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PDE5 Inhibitor Sildenafil Attenuates Cardiac microRNA 214 Upregulation and Pro-apoptotic Signaling after Chronic Alcohol Ingestion in Mice

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Abstract

Abusive chronic alcohol consumption can cause metabolic and functional derangements in the heart and is a risk factor for development of non-ischemic cardiomyopathy. microRNA 214 (miR-214) is a molecular sensor of stress signals that negatively impacts cell survival. Considering cardioprotective and microRNA modulatory effects of sildenafil, a phosphodiesterase 5 (PDE5) inhibitor, we investigated the impact of chronic alcohol consumption on cardiac expression of miR-214 and its anti-apoptotic protein target, Bcl-2 and whether sildenafil attenuates such changes. Adult male FVB mice received unlimited access to either normal liquid diet (control), alcohol diet (35% daily calories intake), or alcohol + sildenafil (1 mg/kg/day, p.o.) for 14 weeks (n=6–7/group). The alcohol-fed groups with or without sildenafil had increased total diet consumption and lower body weight as compared with controls. Echocardiography-assessed left ventricular function was unaltered by 14-week alcohol intake. Alcohol-fed group had 2.6-fold increase in miR-214 and significant decrease in Bcl-2 expression, along with enhanced phosphorylation of ERK1/2 and cleavage of PARP (marker of apoptotic DNA damage) in the heart. Co-ingestion with sildenafil blunted the alcohol-induced increase in miR-214, ERK1/2 phosphorylation, and maintained Bcl-2 and decreased PARP cleavage levels. In conclusion, chronic alcohol consumption triggers miR-214 mediated pro-apoptotic signaling in the heart, which was prevented by co-treatment with sildenafil. Thus, PDE5 inhibition may serve as a novel protective strategy against cardiac apoptosis due to chronic alcohol abuse.

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Authors' Contribution AS, LX, RCK designed the study; AS, LX, FNA, AD performed the experiments and analyzed the data; AS, LX, RCK wrote the manuscript; all authors substantially contributed to the interpretation of the data, critically revised the manuscript, and approved the final version.

Conflicts of interest All authors declare no conflicts of interests related to this study.

Keywords

microRNA; alcoholic cardiomyopathy; PDE5 inhibitor; apoptosis

Introduction

Alcohol abuse remains a serious health problem both in the Western world and in developing countries accruing an estimated 3 million deaths annually according to a report of World Health Organization (WHO) published in 2018 (https://www.who.int/substance_abuse/publications/global_alcohol_report/en/). It is estimated that the per capita alcohol consumption would increase 17.8% by 2030 [1]. Chronic alcohol consumption is associated with organ impairment and failure including the heart with abnormalities characterized by decreased myocardial contractility, arrhythmias and secondary non-ischemic dilated cardiomyopathy [2–4]. Individuals consuming toxic levels of alcohol either manifest with left ventricular (LV) dysfunction mostly with dilated cardiomyopathy [5] or remain asymptomatic with preserved cardiac function [6]. These cardiac perturbations caused by excessive alcohol intake are collectively known as alcoholic cardiomyopathy (ACM). The incidence of ACM is difficult to predict but depends on various factors such as the amount of intake, duration of alcohol drinking habit and individual genetic variability, which play important roles in determining the magnitude of injury to the heart [6]. Several hypotheses have been postulated to explain the deleterious impact of alcohol on the heart, including oxidative stress [3, 7], mainly due to generation of excessive reactive oxygen species (ROS) from alcohol metabolites [7], mitochondrial damage [8] and cell necrosis as well as apoptosis [9, 10]. Recent studies have identified microRNA-214 (miR-214) as a sensitive marker of cardiac stress which has also been shown to play detrimental role in heart disease [11]. miR-214 has been shown to be upregulated in response to pressure overload [12], fibrosis [13] and heart failure (HF) [14].

