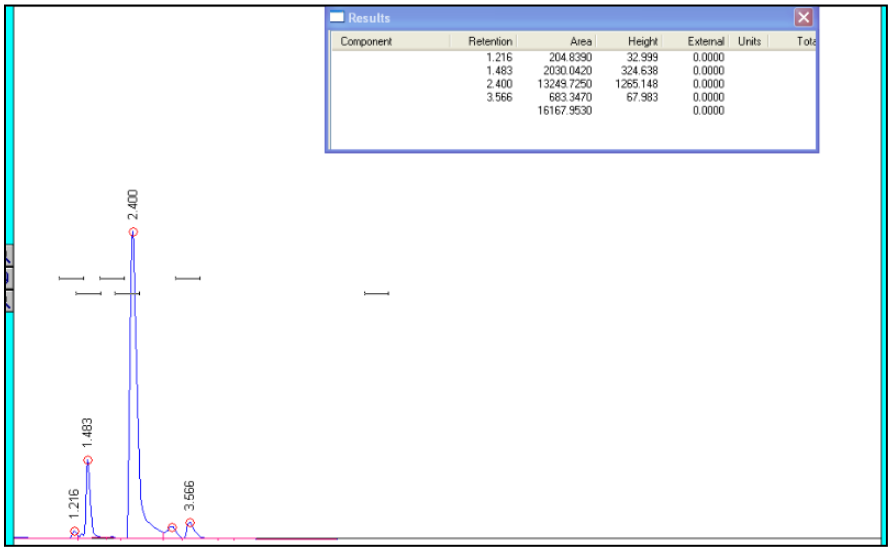

Figure 3: Assay of raw material Amiodarone

Procedure

200 mg Amiodarone solution was administered three times a day which induced injury to the hepatocytes. Blood sample was obtained before, during and after drug administration and it was observed that significant change in marker enzymes were found after 24, 48 and 72 hours of drug administration. When results of control group and amiodarone group were compared then elevation of serum ALT and AST levels were observed in group II as shown in Table 2. However ALP, Protein, bilirubin and uric acid levels show insignificant difference as shown in Tables 3-5. After 48 and 72 hours, elevated LDH, decreased albumin level (after 72 hours) and decreased sodium level was observed in group II when compared with the group I as shown in Tables 3-5 respectively.

Table 2: Comparison of control and amiodarone group on Alanine transaminase (ALT) and Aspartate transaminase (AST)

GROUP No.	Groups	ALT Mean ± SE			AST Mean ± SE		
		(after 24 hours)	(after 48 hours)	(after 72 hours)	(after 24 hours)	(after 48 hours)	(after 72 hours)
I	**Control group**	20.53 ± 2.60	20.92 ± 3.01	22.16 ±3.18	16.69 ± 2.45	15.35 ± 2.86	18.16 ± 2.31
II	**AMD group**	43.07 ± 5.45*	40.72 ± 4.54*	44.80 ± 2.25*	26.43 ± 2.09*	32.01 ± 8.25*	31.07 ± 5.33*

For P value:* represents P<0.05

Table 3: Comparison of control and amiodarone group on alkaline phosphatase (ALP), and Lactate dehydrogenase (LDH)

GROUP No.	Groups	ALP Mean ± SE			LDH Mean ± SE		
		(after 24 hours)	(after 48 hours)	(after 72 hours)	(after 24 hours)	(after 48 hours)	(after 72 hours)
I	**Control group**	49.64 ± 3.55	46.00 ± 3.36	48.05 ±3.28	309.00 ± 20	314.02 ± 17.09	310.08 ± 16.81
II	**AMD group**	85.43 ± 9.46	71.52 ± 17.73	69.23 ± 11.10	304 ± 42.54	746 ± 45.99*	836.90 ± 12.77 *

For P value:* represents P<0.05

Table 4: Comparison of control and amiodarone group on Protein and Albumin

GROUP No.	Groups	Protein Mean ± SE			Albumin Mean ± SE		
		(after 24 hours)	(after 48 hours)	(after 72 hours)	(after 24 hours)	(after 48 hours)	(after 72 hours)
I	**Control group**	6.43 ± 0.25	6.59 ± 0.42	6.39 ± 0.36	5.12 ± 0.19	5.21 ± 0.14	5.24 ± 0.13

For P value:* represents P<0.05

Table 5: Comparison of control and amiodarone group on Bilirubin, Uric acid and sodium

