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Chronic Ethanol Consumption Increases Myocardial Mitochondrial DNA Mutations: A Potential Contribution by Mitochondrial Topoisomerases

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Abstract — **Aims:** Alcoholic cardiomyopathy (ACM) presents as decreased myocardial contractility, arrhythmias and secondary non-ischemic dilated cardiomyopathy leading to heart failure. Mitochondrial dysfunction is known to have a significant role in the development and complications of ACM. This study investigated if chronic ethanol feeding promoted myocardial mitochondrial topoisomerase dysfunction as one underlying cause of mitochondrial DNA (mtDNA) damage and mitochondrial dysfunction in ACM. **Methods:** The impact of chronic ethanol exposure on the myocardial mitochondria was examined in both neonatal cardiomyocytes using 50 mM ethanol for 6 days and in rats assigned to control or ethanol feeding groups for 4 months. **Results:** Chronic ethanol feeding led to significant ($P < 0.05$) decreases in M-mode Fractional Shortening, ejection fraction, and the cardiac output index as well as increases in Tau. Ethanol feeding promoted mitochondrial dysfunction as evidenced by significantly decreased left ventricle cytochrome oxidase activity and decreases in mitochondrial protein content. Both in rats and in cultured cardiomyocytes, chronic ethanol presentation significantly increased mtDNA damage. Using isolated myocardial mitochondria, both mitochondrial topoisomerase-dependent DNA cleavage and DNA relaxation were significantly altered by ethanol feeding. **Conclusion:** Chronic ethanol feeding compromised cardiovascular and mitochondrial function as a result of a decline in mtDNA integrity that was in part the consequence of mitochondrial topoisomerase dysfunction. Understanding the regulation of the mitochondrial topoisomerases is critical for protection of mtDNA, not only for the management of alcoholic cardiomyopathy, but also for the many other clinical treatments that targets the topoisomerases in the alcoholic patient.

INTRODUCTION

Alcoholism remains a significant health problem and represents the third leading cause of preventable deaths (NIH, 2010). More than 4% of the American adults suffer from alcoholism and ~10% of those who drink consume half the alcohol sold in the USA (Mack *et al.*, 2010). Approximately 25% of general adult hospital admissions have problems related to chronic alcohol use (i.e. cirrhosis, cardiomyopathy) (Mack *et al.*, 2010). Further, alcoholics are likely to abuse other substances as well as display higher levels of generalized anxiety disorders and posttraumatic stress disorders than the general population (Regier *et al.*, 1990; Mack *et al.*, 2010). Military personnel are at even greater risk for alcohol abuse compared with the general population which is related to the elevated stress that military personnel might face (Bray *et al.*, 2006). Presentation of patients with ACM is similar to patients presenting with a dilated cardiomyopathy (Fauchier *et al.*, 2000; Awtry and Philippides, 2010). Although a J-shaped curve has been identified for average alcohol consumption and cardiovascular heart disease, there are some qualifiers (Corrao *et al.*, 2000; Di Castelnuovo *et al.*, 2006; Costanzo *et al.* 2010). Even in individuals that have low overall consumption, irregular heavy drinking carries greater risks for cardiovascular disease suggesting that the toxic effects are prolonged (Sempos *et al.*, 2003; Room *et al.*, 2005).

Both acute and chronic ingestion of ethanol is cardiodepressive (Laonigro *et al.*, 2009; Awtry and Philippides, 2010). The toxic effects of alcohol in the cardiovascular system are manifested as decreased myocardial contractility, arrhythmias and secondary non-ischemic dilated cardiomyopathy leading to heart failure (Wilke *et al.*, 1996; Beckemeier and Bora, 1998). There are parallels between alcoholic cardiomyopathy and other cardiomyopathies including diabetes and doxorubicin cardiotoxicity, which are associated with abnormal cardiac function, accelerated apoptosis and loss of cardiac mass (Fiordaliso *et al.*, 2000; Frustaci *et al.*, 2000; Fang *et al.*, 2004, 2005).

Mitochondrial dysfunction has a significant role in the development and complications of alcoholic cardiomyopathy (Kim *et al.*, 2001; Vendemiale *et al.*, 2001; Cahill *et al.*, 2005; Hajnoczky *et al.*, 2005; Piano *et al.*, 2007). Chronic alcohol exposure accelerates mitochondrial dysfunction and apoptosis across different organs including the heart, liver and pancreas (Vendemiale *et al.*, 2001; Cahill *et al.*, 2002; Hajnoczky *et al.*, 2005; Lee *et al.* 2010). Most studies have independently examined the impact of alcohol use on hepatic mitochondria or examined degradation of cardiac function in response to chronic ethanol exposure (Vendemiale *et al.*, 2001; Cahill *et al.*, 2002; Hajnoczky *et al.*, 2005; Lee *et al.* 2010). Far fewer studies have examined alcohol's impact on the myocardial mitochondria. Given the near total dependence of the myocardium on aerobic metabolism, this represents a significant gap in our knowledge. Some studies have reported that the deleterious effects of ethanol are a function of increased oxidant stress. It is widely accepted that increased oxidative stress directly attacks mitochondrial DNA (mtDNA), as the sole mechanism for mtDNA damage. Our recent studies in models of diabetes alter that paradigm to include a significant role for mitochondrial topoisomerases in the propagation of mtDNA damage as an underlying cause of diabetic cardiomyopathy (Medikayala *et al.*, 2011; Hicks *et al.*, 2013). These findings are directly applicable to alcohol-induced cardiomyopathies. To date, no reports have examined the impact of ethanol presentation on mitochondrial topoisomerase function in the heart. Thus the present paper seeks to determine if chronic ethanol feeding promotes myocardial mitochondrial topoisomerase dysfunction as one underlying cause of mtDNA damage and mitochondrial dysfunction. Our findings have implications for the management of ACM as well as other clinical treatments that targets the topoisomerases in the alcoholic patient.

