CARDIOPROTECTIVE EFFECT OF COENZYME Q_{10} ON CARDIAC MARKER ENZYMES AND ELECTROCARDIOGRAPHIC ABNORMALITIES IN DIGOXIN INDUCED CARDIOTOXICITY IN WISTAR RATS

Arvindkumar E. Ghule, Subhash L. Bodhankar*

Department of Pharmacology, Poona College of Pharmacy, Bharati Vidyapeeth University, Pune, India.

Summary

The objective of the present study was to investigate effect of Coenzyme Q_{10} supplementation (100 mg/kg), on digoxin induced cardiotoxicity in rats. Male wistar rats (250-300g) were divided into 2 groups of eight animals each. Group I- control group (Olive oil 2 ml/kg/day p.o. for 18 days). and group II- Coenzyme Q_{10} group (Animals received CoQ_{10} (100 mg/kg/day p.o. for 18 days) and on 18^{th} day in both group digoxin was administered in anesthetized animal by jugular vein till the cardiac arrest occurred. Electrocardiogram (ECG) was recorded in anaesthetized rats. Blood was withdrawn via two way cannula exactly at the start of cardiac arrest which was monitered by electrocardiographic observation. Estimation of serum creatine kinase-MB isoenzyme (CK-MB), lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) were performmed. The hearts of 6 animals were selected for estimation of superoxide dismutase (SOD), reduced glutathione (GSH), lipid peroxidation and total protein. The histopathology of hearts of remaining two animals was carried out. In control animals cardiac arrest was produced by 3820 ± 101.98 µg/kg whereas in drug treated group cardiac arrest occurred at 4460 ± 396.99 µg/kg. Drug treatment nonsignificantly reduced serum CKMB and AST while that of LDH was significantly decreased. Coenzyme Q_{10} treatment significantly increased SOD and GSH, whereas Concentration of MDA was significantly decreased. Histopathology showed protection against digoxin induced Cardiotoxicity. Prolong time of cardiac arrest along with significant decrease in serum LDH , heart MDA and increase in GSH and SOD as well as result of histopathological analysis indicated Cardioprotective activity of Coenzyme Q_{10} treatment in digoxin Cardiotoxicity.

Keywords: Antioxidants, Cardiac arrhythmia, cardiac toxicity, Coenzyme Q_{10}, and ROS.

***Correspondance address** :
Dr. S.L. Bodhankar**,**
Professor and Head**,**
Department of Pharmacology,
Poona College of Pharmacy,
Bharati Vidyapeeth University,
Erandawane, Pune-411038, India.
Tel: +91-20-25437237,
Fax: +91-20-25439383,
E-mail: sbodh@yahoo.com