Group No.	Groups	Bilirubin Mean ± SE			Uric acid Mean ± SE			Sodium Mean ± SE		
		(after 24 h)	(after 48 h)	(after 72 h)	(after 24 h)	(after 48 h)	(after 72 h)	(after 24 h)	(after 48 h)	(after 72 h)
I	**Control group**	0.63 ± 0.034	0.62 ± 0.048	0.61 ± 0.032	6.39 ± 0.98	5.65 ± 0.86	6.02 ± 0.88	130.14± 15.47	123.29 ± 17.23	127.00 ± 14.52
II	**AMD group**	0.65 ± 0.14	0.65 ± 0.09	0.65 ± 0.13	6.03 ± 1.23	8.73 ± 0.85	11.57 ± 1.32	168.75 ± 26.12	81.97 ± 20.69*	29.74 ± 8.81*

For P value:* represents P<0.05

Table 6: Comparison of control and amiodarone group on haematological parameters [White blood cell count (WBC), Lymphocytes (LYM), Minimum inhibitory dilution (MID), Granulocytes (GRA), Lymphocytes percentage (LY%), Red blood cells (RBC), Hemoglobin (HG), Hematocrit (HCT), Mean platelet volume (MPV), Platelet distribution width (PDWc), MCV (Mean corpuscular volume), PLT (Platelets), PCT (Platelet Crit), Mean corpuscular hemoglobin (MCH) and Mean corpuscular hemoglobin concentration (MCHC)]

Parameter	Control Group	AMD group		
		after 24 hrs	**after 48 hrs**	**after 72 hrs**
WBC	5.58 $\times 10^9$/l	5.33 $\times 10^9$/l	4.65 $\times 10^9$/l	6.05 $\times 10^9$/l
		(↓ 4.48%)	(↓ 16.67%)	(↑ 8.42%)
LYM	2.53 $\times 10^9$/l	2.65 $\times 10^9$/l	3.08 $\times 10^9$/l	2.97 $\times 10^9$/l
		(↑ 4.74%)	(↑ 21.74%)	(↑ 17.39%)
MID	0.12 $\times 10^9$/l	0.53 $\times 10^9$/l	0.25 $\times 10^9$/l	0.6 $\times 10^9$/l
		(↑ 341.6%)	(↑ 108.3%)	(↑ 400.0%)
GRA	1.03 $\times 10^9$/l	1.44 $\times 10^9$/l	1.33 $\times 10^9$/l	2.5 $\times 10^9$/l
		(↑ 39.81%)	(↑ 29.13%)	(↑ 142.72%)
LY%	47.10%	50.50%	68.40%	64.10%
		(↑ 8.07%)	(↑ 45.22%)	(↑36.09%)
GR%	50.90%	39.90%	27.00%	29.20%
		(↓ 21.61%)	(↓ 46.95%)	(↓ 42.63%)
RBC	2.98 $\times 10^{12}$/l	4.36 $\times 10^{12}$/l	5.16 $\times 10^{12}$/l	5.78 $\times 10^{12}$/l
		(↑ 46.31%)	(↑ 73.15%)	(↑ 93.96%)
HGB	5.5 g/dl	8.7 g/dl	10.3 g/dl	6.4 g/dl
		(↑ 58.18%)	(↑ 87.27%)	(↑ 16.36%)
HCT	16.70%	24.15%	28.00%	30.20%
		(↑ 44.61%)	(↑ 67.66%)	(↑ 80.84%)
MCV	56 ft	55 ft	54 ft	53 ft
		(↓ 1.79%)	(↓ 3.57%)	(↓ 1.79%)
MCH	18.65 pg	19.9 pg	19.4 pg	12.5 pg
		(↑ 6.70%)	(↑ 4.02%)	(↓ 32.98%)
MCHC	33.1 g/dl	36.05 g/dl	35.9 g/dl	23.4 g/dl
		(↑ 8.91%)	(↑ 8.46%)	(↓ 29.31%)
RDWc	12.70%	12.80%	13.60%	14.60%
		(↑ 0.79%)	(↑ 7.09%)	(↑ 14.96%)
PLT	157 $\times 10^9$/l	39 $\times 10^9$/l	45 $\times 10^9$/l	73 $\times 10^9$/l
		(↓ 75.16%)	(↓ 71.33%)	(↓ 53.50%)
PCT	0.07%	0.02%	0.02%	0.03%
		(↓ 71.43%)	(↓ 71.43%)	(↓ 57.14%)
MPV	4.2 ft	4.6 ft	4.3 ft	4.8 ft
		(↑ 9.52%)	(↑ 2.38%)	(↑ 14.9%)
PDWc	28.90%	29.20%	30.30%	34.20%
		(↑ 1.04%)	(↑ 4.84%)	(↑ 18.34%)
Lyse	0.50 ml	0.50 ml	0.50 ml	0.50 ml