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METHODS

Male Wistar (3–6 months old) were used throughout this study. Where indicated ethanol feeding was 10% (vol:vol) in drinking water. The efficacy of the ethanol feeding protocol was verified by measurement of blood alcohol levels from blood samples drawn between 0800 and 0900. Alcohol levels were measured using a NAD-ADH protocol as described by the manufacturer (N1760; Sigma-Aldrich, St. Louis, MO, USA). Experimental protocols had institutional approval and animals were maintained in accordance with APS's *Guiding Principles in the Care and Use of Animals* and the *Guide for the Care and Use of Laboratory Animals* (National Research Council, revised 1996).

Cell culture

Neonatal cardiomyocytes from 1- to 3-day-old Wistar rats were prepared using collagenase IV, as we have previously described (Edwards *et al.*, 1992, 1994). Following preparation cells were plated overnight LG-DMEM + 10%FBS+ 0.1 mmol/l BrdU+ 1.0 mmol/l D-valine overnight before switching to the experimental media (LG-DMEM + 1% FBS + 1× NEAA +2 mmol/l glutamine), where indicated ethanol was added to the culture media.

Cardiovascular function

Echocardiography and pressure–volume loop analysis were used to evaluate cardiovascular function. Echocardiography was assessed using an Accustom Sequoia C256 system as described previously (Kinugawa *et al.*, 2005). In brief, animals were anesthetized using 1% isoflurane/100% O_2 metered through an Isotec4 vaporizer (VetEquip, Pleasanton, CA, USA) and maintained on a heated pad throughout the protocol. Once asleep, animals were allow to stabilize for 10 min before baseline measurements were collected. Following this dobutamine was injected (50 µg/kg *i.p.*) and measurements were collected after 5 min. Pressure–volume loop analysis allows for high fidelity recording of left ventricle function *in vivo* (Kass *et al.*, 1986). In brief, animals were anesthetized using 3% isoflurane/100% O_2 and maintained on a heated pad throughout the protocol. Surgical preparation included: (a) exposing the abdomen to lasso the inferior vena cava just proximal to the insertion of the right renal vein, (b) cannulation of the left external jugular vein for saline or dobutamine infusion, cannulation of the right carotid using a Millar SPR-838 transducer. Once the animal was instrumented, isoflurane was adjusted to 1% and the animal allowed to stabilize for 10 min before baseline occlusion to alter preload was performed. Following this, dobutamine (5.0 µg/kg/min *i.v.*) was infused and occlusion was performed after 12 min. At the end of the date collection period the isoflurane concentration was increased to 5% and after 5 min the hearts were resected for tissue harvest. Signal acquisition was done using a Millar Pressure–Volume Catheter (PR-838) and signal conditioning performed by the MPVS Ultra® System (Millar, Houston, TX, USA). Subsequent data analysis was performed using LabChart 7 Pro (ADI Instruments, Colorado Springs, CO, USA).

Cellular and mitochondrial function

Cytochrome oxidase (Complex IV) was measured by following the oxidation of reduced cytochrome C at A_{550}, as originally described by Wharton and Tzagoloff (Wharton and Tzagoloff, 1967; Medikayala *et al.*, 2011). Succinate dehydrogenase was determined from the cleavage of the tetrazolium salt MTT (3-4,5-dimethyliazol-2-yl)-2,5-diphenyl tetrazolium bromide (Denizot and Lang, 1986). ATP levels were determined by the CellTiter-Glo luminescent assay (Promega, Madison, WI, USA). Changes in mitochondrial membrane potential were estimated using JC1 (5,5′,6,6′-tetrechloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide). We determined shifts in the distribution of the aggregated (red) JC1 in healthy mitochondria to monomeric (green) JC1 in degenerating mitochondria that exhibited a loss of membrane potential. In brief, cells were incubated with 1 µM JC1 (Anaspec, Fremont, CA, USA) for 30 min at 37°C, before the cells were trypsinized to release them from the plate. Cells were washed twice in filtered (0.2 µ) PBS and resuspended in PBS. The red and green fluorescence was determined using a Guava Flow Cytometer and gated to count cells >10 µ; instrument gains were initially set using unstained cells and the Red:Green ratio was derived from all gated cells >10 µ taking the Red geometric mean: green geometric mean.

Western blot analysis

Western blot analysis on left ventricle homogenates was performed as previously described (Rafalski *et al.*, 2007). The primary antibodies used were COX 4 subunit (1:1000 dilution; Molecular Probes, Grand Island, NY, USA) or VDAC (1:500; Rockland, Gilbertsville, PA, USA). The secondary antibody was an anti-rabbit HRP (1:4000 dilution: Amersham, Buckinghamshire, UK) and used in combination with a Pierce ECL kit. For band quantification, care was taken to ensure that band density remained within the linear range and did not saturate the film, by performing exposures of different times. Band density was quantified using the AlphaEaseFC software (AlphaInnotech, San Leandro, CA, USA).

Mitochondrial isolation

Mitochondria that were devoid of nuclei were isolated by differential centrifugation as we have demonstrated previously (Edwards, 2008; Medikayala *et al.*, 2011). In brief, left ventricles from animals were minced using fine scissors before being put into a dounce homogenizer. Cultured cells were collected in ice-cold PBS and centrifuged (300 g, 5 min at 4°C). The minced tissues or cell pellets were suspended in mitochondrial isolation buffer (250 mmol/l Sucrose, 10 mmol/l Tris-Cl pH 7.5, 1 mmol/l EDTA, 1 mmol/l EGTA, 1.5 mmol/l $MgCl_2$, 10 mmol/l KCl, 1 mmol/l DTT, 1 mmol/l PMSF, 1× protease inhibitors; Sigma P-8340) and homogenized using a dounce homogenizer (10 strokes 'A' pestle and 10 strokes 'B' pestle). The extracts were then subjected to successive rounds of centrifugation: (a) 300 g, 5 min at 4°C times 3, (b) 1000 g, 5 min at 4°C times 2, (c) 2000 g 5 min at 4°C times 1, (d) 13,000 g, 10 min at 4°C times 3. Mitochondrial pellets were resuspended in buffer (50 mmol/l Tris-Cl pH 7.5, 0.5 mmol/l EDTA, 0.5 mmol/l EGTA, 1 mmol/l DTT, 10% glycerol, 1 × protease inhibitors) and lysed on ice for 30 min before protein concentration was determined by the Bradford method (BioRad, Hercules, CA, USA). Where a nuclei fraction was isolated, the pellet isolated from the first 1000 g spin was used. The cytosolic fraction was derived from the supernatant of the first 13,000 g spin and was cleared of other membrane fractions by an additional centrifugation step (15,000 g, 60 min at 4°C).