Introduction

The main pharmacologic effect of digitalis compounds is their property to increase the contractile strength of heart muscle in a dose-dependent way by positive inotropic effect. This property has been the mainstay of treatment of heart failure during the last 200 years. In addition to its inotropic effect, digitalis produces severe toxicity in the gastrointestinal and nervous systems, as well as in the heart itself [1]. Both inotropic and toxic effects have been attributed principally to total or partial inhibition respectively, of the responsible enzyme for Na+ and K+ transport through the cell membrane, that is, Na+–K+-dependent adenosintriphosphatase (Na+/K+-ATPase) [2,3]. It has been proposed that the toxicity mechanism is due to a decrease in K+ and an overload of Ca2+ intracellularly due to persistent inhibition of the Na+-pump, producing electrophysiological changes such as reduction in resting potential and a rise in cell automatism that leads to homeostasis loss and cell death [4]. It is reported that digitalis at toxic concentration (N10 nM) produced morphologic (nuclear condensation and fragmentation) and biochemical (release of mitochondrial cytochrome c, caspase-9 and -3 activation, nuclear DNA degradation) changes related with apoptosis in HeLa cells. CoQ_{10} which is also known as ubiquinone, is a lipid-soluble, vitamin-like substance presented in the hydrophobic interior of phospholipid bilayer of virtually all the cellular membranes [5]. The most well-known function of CoQ_{10} is enhancement of mitochondrial activity related to the synthesis of adenosine triphosphate (Turunen et al. 2004). In addition, CoQ_{10} plays a role in inhibiting lipid peroxidation by either scavenging ROS directly or in conjunction with α-tocopherol. Principal function of CoQ_{10} is to act as an electron carrier between the NADH and succinate dehydrogenases and the cytochrome system [6]. During mitochondrial electron transport, ubiquinone also occurs as semiquinone and ubiquinol, the fully reduced form of ubiquinone. Semiquinone has a role in the generation of superoxide anions during mitochondrial respiration [7], whereas ubiquinol functions as an intracellular antioxidant, presumably by preventing both the initiation and propagation of lipid peroxidation. CoQ_{10} appears to be involved in the coordinated regulation between oxidative stress and antioxidant capacity of heart tissue, when the heart is subjected to oxidative stress in various pathogenic conditions [8]. In view of the role of mitochondrial enzymes in the generation of oxidative system and ensuring cardiotoxicity. It was hypothesized that drug having antioxidant activity may impart cardiotoxicity.

Materials and Methods

Animals: Male wistar albino rats (250-300 g) were obtained from National Toxicological Centre (NTC) Pune. Rats were housed under standard housing conditions of 25ºC, relative humidity 60% and photo period of 12 h dark/12 h light. Pellet diet (Chakan Oil Mills, Pune, India) and water were provided *ad libitum*. All experimental protocols including the animal studies were approved by the Institutional Animal Ethics Committee (IAEC) of Poona College of pharmacy, Bharati Vidyapeeth University, Pune, India.

Chemicals and sample: The Coenzyme Q_{10} (Medicines private limited, Mumbai) and digoxin injection (Samarth Life Sciences private limited, Mumbai) were procured from respective companies. Epinephrine hydrochloride, super oxide dismutase (SOD) and malondialdehyde were purchased from sigma Chemical Co., USA. Reduced glutathione (GSH), 5, 5′-dithiobis (2-nitro benzoic acid) (DTNB) and thiobarbituric acid (TBA) were obtained from Hi media; India.

All chemicals used were of analytical grade. CK-MB kit was purchased from Randox Laboratories Ltd., Ardmore, Diamond Road, Crumlin, Co. Antrim, United Kingdom, BT29 4QY and LDH as well as AST from Ecoline, Merck Ltd, Worli, Mumbai -400 018.

Experimental procedure: The dose schedule of digoxin was selected on the basis of previous pilot dose response study. Male wistar rats (250-300g) were divided into 2 groups of eight animals each. Group I- control group (Olive oil 2 ml/kg/day p.o. for 18 days). and group II- Coenzyme Q_{10} group (CoQ_{10} (100 mg/kg/day p.o. for 18 days) and on 18^{th} day in both group digoxin was administered in anesthetized animal by jugular vein till the cardiac arrest occurred. The animals were anesthetized with urethane. ECG was recorded using 8 channels Power Lab System (AD Instruments Pty Ltd, Unit 13, 18-22 Lexington Drive, Bella Vista NSW 2153, Australia). Blood was centrifuged at 7500 rpm for 15 minutes at 0ºC. Then serum was transferred by using micropipette in eppendorfs tubes and stored at 4ºC till analyzed. Immediately after cardiac arrest animals hearts were isolated and weighed. Two hearts from two animals were randomly selected for histopathology. The hearts from remaining six animals were cut in to small pieces, placed in chilled 0.25M sucrose solution and blotted on a filter paper. The tissues were then homogenized in 10% chilled tris hydrochloride buffer (10mM, pH 7.4) by tissue homogenizer (Remi Motors, Mumbai, India 400 058) and centrifuged at 7500 rpm for 15 minutes at 0ºC using Eppendorf 5810-R high speed cooling centrifuge. The clear supernatant was used for the estimation of SOD, GSH, MDA content and total protein.