Administration of 200 mg amiodarone markedly decrease white blood cell count (WBC) after 24 and 48 hours but after 72 hours of amiodarone WBC count is increased when compared with control group. Lymphocytes (LYM) count also increased. Minimum inhibitory dilution (MID), Granulocytes (GRA), Lymphocytes percentage (LY%), Red blood cells (RBC), Haemoglobin (HG), Hematocrit (HCT), Mean platelet volume (MPV) and Platelet distribution width (PDWc) increased after amiodarone administration. However MCV (Mean corpuscular volume), PLT (Platelets), PCT (Platelet crit) decreased while MCH and MCHC first increased and then decreased 72 hrs of amiodarone administration as shown in Table 6. Percentage of increase or decrease of parameters is shown in paranthesis. Histopathological comparison of liver of control and amiodarone group is shown in Table 7 which showed that amiodarone dilated the vascular channels and portal tract with the presence of fibrosis, fibroblasts and inflammation. However amiodarone also caused steatosis, necrosis and ballooning degeneration as shown in Figures 4-7.

Table 7: Histopathological findings of control and AMD group

Parameters	Control Group	Amiodarone Group
Vascular channels	Normal	Dilated
Fibrosis	NO	YES
Fibroblasts	NO	YES
Periportal inflammation	NO	YES
Portal Tract	Normal	Widened
Necrosis	NO	Coagulative and Periportal necrosis
Ballooning degeneration	NO	YES
Steatosis	NO	YES

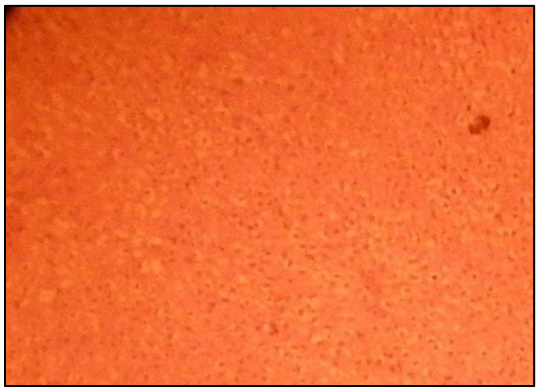

Figure 4: Hematoxylin and eosin staining of liver of control group ×10

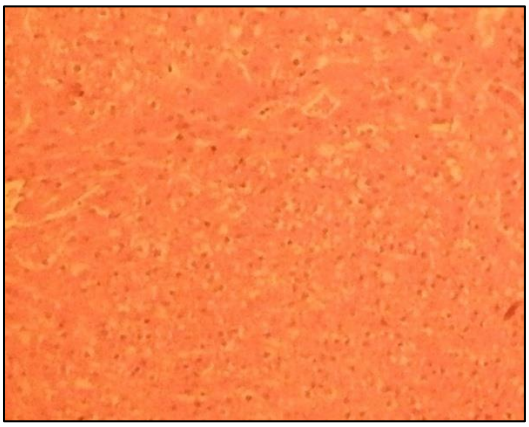

Figure 5: Hematoxylin and eosin staining of liver of control group ×10

Amiodarone is a class III anti-arrhythmic drug used in the treatment of ventricular fibrillation and in refractory angina. It shows multiple therapeutic uses because of its various pharmacological and electrophysiological actions. Amiodarone is also associated with various adverse effects along with idiosyncratic hepatotoxic reactions [11].

In this study, 200 mg amiodarone solution was administered intraperitoneally and liver toxicity was investigated. Amiodarone and its active metabolites accumulate in liver and kupffer cells and causes phospholipidosis. It abrupts the mitochondrial oxidation and causes uncoupling of oxidative phosphorylation. Nuclear disassembly may occur because of oxidative stress produced by free radicals. Oxidative stress leads to the leakage of enzymes from the cells [12]. Therefore elevation of serum ALT and AST enzyme activities in group II indicates damage to the liver as shown in Table 2.