mtDNA damage

Total DNA was extracted from cultured cardiomyocytes or 1–5 mg left ventricle using Sigma Extract-n-Amp (Sigma, St. Louis, MO, USA). A LRPCR protocol was used to assess mtDNA damage by real-time QPCR using a Stratagene Mx3000P as we have described previously (Edwards, 2008; Medikayala *et al.*, 2011). In brief, any lesion (strand breaks, base modifications and apurinic sites) will stop a thermostable DNA polymerase capable of generating a long DNA product (Edwards, 2008). This amplification was compared to that of a short PCR (SRPCR) product (150–250 bp) that was unlikely to contain any lesions. A second short range PCR was performed using primers to the genomic gene β-actin and used to derive the mitochondrial copy number. SRPCR was done using a Brilliant QPCR Master Mix, while LRPCR was done using *PfuUltra*™ II Fusion HS DNA polymerase (Stratagene, La Jolla, CA, USA). The primers for the LRPCR reaction were 5′-GCCAGGACCAAACCTTTGTGTTTA-3′ forward and 5′-GGACTAGCCCCATTCCACTAC-3′ reverse. Primers used for the SRPCR and β-actin PCR reactions were as we have previously described (Edwards, 2008). Quantification of mtDNA damage and mitochondrial copy number were derived by the 2ΔCt method, from the comparison of LRPCR: SRPCR and SRPCR:β-actin, respectively.

DNA cleavage assay

DNA cleavage was determined by degradation of linear DNA using a Cy5- fluorescent probe and modified from our previous description (Medikayala *et al.*, 2011). In brief, a linear mtDNA was amplified by a PCR reaction using an internally labeled Cy5-labeled primer: forward: 5′-AAATTTCCCG ACACAAAATCTTTCC$^{(Cy5)}$TCCTAACTAAACCCTCTTTA CTTGC-3′ and the reverse primer was: 5′-CTCTTGGTAAGT AAATTTCTTTCTCC-3′ using mtDNA as the template to generate a 1274 bp probe. To perform the cleavage assay, isolated mitochondria (1–10 μg protein) were incubated in buffer (50 mmol/l TrisCl pH 7.5, 100 mmol/l KCl, 0.5 mM EDTA, 0.5 mmol/l DTT, 30 μg/ml BSA) to a final volume of 20 μl. The reaction was cooled on ice and at time zero the labeled DNA was added. The reaction was incubated for 30 min at 37°C to which 6 μl loading buffer was added. The inhibition experiments were performed as described previously where the extracts were incubated (± inhibitor) for 60 min at 37°C before the DNA was added (Medikayala *et al.*, 2011). 7 μl was electrophoresed on a 4% polyacrylamide gel and visualized using a Storm 840 PhosphoImager (Molecular Dynamics, CA, USA). Band density was quantified using the AlphaEaseFC software (AlphaInnotech, San Leandro CA, USA). The DNA relaxation protocol was modified from that described by Low *et al.* (2003). In brief, mitochondrial extracts (1–10 μg) were incubated in buffer (50 mM TrisCl pH 8.0, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM DTT) and to this 600 ng supercoiled pBR322 plasmid was added to a total volume of 20 μl. The mixture was incubated for 10 min at 37°C and the reaction stopped by the addition of 4 μl of a 5%sarcosine/6× SDS buffer and the reaction placed on ice until 5 μl was loaded onto a 1% agarose/T AE gel. Bands were imaged at 260 nm using a ChemiImager 5550 and density quantified using the AlphaEaseFC software (AlphaInnotech, San Leandro, CA, USA).

Statistical analysis

Where appropriate ANOVA analyses were performed using NCSS Software (NCSS, Kaysville, UT, USA). *Post hoc* analysis was done using a Fisher's LSD analysis. Values presented are mean ± SEM, and statistical significance was set at $P < 0.05$, unless otherwise indicated.

RESULTS

We have investigated the effect of prolonged ethanol exposure on the heart both *in vivo* by chronic feeding and *in vitro* by the addition of ethanol to cardiomyocytes in primary culture. Neonatal cardiomyocytes were maintained in the absence or presence of increasing concentrations of ethanol. The media was changed daily as our preliminary experiments indicated that at 100 mM ethanol ~50% of the ethanol was consumed in a 24 h period (data not shown). Six days of ethanol treatment led to a dose-dependent compromise of mitochondrial function as evidenced by a significant decrease in succinate dehydrogenase activity and decreased ATP levels (Fig. 1). JC1 has been used as an indicator of mitochondrial membrane