Serum parameters: Serum levels of creatine kinase-MB isoenzyme (CK-MB), lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) enzymes were measured by automated chemistry analyzer, Micro lab 300, Merck, using reagent kits.

Tissue Parameters

Lipid peroxidation assay (MDA content) : This assay was used to determine thiobarbituric acid-reactive substances (TBARS) level as described by Slater and Sawyer [9]. Supernatant of tissue homogenate (2 ml) was collected in eppendorfs and 2.0 ml of freshly prepared 10% w/v trichloroacetic acid (TCA) was added to it. The mixture was allowed to stand in an ice bath for 15 minutes. After 15 minuets, followed by centrifugation at 2500 rpm for 15 minutes at 0°C. Two ml of clear supernatant solution was mixed with 2.0 ml of freshly prepared 0.67%w/v thiobarbituric acid. The resulting solution was heated in a boiling water bath for 10 minutes. It was then immediately cooled in an ice bath for 5 minutes. The absorbance of colour developed was measured by UV/VIS spectrophotometer (JASCO-V-530, Japan) at 532 nm using 1, 1, 3, 3-tetraethoxypropane as a standard.

Estimation of GSH: The assay of GSH was carried out by Moron *et al* [10]**.** 1.0 ml of tissue homogenate (supernatant) and 1 ml of 20% TCA were mixed and centrifuged at 2500 rpm for 15 minutes at 0°C. In 0.25 ml of supernatant, 2 ml of 5, 5´-dithiobis (2-nitro benzoic acid) (0.6M) reagent was added. The final volume was made up to 3 ml with phosphate buffer (pH 8.0). The colour developed was read at 412 nm against reagent blank. Different concentrations (10-50µg) of standard glutathione were processed as mentioned above for constructing standard curve. The amount of reduced glutathione was expressed as µg of GSH /gm of protein.

Estimation of SOD activity: The SOD activity was determined by the method of Misera and Fridovich [11]. 0.5 ml of heart homogenate, 0.5 ml of cold distilled water, 0.25 ml of ice-cold ethanol and 0.15 ml of ice -cold chloroform were mixed well using cyclomixer for 5 minutes and centrifuged at 2500 rpm for15 minutes at 0°C. To 0.5 ml of supernatant, 1.5 ml of carbonate buffer (pH 10.2) and 0.5 ml of 0.4 M ethylenediaminetetraacetic acid (EDTA) solutions were added. The reaction was initiated by the addition of 0.4 ml of epinephrine bitartarate (3mM) and the change in optic density /minute was measured at 480 nm against reaction blank. SOD activity was expressed as units /mg protein. Change in optical density per minute at 50% inhibition of epinephrine to adrenochrome transition by the enzyme was taken as the enzyme unit. Calibration curve was prepared by using 10-125 units of SOD.

Determination of total proteins: Protein concentrations were determined using the method of Lowry *et al* [12]. Diluted fraction aliquots (0.1ml) from supernatant were taken in test tubes. To this 0.8 ml of 0.1M sodium hydroxide and 5ml of Lowry C reagent (freshly prepared mixture of 1 ml of (0.5%w/v) Copper sulphate in 1% sodium potassium tartarate into 50 ml of (2% w /v) sodium carbonate in 0.1M sodium hydroxide) was added and the solution was allowed to stand for 15 minutes. Then 0.5ml of 1N Folin´s phenol reagent was added and the contents were mixed well by vortex mixer. Colour developed was measured at 640 nm against reagent blank containing distilled water instead of sample. Different concentrations (40-200µg) of standard protein bovine serum albumin (BSA) were processed as mentioned above for preparation of standard curve. The values were expressed as mg of protein /g of wet tissue (mg /g).