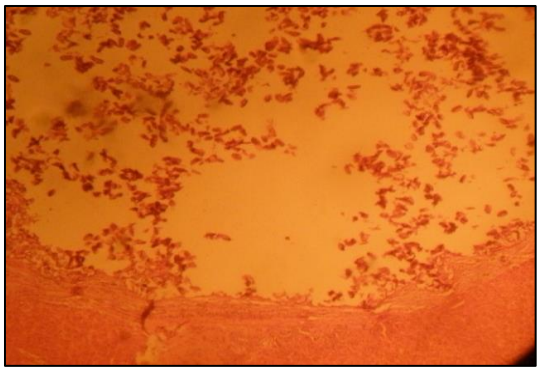

Figure 6: Hematoxylin and eosin staining of liver of amiodarone group ×10

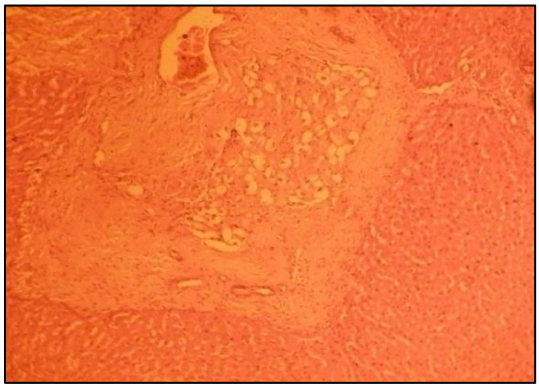

Figure 7: Hematoxylin and eosin staining of liver amiodarone group ×10

DISCUSSION

Chronic amiodarone administration causes hepatic dysfunction from benign to fatal hepatitis and cirrhosis. Thus may leads to apoptosis and necrosis of the hepatocytes. However the exact injury mechanism is unknown [13] and any damage or disease of the liver effect amiodarone metabolism which may further worsening the condition and causes amiodarone induced hepatotoxicity [14]. Amiodarone also activates magnesium dependent neutral sphingomyelinase (N-SMase) mechanism by decreasing the level of glutathione. N-SMase then activates protein kinase/c-jun kinase (SAPK/JNK) signaling pathway by increasing the level of ceramide and thus lead to apoptosis of hepatocytes [15].

In group II, increased serum LDH level was observed with highly significant P value when compared with the control group as shown in Table 3. LDH actually used as hypoxic marker and thus increased LDH level indicates transcription under anaerobic environment [16].

Hyponatremia was observed with amiodarone in group II when compared with group I. Mutation of aquaporins and suppression of ADH hormone may occur with amiodarone administration. This is turn will lead to suppression of adenyl cylase and inability of vasoreceptors (V2) to insert into the apical membrane of distal tubule and collecting duct epithelial cells. This will result into decreased water reabsorption and increased water and electrolytes excretion, thus lead to hyponatremia by amiodarone [17].

Decreased albumin level was observed in group II as shown in Table 4 because of decreased albumin synthesis by damaged hepatocytes. However it may also because of malnutrition and malabsorption [18]. Endoplasmic reticulums (ER) in hepatocytes are responsible for synthesis of proteins. ER stress was observed due to release of various intracellular signaling mediators which then decrease albumin synthesis [19,20].

Decreased uric acid excretion was observed in Table 5 with amiodarone administration because of tubular alteration with the partial loss of brush boarder microvilli and necrotic tubular epithelium [21]. Histopathologic examination of liver further confirmed the hepatotoxic effect of amiodarone. Leucocytosis was observed after 72 hours of amiodarone administration in group II as shown in Table 6. It may because of hypersensitivity or allergic reactions. Increased in granulocytes and lymphocytes count may because of release of inflammatory mediators by immune system. Activation of the immune system may further cause oxidative stress by production of free radicals. Erythrocytosis, increased in hemoglobin and hematocrit values were also observed with amiodarone. As amiodarone move the hepatocytes towards hypoxic condition thus upregulates the secretion of erythropoietin from the kidney which then compensates hypoxia by stimulate the production of red blood cells from bone marrow. Amiodarone resulted into thrombocytopenia as shown in table because of reduced level of thrombopoietin, which is produced by the liver and also it directly depress the vitamin K dependent clotting factor resulting into abnormalities into the blood flow and increased prothrombin time [22].

CONCLUSION

Amiodarone induced biochemical, hematological and histological changes by interrupting the balance between oxidant and antioxidant system. Thus it was concluded that hepatotoxicity was induced by amiodarone.

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