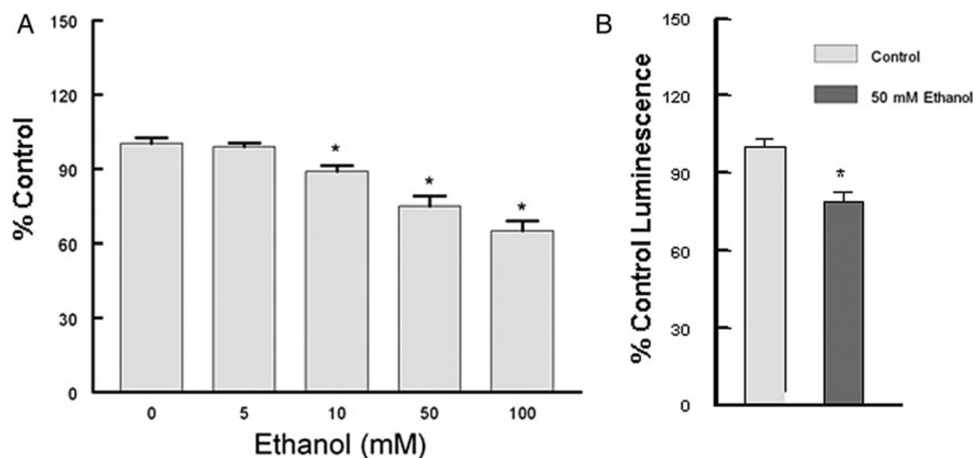
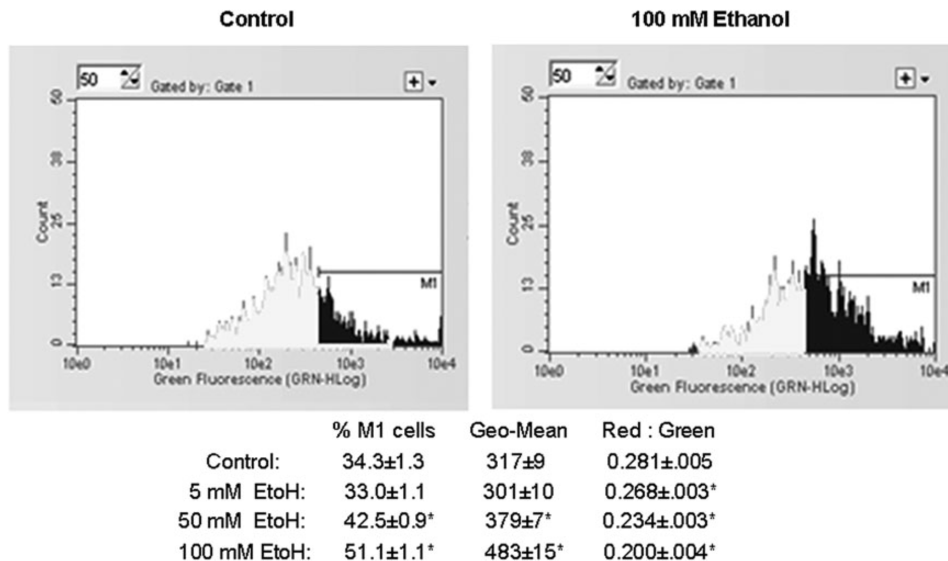


Fig. 1. Ethanol decreases cardiomyocyte mitochondrial function. (**A**) Neonatal cardiomyocytes were exposed to increasing ethanol concentrations for 6 days. Succinate dehydrogenase activity was determined by a MTT protocol (Medikayala *et al.*, 2011). (**B**) Ethanol decreased ATP levels in neonatal cardiomyocytes exposed to 50 mM ethanol for 6 days. Values presented are mean ± SEM and normalized to water fed controls. *$P < 0.05$ compared with control.



	% M1 cells	Geo-Mean	Red : Green
Control:	34.3±1.3	317±9	0.281±.005
5 mM EtoH:	33.0±1.1	301±10	0.268±.003*
50 mM EtoH:	42.5±0.9*	379±7*	0.234±.003*
100 mM EtoH:	51.1±1.1*	483±15*	0.200±.004*

Fig. 2. Ethanol treatment significantly altered JC1 fluorescence. Following 6 days of ethanol treatment, cardiomyocytes were stained with 1 μM JC1 as described in Methods. Using flow cytometry, cells were gated to count cells >10 μ. Increases in green fluorescence are indicative of mitochondrial depolarization as determined from the percentage of cells in the M1 region (% M1), the geometric mean of all gated cells (Geo-Mean), and the shift in the Red:Green ratios derived from the Red:Green geometric means. Values presented are mean ± SEM. *$P < 0.05$ compared with water fed control.

potential, as depolarization is thought to be an early event of mitochondrial dysfunction (Reers *et al.*, 1995). Using flow cytometry cells were gated to count the larger (>10 μ) cells, and ethanol significantly increased the percentage of depolarized (green) mitochondria as well as the geometric mean of green fluorescence (Fig. 2). Qualitatively similar results were obtained from the analysis of all cells (data not shown). In combination, these results clearly indicate ethanol-induced mitochondrial dysfunction. Concomitant with mitochondrial dysfunction we observed a significant increase in mtDNA damage (Fig. 3). These experiments in cultured cardiomyocytes indicate a direct effect of ethanol on cardiomyocyte metabolism that is not dependent upon signaling from a remote source.

The efficacy of the ethanol feeding protocol was verified by measurement of blood alcohol levels. Water fed animals had a blood alcohol concentration of 6.7 ± 1.9 mg%, while ethanol animals fed had a blood alcohol level of 46.4 ± 1.9 mg%; these values comparable to other studies (Brown *et al.*, 1998).

Cardiac function was followed longitudinally by echocardiography. A time-dependent degradation of fractional shortening was observed that was significantly decreased only after 4 months (~12 human years) of ethanol consumption (Fig. 4A). At 4 months both fractional shortening and ejection fraction were significantly depressed (Fig. 4B). In contrast, following a submaximal (50 μg/kg) injection of dobutamine, no differences were observed between the two groups suggesting the cardiac reserve was not yet significantly compromised (Fig. 4B). By pressure–volume loop analysis, we observed results similar to the echocardiography results as basal function was depressed as evidenced by significant decreases in the cardiac output index and ejection fraction (Fig. 5A and B). Although the cardiac output index was increased in response to dobutamine infusion in the control group, it was blunted in the ethanol fed animals (Fig. 5A). Ethanol feeding also significantly altered Tau (Fig. 5D) suggesting that diastolic function was compromised.