Histopathological studies: Hearts were quickly removed, preserved in 10% formalin, processed and embedded in paraffin. Four µm thick paraffin sections were cut on glass slides and stained with hematoxylin and eosin (H&E) reagents and observed under light microscope to evaluate myocardium injury.

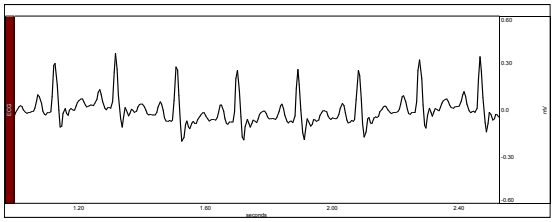
Statistical analysis: The statistical analysis was done by software package of Graph pad prism (version 4.03). All values were expressed as mean ± SEM. Data was analysed by two way ANOVA followed by *post hock* Mann Whitney test and Bonferroni post tests. Survival curves analysis was carried out by Logrank Test. $P < 0.05$ was considered statistically significant.

Results

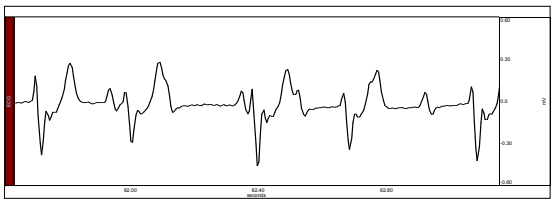
Effect of Coenzyme Q_{10} (100mg/kg) on digoxin induced cardiac arrhythmia & cardiac arrest in male wistar Rats : The results of present investigation indicated that the mean dose of digoxin require to produce ectopic beats was 3660 µg/kg, ventricular fibrillation 3700 µg/kg and cardiac arrest 3820 µg/kg in 6 male wistar Rats. The test drug CoQ_{10} (100mg/kg by oral route) pretreated for 18 days as per acute treatment before digoxin administration, that was found to increase the dose of digoxin required to produce ectopic beats (4380 µg/kg), ventricular fibrillation (4420 µg/kg) and cardiac arrest (4460 µg/kg). The results thus indicated that CoQ_{10} is effective in prolonging the arrhythmogenic effect of digoxin and death by cardiac arrest (Figure 1 and 2).

Figure 1: ECG recording after administration of various concentration of digoxin in vehicle and CoQ_{10} treated male Wistar rats.

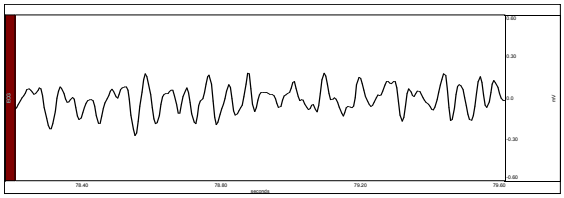
(Vehicle +digoxin group)



(1) Normal response (before digoxin administration)

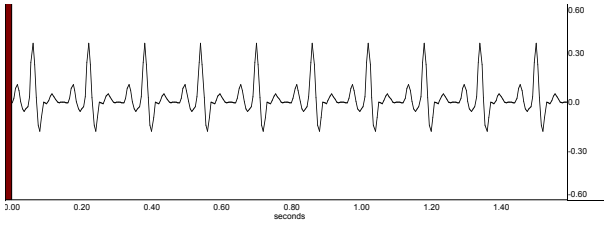


(2) After 3660 µg/kg of digoxin administration showing ectopic beats.

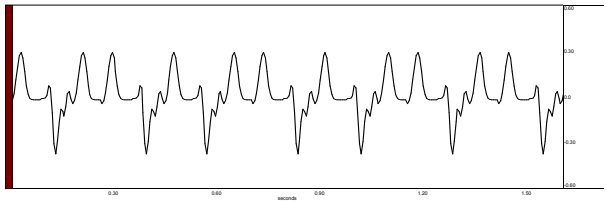


(3) After 3700 µg/kg of digoxin administration showing ventricular fibrillation .