However, the role of miR-214 in the regulation of cellular function differs between diseased and normal conditions [15, 16]. Recent reports suggested that miR-214 is cardioprotective during acute myocardial infarction (MI) [17, 18]. Despite previous efforts to understand the molecular mechanisms and treatment options for ACM, there is virtually no therapeutic modality which has significant impact in treating this disease. Chronic ethanol consumption results in metabolic perturbations in the cell through enhancing oxidative stress [3, 7], cell necrosis and apoptosis [9, 10]. Alcohol-induced activation of hepatocyte apoptosis was alleviated by resveratrol via SIRT1-dependent inhibition of endoplasmic reticulum (ER) stress, caspase-12, and phosphodiesterase (PDE) activity [19]. Interestingly, we previously demonstrated that both sildenafil and resveratrol protected against cardiac ischemia-reperfusion injury via activation of SIRT1 in mice [20]. Other studies from our group also demonstrated that sildenafil protects against myocyte cell loss following ischemia/re-oxygenation [21, 22], myocardial infarction [23, 24], as well as doxorubicin-induced cardiomyopathy [25]. In this context, we hypothesized that PDE5 inhibitor, sildenafil may be a potential cardioprotective modality in ACM. Moreover, a recent publication also suggested that PDE-5 inhibition can modify miR signature profile through modulation of NO-cGMP pathway [26]. Also alcohol consumption has been

documented to cause epigenetic changes in alcoholic liver diseases [27] and to modulate miR expression and cell cycle [28]. Based on this background, we hypothesized that cardiac miR-214 and its associated apoptotic pathway may be affected following chronic alcohol consumption, whereas PDE5 inhibition with sildenafil might modulate such a pathologic process. Therefore, the current study was designed to determine the impact of long-term alcohol feeding on cardiac function and cardiac expression of miR-214 and its associated apoptotic signaling molecules. We further determined whether sildenafil can exert beneficial effects against alcoholic cardiotoxicity and attenuate the miR-214-related changes triggered by chronic alcohol consumption.

Materials and Methods

Animals and Liquid Diet Treatment

All animal procedures were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee (IACUC) and experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (8th edition, National Academies Press, 2011).Nineteen adult male Friend Virus-B (FVB) mice (age 12–14 weeks) were purchased from Harlan (Indianapolis, IN, USA). Mice were randomly grouped to receive unlimited access to one of the following 3 liquid diet formula purchased from BioServ (Frenchtown, NJ, USA) as the sole source of food and water for the entire 14 weeks duration of the study. The mice in *Control* group (n=6) received normal rodent liquid diet (Product# F1259SP) and *Alcohol* group (n=7) received alcohol-enriched diet (Product # F1258SP, in which alcohol provides 35% of daily energy intake). *Alcohol+Sil* group (n=6) received alcohol-enriched diet added with water-dissolved sildenafil solution (final sildenafil dose of 1 mg/kg/day). This sildenafil dose was determined by the daily consumption of liquid diet volume, which was monitored daily throughout the entire 14-week treatment period. Two animals were housed in each cage and the volume of liquid diet consumption by each mouse was estimated as a half of the total volume intake per cage. Body weight of mice was recorded every week.

Assessment of Left Ventricle Function by Echocardiography

Cardiac function was evaluated using a Vevo770 imaging system (Visual Sonics Inc.) equipped with a 30 MHz linear transducer. Mice were lightly anesthetized with sodium pentobarbital (30 mg/kg; i.p.) and the echocardiographic procedure was carried out to measure LV end-diastolic diameter (EDD), end-systolic diameter (ESD), anterior wall diastolic thickness (AWDT), and posterior wall diastolic thickness (PWDT) for three consecutive cycles in M-mode using methods adopted by the American Society of Echocardiography. LV fractional shortening (FS) was calculated as (LVEDD-LVESD)/ LVEDD*100. Ejection fraction (EF) was calculated with the Teichholz formula. Heart rate was measured and averaged for 3 cardiac contractile cycles.

RNA Isolation and Real-time PCR

Total RNA was isolated from heart tissues using miRNeasy kit (Qiagen, Germantown, MD, USA) for miRNA analysis. miR-214 specific stem loop primer was used to generate miR-214 cDNA using microRT reverse transcription kit (Applied Biosystems, USA)

according to the manufacturer's recommendation. The obtained cDNA product was used to quantify miR-214 expression level with TaqMan amplicon specific probes by real time qPCR using Roche 480 III cycler (Roche Diagnostics Corp.) and sno-202 was used as an internal control. PCR cycles were as follows: Initial denaturation at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. The relative miRNA expression was calculated using $2^{-\Delta\Delta}$Ct method and normalized to the expression of sno-202.