Similar to our results in cardiomyocytes, we observed that chronic ethanol consumption led to the degradation of myocardial

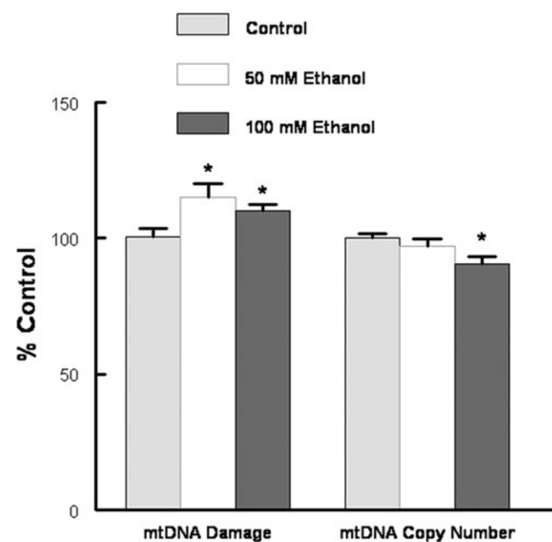


Fig. 3. Ethanol treatment increased mtDNA damage. Cardiomyocytes were exposed to 50 or 100 mM ethanol in DMEM/1%FBS for 6 days. mtDNA damage and mitochondrial copy number was assessed by a QPCR protocol as described in Methods. Values are normalized to water fed control and are mean ± SEM. *$P < 0.05$ compared with the control.

mitochondrial function. Ethanol feeding significantly decreased cytochrome oxidase activity that in part was caused by a significant decrease in the mitochondrial encoded Cytochrome Oxidase Subunit 1 (Fig. 6A). Concomitant with these observations we observed a significant increase in mtDNA damage (Fig. 6B), but without changes in mitochondrial copy number.

We have previously reported that mitochondrial topoisomerase dysfunction contributed to a decline in mtDNA integrity and mitochondrial competence (Medikayala *et al.*, 2011; Hicks *et al.*, 2013). To evaluate if mitochondrial topoisomerase function was altered by ethanol feeding, isolated left ventricle mitochondrial extracts, devoid of nuclear contaminants,

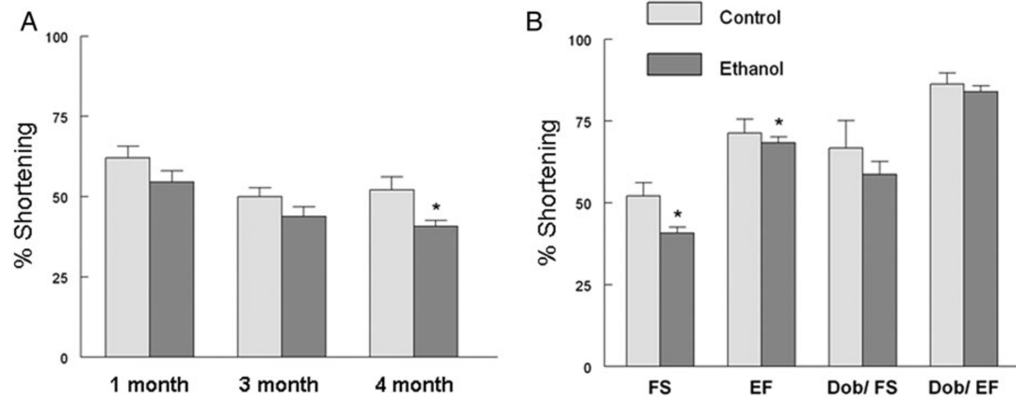


Fig. 4. Ethanol feeding progressively compromised cardiac function. (**A**) Basal fractional shortening was measured by M-Mode echocardiography after 1, 3 and 4 months of ethanol feeding. (**B**) M-Mode analysis echocardiography after 4 months of ethanol feeding. Dobutamine was injected (50 µg/kg *i.p*) and measurements made after 5 min. Values presented are mean ± SEM $*P < 0.05$ compared with water fed control animals.