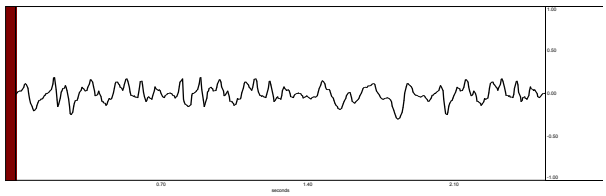
(CoQ_{10} + digoxin group)



(1) Normal response (before digoxin administration)

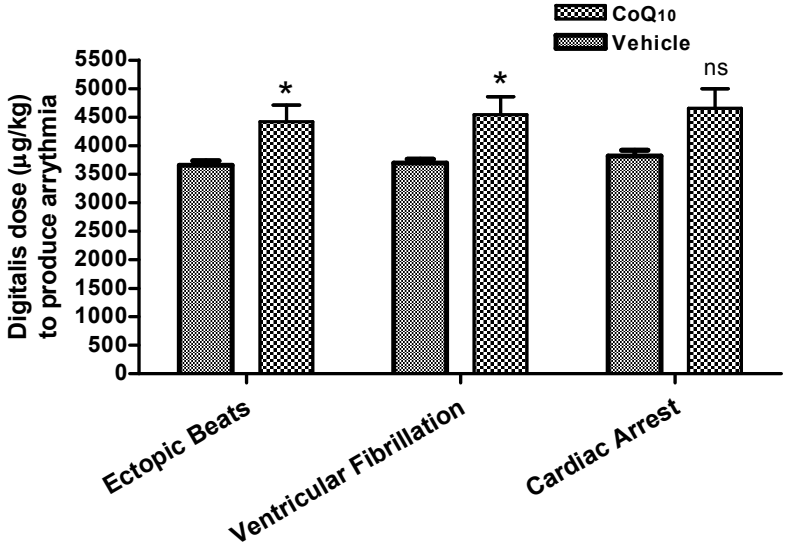


(2)After 4380 µg/kg of digoxin administration showing ectopic beats.



(3) After 4420 µg/kg of d digoxin administration showing ventricular fibrillation .

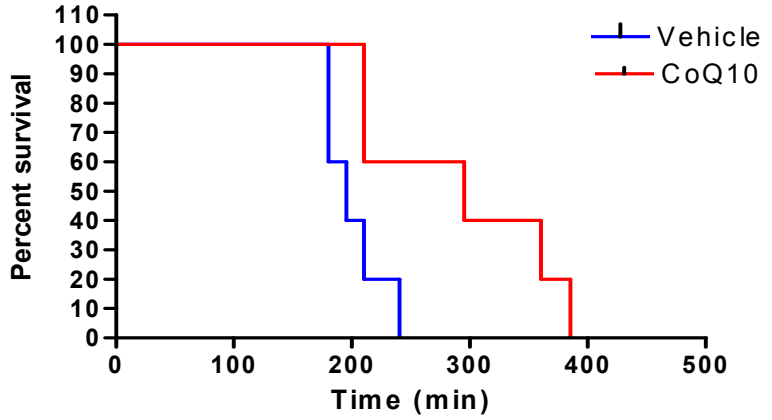
Figure 2: Effect of toxic concentration of digoxin in CoQ_{10} (100 mg/kg) and vehicle treated male Wistar rats.



Values are expressed as mean ± SEM., (n=6) Data analyzed by Mann Whitney test, * $p<0.05$ as compared with vehicle group, ns-non significant.

Effect of CoQ_{10} (100mg/kg) on time factor and Survival Curves in digoxin induced cardiotoxicity: The median survival time for digoxin induced cardiac arrest was 195 min. whereas for CoQ_{10} group was 295 min (Figure 3). The result thus indicated the longevity of life span of CoQ_{10} treated animals.