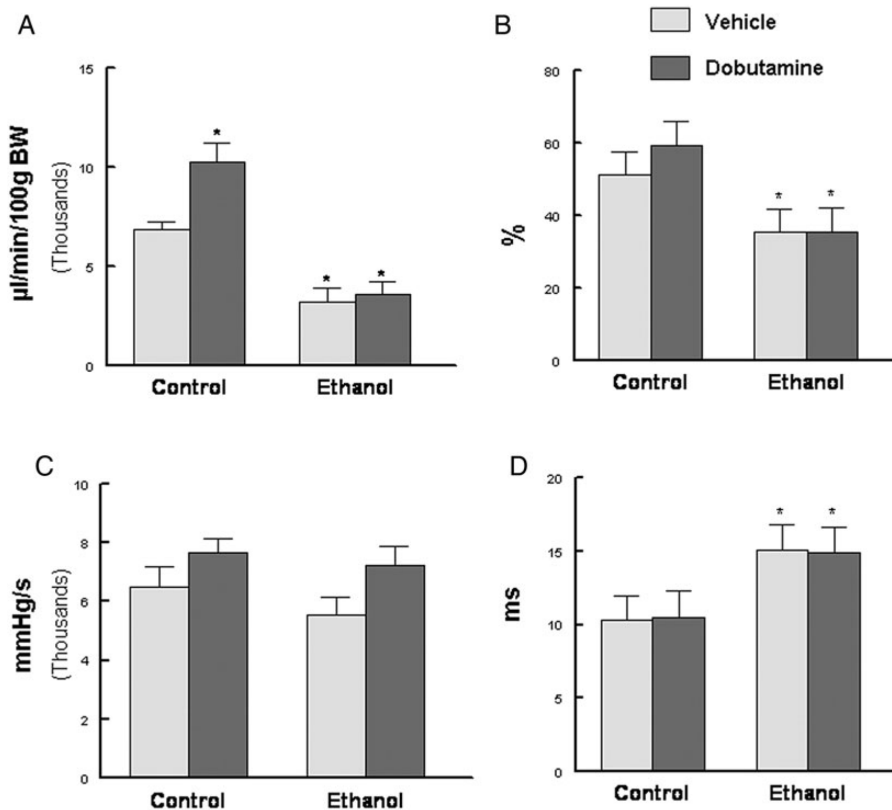


Fig. 5. Chronic ethanol feeding compromised cardiac function. (**A**) cardiac output index (µl/min/100 g BW), (**B**) ejection fraction, (**C**) dP/dt_{max} (mmHg/sec), (**D**) Tau (ms). Animals were anesthetized using 1% isoflurane and a Millar pressure–volume catheter used to make CV functions tests. Dobutamine was infused (5.0 µg/kg/min *i.v*) and measurements made after 5 min. Values are mean ± SEM; $*P < 0.05$ compared with control/vehicle animals.

were used. We measured mitochondrial topoisomerase DNA relaxation and DNA cleavage activity (Fig. 7). Incubation of supercoiled DNA with the mitochondrial extracts generated the typical isopane pattern of topoisomerases (Fig. 7A; ln: 3 and 4). Measurement of either mtDNA relaxation or DNA cleavage activity demonstrated that chronic ethanol feeding significantly increased mitochondrial topoisomerase-dependent strand breakage in DNA compared with water fed controls (Fig. 7B and D). To determine if ethanol could directly alter mitochondrial topoisomerase activity, mitochondrial extracts were preincubated with ethanol or maleimide (topoisomerase inhibitor). Acute exposure of ethanol did not significantly alter mitochondrial topoisomerase DNA cleavage (Fig. 8A). In contrast, 1 µM maleimide did significantly increase DNA cleavage of the mitochondrial topoisomerases (Fig. 8B).

DISCUSSION

Both acute and chronic ingestion of ethanol are cardiodepressive (Kim *et al.*, 2001; Vendemiale *et al.*, 2001; Hajnoczky *et al.*, 2005; Laonigro *et al.*, 2009; Larosche *et al.*,

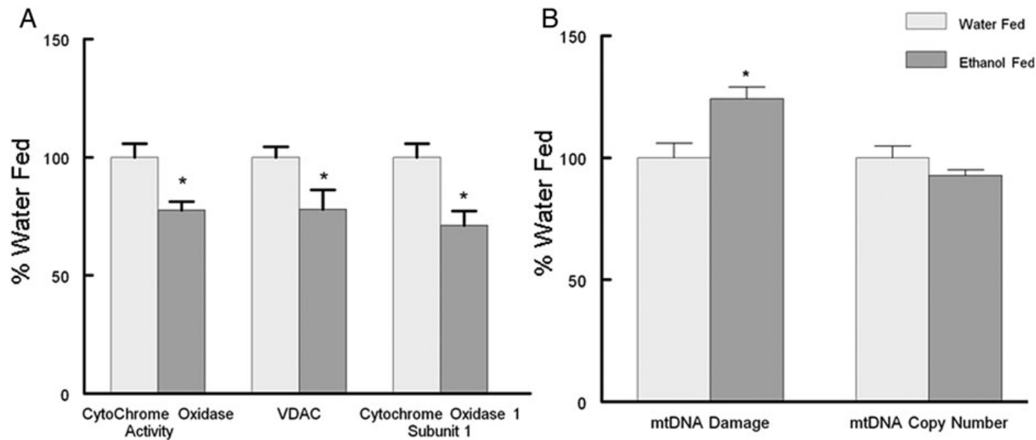


Fig. 6. Ethanol feeding induces myocardial mitochondrial dysfunction and mtDNA damage. (**A**) Left ventricle Cytochrome Oxidase enzyme activity and the mitochondrial proteins VDAC (nuclear encoded) and Subunit 1 Cytochrome Oxidase (mitochondrial encoded) by western blot. (**B**) Mitochondrial DNA damage was determined by a long range QPCR assay we developed (Edwards, 2008) and is the ratio of LRPCR:SRPCR. mtDNA copy number is the ratio of SRPCR: β-actin QPCR as described in Methods. Values are mean ± SEM and normalized to water fed control animals. *$P < 0.05$ compared with control animals.