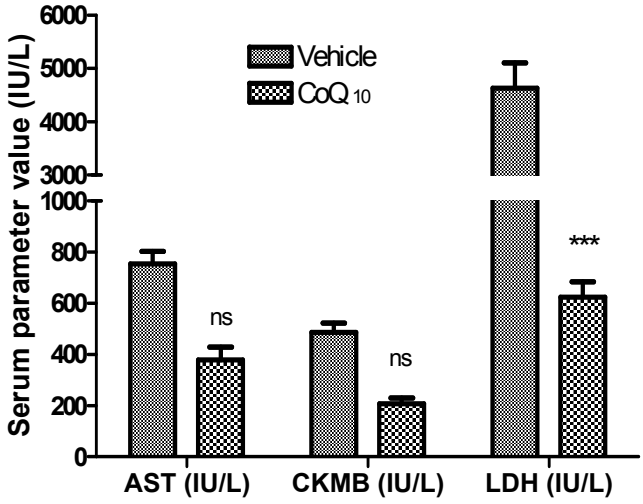
Figure 3: Effect of CoQ_{10} (100mg/kg) on time factor and survival curves in digoxin induced cardiotoxicity.



Data analyzed by Logrank Test, (n=6) * $p<0.05$ as compared with vehicle group. Median survival Time for cardiac arrest for Vehicle group was 195 min, median survival time for cardiac arrest for in CoQ_{10} group was 295 min.

Serum enzymes concentration: The CoQ_{10} pretreatment resulted in non significant decreased in serum CK-MB and AST levels compared to that of control group. Whereas the levels of LDH in CoQ_{10} group was significantly ($p < 0.001$) decreased as compared to control group (Figure 4).

Figure 4: Effect of CoQ_{10} (100mg/kg/day for 18 day) on levels of AST, CK-MB and LDH in serum of male wistar rats receiving toxic dose of digoxin.

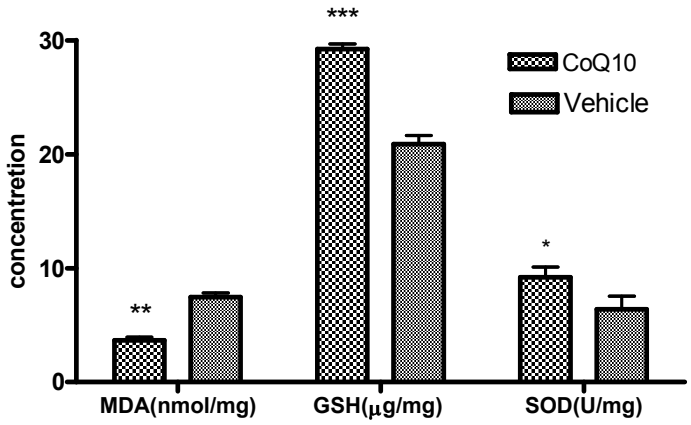


Values are expressed as mean ± SEM Data analyzed by two way ANOVA., followed by Bonferroni post tests. *** $p<0.001$ as compared with Vehicle group. ns- non significant as compared with Vehicle group.

Tissue enzymes concentration: CoQ_{10} treatment produced significant increase in GSH ($p < 0.001$) and SOD ($p < 0.05$) as compared to control group, whereas concentration of MDA was found to be significantly ($p<0.01$) decreased (Figure 5).

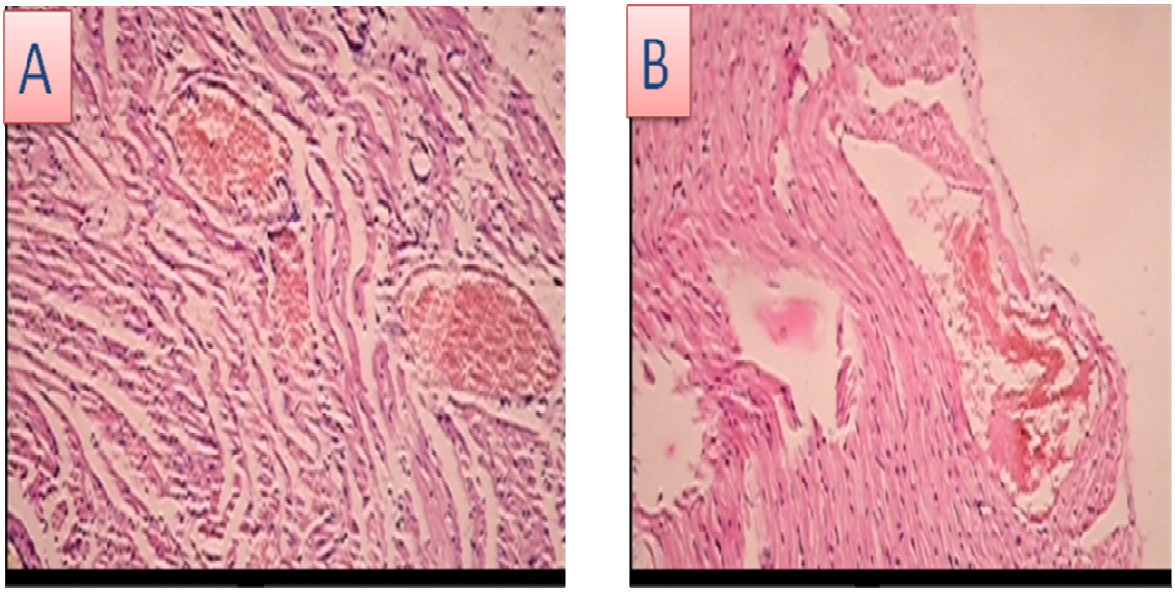
Histopathological changes: Histopathology study of CoQ_{10} treated heart showed moderate to severe damage, necrosis nuclear pyknosis, cytoplasmic eosinophilia, vacuolization and vascular congestion, whereas pretreatment of CoQ_{10} showed mild Inflammation, nuclear pyknosis, cytoplasmic vacuoles and cytoplasmic eosinophilia (Figure 6). Thus CoQ_{10} offered weak protection against digoxin induced cardiotoxicity.

Figure 5: Effect of CoQ_{10} (100mg/kg) on antioxidant enzymes in heart of male wistar rats receiving toxic dose of digoxin.



Data analyzed by two way ANOVA followed by Bonferroni post tests. *** $p<0.001$, ** $p<0.01$, $p<0.05$ as compared with Vehicle group. ns-non significant as compared with Vehicle group.

Figure 6: Histopathological changes of rat heart in digoxin induced cardiotoxicity pretreated with vehical and CoQ_{10}



A- Vehicle + digoxin treated group - showing sever necrosis of heart muscle fibers along with focal mass and fragmentation, sever Inflammation, nuclear pyknosis and cytoplasmic vacuoles in the rat heart. myocardial cells showed abnormal changes like vascular congestion as well as inflammation. **B**: Treated with CoQ_{10} (100 mg/kg for 18 day) + digoxin, showing mild type of Inflammation, nuclear pyknosis and cytoplasmic vacuoles than Vehicle + digoxin treated animals. Magnification 40X and 100X.

Discussion

Cardiac glycosides have been used for the treatment of congestive heart failure for centuries [13]. Heretofore, the molecular mechanism for these cardiac glycosides has been ascribed to their effect on ion concentrations initiated through inhibition of the Na^+-K^+ATPase [14]. It is reported that sodium pump inhibition initiates a signal cascade that does not require observable changes in intracellular sodium concentrations that might be anticipated from inhibition of the enzymatic function of the Na^+-K^+ATPase. Furthermore, it is found the generation of reactive oxygen species (ROS) to be an essential part of this signal cascade [15]. It has also have observed that the administration of either purified digitalis-like substances (DLS) such as ouabain or the serum from uremic patients, which is known to contain increased concentrations of these DLS, produces acute diastolic dysfunction in isolated rat cardiac myocytes studied in culture [16].