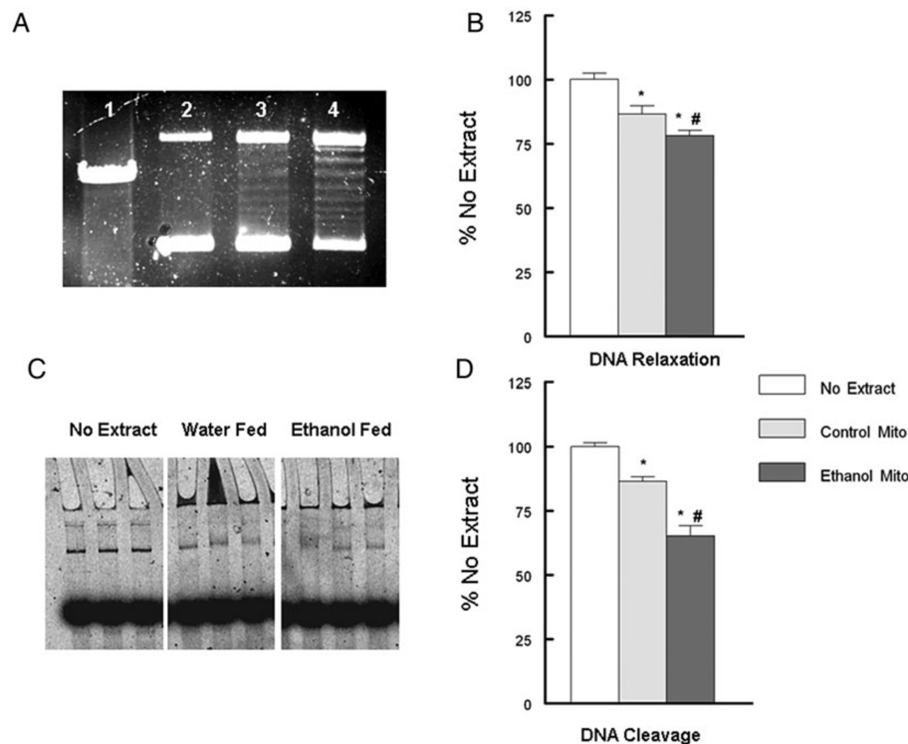


Fig. 7. Ethanol feeding altered myocardial mitochondrial topoisomerase function. (**A**) DNA relaxation of supercoiled plasmid DNA ln 1: linearized pBR322, ln 2: no extract, ln 3; 5.0 µg mitochondrial extract, ln 4, 10 µg mitochondrial extract. (**B**) Quantification of DNA relaxation as described in Methods. (**C**) Representative figure of DNA cleavage assay. (**D**) Quantification of DNA cleavage as described in Methods. Values are mean ± SEM and normalized to no mito controls. *$P < 0.05$ compared with no mito, #$P < 0.05$ compared with control Mito.

2009; Awtry and Philippides, 2010). Mitochondrial dysfunction has a significant role in the development and complications of alcoholic cardiomyopathy (Kim *et al.*, 2001; Vendemiale *et al.*, 2001; Cahill *et al.*, 2005; Hajnoczky *et al.*, 2005; Piano *et al.*, 2007). This is in addition to the known detrimental effects of long-term ethanol consumption on hepatic mitochondria (Fromenty *et al.*, 1995; Mateos *et al.*, 1995; Mansouri *et al.*, 1997; Cahill *et al.*, 2002; Demeilliers *et al.*, 2002). The major findings of the present study are that increased ethanol presentation, both *in vitro* and *in vivo*, significantly elevated mitochondrial topoisomerase DNA cleavage activity that was associated with mtDNA damage and mitochondrial dysfunction.

Presentation of patients with alcoholic cardiomyopathy (ACM) is similar to patients presenting with a dilated cardiomyopathy (Fauchier *et al.*, 2000; Awtry and Philippides, 2010). Lazarevic *et al.* (2000) observed an increase in isovolumic relaxation time, deceleration time of the early diastolic filling velocity and late diastolic filling velocity (Lazarevic *et al.*, 2000). In the present study, the ethanol-induced

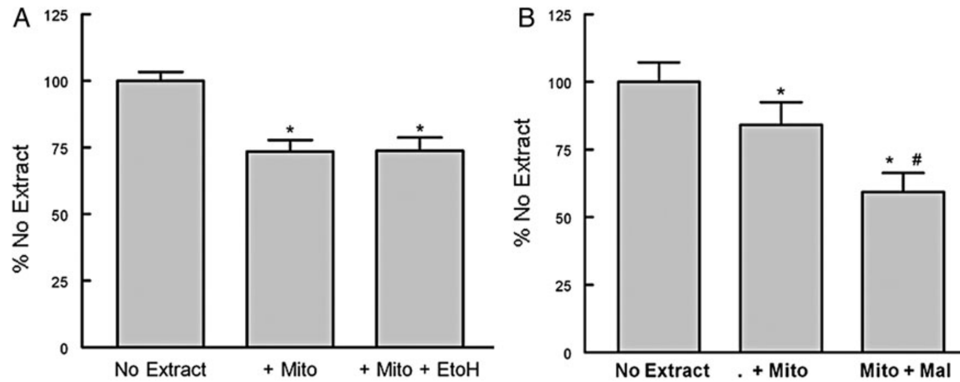


Fig. 8. Ethanol does not directly alter mitochondrial topoisomerase function. (**A**) Myocardial mitochondria extracts were incubated in the absence or presence of 50 mM ethanol. (**B**) Myocardial mitochondria extracts were incubated in the absence or presence of 1 μM maleimide. Mitochondrial were incubated for 60 min at 37°C in the absence or presence of the ethanol or maleimide before linear DNA was added and the incubation continued for another 30 min at 37°C. The samples were then placed on ice and loading buffer added and an aliquot run on a 4% polyacrylamide gel. Values are mean ± SEM and normalized to no extract controls. $^*P < 0.05$ compared with no extract, $\#P < 0.05$ compared with + Mito.

increases in Tau are consistent with impaired diastolic function (Fig. 3D). In addition to diastolic impairment, progressive degradation of cardiovascular function has been evidenced by significant decreases in ejection fraction and fractional shortening in patients as well as reduced sensitivity to dobutamine (Segel, 1988; Urbano-Marquez *et al.*, 1989; Kim *et al.*, 2001). These results are consistent with our findings of a reduced response to dobutamine injections on the cardiac output index (Fig. 5).

At the cellular level, many studies have reported that chronic alcohol consumption results in abnormalities in myocardial extracellular and intracellular structures, including the mitochondria (Alexander, 1967; Tsiplenkova *et al.*, 1986; Meehan *et al.*, 1999; Ling *et al.*, 2011). In the present study, ethanol feeding caused a significant decline in mitochondrial function as evidenced by significantly decreased mitochondrial membrane potential, cytochrome oxidase activity, and the mitochondrial proteins, VDAC and cytochrome oxidase subunit 1.