The cellular mechanisms for direct effects of cardiac glycosides are not yet fully resolved. Inhibition of sarcolemmal Na^+, K^+- ATPase (sodium pump) is involved in toxicity and probably to a large extent in therapeutic efficacy. [1,2]. Sodium pump inhibition by cardiac glycosides leads ultimately to increased intracellular Ca^{2+} concentration through Na^+/Ca^{2+} exchange and associated increase in slow inward Ca^{2+} current as well as transient Ca^{2+} current. Increased activity of Na^+/H^+ exchange is implicated in amplifying the intracellular Ca^{2+} concentration as a result of digitalis induced increased inotropy with consequent fall in intracellular pH. Na^+/H^+ exchange also works as a major pathway of Na^+ entry and subsequent intracellular Ca^{2+} overload in myocardial tissue, especially in ischaemia/reperfusion dysfunction [17].

In the present investigation, CoQ_{10} treatment resulted in more quantity of digoxin required to produce ectopic beats (4380 µg/kg), ventricular fibrillation (4420 µg/kg), and cardiac arrest (4460 µg/kg). The significant (p value <0.05) prolongation of onset of digoxin induced ectopic beat as well as cardiac arrhythmia may be due to the cardioprotective activity of CoQ_{10}. However non significant difference in the end result i.e. cardiac arrest indicated that CoQ_{10} did not completely antagonize digoxin toxicity. Therefore CoQ_{10} may not be regarded as antidote for digoxin induced cardiotoxicity. Significant delay in the onset of ectopic beats and cardiac arrhythmia can be regarded as beneficial effect of CoQ_{10}.

It is described that physiological/pathophysiological scheme in which ROS such as peroxynitrite can actually increase SERCA (sarco-endoplasmic reticular Ca^{2+}) activity by *S*-glutathiolation at a reactive Cys674 residue [18]. The author proposed that this process is impaired in the setting of atherosclerosis by irreversible oxidation of the key reactive thiols on SERCA. The signaling roles of intra cellularly generated ROS in cardiac myocytes have been suggested by previous studies when myocytes were subjected to hypoxia or hypoxia reoxygenation. [15]. In the present investigation, digitalis administration in vehicle treated animal's showed increased MDA content (an index of lipid peroxidation) and reduction in cardiac tissue level of protective biological antioxidant enzymes like GSH and SOD, thereby indicating oxidative stress. These results correlate with previous studies which have demonstrated the involvement of oxidative stress and lipid peroxidation in digoxin induced cardiotoxicity [19]. The result indicated that treatment with CoQ_{10} significantly increases the concentration of GSH and SOD, and decreased MDA level significantly. Thus indicating cardioprotective effect.

When myocardial cells, containing LDH and CK are damaged or destroyed due to deficient oxygen supply or glucose, the cell membrane becomes permeable or may rapture, which results in the leakage of enzymes. This accounts for the decreased activities of LDH and CKMB in heart tissue of rats treated with digoxin. It is now generally accepted that activity measurement of enzymes such as AST, CKMB and LDH can be used in the assessment of myocardial injury.

In present study CoQ_{10} pretreatment resulted in non significant decreased in biological serum markers CK-MB and AST as compared to that of control group. Whereas the levels of LDH in CoQ_{10} group was significantly reduced, thus indicating cardioprotective effect.

Histopathology of rat heart of vehicle group showed moderate to severe damage in the form of necrosis, nuclear pyknosis, cytoplasmic eosinophilia, vacuolization and vascular congestion, whereas treatment of CoQ_{10} showed mild type of myocardial damage, necrosis and vascular congestion. The histopathological finding correlated with changes in biochemical marker. It is thus concluded that CoQ_{10} pretreatment offered weak protection against digoxin induced cardiotoxicity.

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