In both cultured cells and in chronic feeding experiments, elevated ethanol presentation increased mtDNA damage, findings that are consistent with previous reports in alcoholic patients (Teragaki *et al.*, 2000). In contrast, mitochondrial copy number was decreased only in cultured cardiomyocytes exposed to 100 mM ethanol, but no changes were observed in the left ventricle. Previous studies have demonstrated that ethanol feeding significantly decreased mitochondrial DNA and RNA levels as well as decreasing the number of functionally active mitochondrial ribosomes (Kou and Cohen, 1998; Cahill *et al.*, 1999; Cahill and Cunningham, 2000). This suggests that any compensatory response to the decline in cardiovascular function may have been ineffectual as a result of the increase in mtDNA damage or alteration of mtDNA sequence. Although mitochondria are poor at repairing UV-induced damage, they are capable of other forms of DNA damage repair (Clayton *et al.*, 1975; LeDoux *et al.*, 1992; Druzhyna *et al.*, 2008). Thus, the significant increase in mtDNA damage that we observed indicated the rate of mtDNA damage overwhelmed the functional mtDNA repair capability. As we have previously reported, mtDNA damage may also take the form of nucleotide substitutions that significantly alter the coding sequence of the mitochondrial proteins (Hicks *et al.*, 2013). The consequences of mtDNA mutations have been shown to significantly alter OXPHOS assembly (Pello *et al.*, 2008; Gil Borlado *et al.*, 2010). A mutation in a patient in the cytochrome b (A15533G) region resulted in a presentation of lactic acidosis and mild mental decay. Using transmutational cybrids, this group observed significant alterations in the rate of Electron Transport Complex (ETC) assembly (Gil Borlado *et al.*, 2010). Mimicking other mutations common to LHON also significantly altered the rates of ETC assembly (Pello *et al.*, 2008). Another consequence of altered mtDNA sequence is the interdependency of the ETC Complexes for their stability within the mitochondrial membrane (Lamantea *et al.*, 2002; Ugalde *et al.*, 2003; Acin-Perez *et al.*, 2004; Edgar *et al.*, 2009). Collectively these studies point to the critical role of mtDNA fidelity has on the mitochondrial function within the myocardium.

It is widely accepted that mitochondrial-derived oxidant stress is the sole endogenous cause of mtDNA damage. However, a number of reports have found significant mitochondrial dysfunction or mtDNA damage in the absence of oxidant stress (Hiona *et al.*; Zhang *et al.*, 2000, 2005; Kujoth *et al.*, 2005; Trifunovic *et al.*, 2005). Our recent papers are also at variance with this concept to show that mitochondrial topoisomerase dysfunction promotes mtDNA damage in the diabetic heart (Medikayala *et al.*, 2011; Hicks *et al.*, 2013).

Topoisomerases resolve the topological difficulties of DNA replication by allowing the double helix to pass through itself. In post mitotic cells, the mitochondrial genome is thought to replicate about once a month and resolving the topology of mtDNA replication would be insurmountable in the absence of the topoisomerases (Cortopassi and Wang, 1995). Although topoisomerase activity is essential to the cell and generally protective, it may also be genotoxic (Deweese and Osheroff, 2008). The impact of myocardial mitochondrial topoisomerases on mitochondrial function has been demonstrated on several levels including the use of topoisomerase inhibitors, estimation of molecular weight for DNA cleavage activity and immunoprecipitation of mitochondrial extracts with topoisomerase antibodies (Medikayala *et al.*, 2011; Hicks *et al.*, 2013).

Topoisomerase inhibitors or poisons act via a variety of mechanisms to alter topoisomerase function. Some, such as maleimide, permit DNA binding and cleavage, but not the religation or release of the relaxed DNA structure. This has the consequence of inducing DNA strand breaks (DSB) without a comparable increase in DNA religation. This dysfunction is the underlying basis for their use as antineoplastic or antibiotic

drugs. We have previously reported that sobuzoxane and novobiocin (topoisomerase II inhibitors), or hydroxycamptothecin (topoisomerase I inhibitor) exacerbated myocardial mitochondrial topoisomerase-dependent DNA cleavage (Medikayala *et al.*, 2011; Hicks *et al.*, 2013). In the present study, acute exposure to ethanol did not directly alter mitochondrial topoisomerase function, while in contrast maleimide did. That ethanol does not have a direct effect indicates that its effects are mediated through another agent. Chronically elevated ethanol presentation significantly increased both DNA relaxation and DNA cleavage activity of the myocardial mitochondria topoisomerases. As we and others have previously discussed oxidant stress alters topoisomerase function to inhibit the religation step which promotes DNA strand breaks (Li *et al.*, 1999; Medikayala *et al.*, 2011; Hicks *et al.*, 2013). These observations are consistent with ethanol-induced mitochondrial topoisomerase dysfunction resulting in an imbalance between DNA strand breaks and religation. Our findings suggest that alcoholism follows a similar pathophysiologic mechanism in the heart's mitochondria, to that described for diabetes and some topoisomerase inhibitors (Medikayala *et al.*, 2011; Hicks *et al.*, 2013).

SUMMARY

Mitochondrial dysfunction has a significant role in the development and complications of alcoholic cardiomyopathy and the present study reiterates this (Wieland and Lauterburg, 1995; Kim *et al.*, 2001; Pagel *et al.*, 2002; Cahill *et al.*, 2005; Piano *et al.*, 2007; Guiraud *et al.*, 2008). Ethanol feeding compromised mitochondrial function in part as a result of a decline in mtDNA integrity. Separate from a direct impact of oxidative stress on mtDNA, ROS-induced alteration of mitochondrial topoisomerase function appeared to accelerate and propagate an increase in mtDNA damage. This indicates that mitochondrial topoisomerase dysfunction will have important consequences for myocardial function. Topoisomerases are critical for all cells and mitochondria could not survive without them. Topoisomerases are the focal point for many antibiotic and antineoplastic reagents, and their regulation is central to the clinical management of several diseases. These findings have broad implications for the management of the alcoholic patient. Understanding the regulation of the mitochondrial topoisomerases is critical for protection of mtDNA, not only for the management of alcoholic cardiomyopathy, but also for the many other clinical treatments that targets the topoisomerases in the alcoholic patient.

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