Western Blot Analysis

The total protein was extracted from ventricular tissue via homogenization in HEPES lysis buffer containing 10 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 0.1% SDS, protease and phosphatase inhibitors (Cell Signaling Technology, Danvers, MA) and was sonicated for 30 seconds. Protein concentration of the supernatant was evaluated using Bradford BSA protein assay reagent (Bio-Rad Laboratories, Hercules, CA). Equal amounts (60 μg per lane) of proteins or pre-stained molecular weight markers (Precision plus, BIO-RAD) was separated on 4–20 % SDS-polyacrylamide gels and then transferred electrophoretically to nitrocellulose membranes (0.2 μm pore size, Bio-Rad). Membranes were incubated for 1 hour in blocking solution containing 5% milk in Tris-buffered saline (TBS), and incubated overnight at 4°C with one of the rabbit polyclonal antibodies: anti-P-ERK 1/2 (Thr-177/160; dilution 1:500, Santa Cruz Biotechnology), anti-ERK1/2 (dilution 1:500, Santa Cruz biotechnology), anti-beta Actin (dilution 1:1000;Santa Cruz Biotechnology) anti-Bcl-2 (dilution 1:1000, Cell Signaling Technology); anti-Bax (dilution 1:1000, Cell Signaling Technology) and anti-PARP (dilution 1:1000; Cell Signaling Technology). Membranes were then washed briefly three times in TBS and the blots were then incubated for 1 hour with horseradish peroxidase (HRP)–conjugated secondary antibody (1:3000). Antibody binding was detected using enhanced chemiluminescence (Amersham Pharmacia, Pittsburgh, PA), and film was scanned and the intensity of immunoblot bands was quantified using Image J software (Bethesda, NIH).

Bioinformatics Analysis

Potential targets of miR-214 were predicted using the bioinformatics database miRBase by miRanda algorithm (http://www.mirbase.org) and validated microRNA target list was obtained using miRWalk (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/).

Statistical Analysis

All results are expressed as mean ± SE. For any of the reported parameters, the statistical comparison was made among the three experimental groups using one-way ANOVA, followed by Student-Newman-Keuls post-hoc test for pair-wise comparison. Probability value of $P<0.05$ was considered statistically significant.

Results

Effect of Chronic Alcohol Ingestion on Food Intake and Organ/Body Weight

Mice fed with alcohol (with or without sildenafil) consumed significantly higher volume of the liquid diet compared with the non-alcohol control liquid diet during the entire 14-week

experiment period (Fig. 1A). There was an inverse relationship between volume of food intake and body weight reduction in alcohol-treated mice as compared with controls (Fig. 1B), i.e. both *Alcohol* and *Alcohol+Sil* groups had significantly lower body weight at the end of 14 weeks as compared to *Control* group (Fig. 2A; $P<0.05$).There was no difference in body weight between *Alcohol* and *Alcohol+Sil* groups. In addition, the heart weight measured at the end of protocol showed a slight decrease in the alcohol-treated groups as compared with controls (Fig. 2B), although the ratio of heart weight to body weight did not change significantly among the 3 groups (Fig. 2C). The liver weight was not markedly different among the treatment groups although the liver to body weight ratio was significantly higher in *Alcohol* and *Alcohol+Sil* groups as compared with the *Control* (Fig. 2D). Chronic alcohol feeding did not change the ratio of kidney weight to body weight (Fig. 2E).

Effect of Chronic Alcohol Ingestion on Cardiac Contractile Function

Echocardiography results indicated a comparable heart rate among the 3 groups (see Fig. 3A–3C for representative echocardiographic images). LV ejection fraction (LVEF), a measure of ventricular contractility did not differ between the control and alcohol-treated groups (Fig. 3D). Other LV functional parameters such as End-Systolic Diameter (ESD), and End-Diastolic Diameter (EDD) were also similar among the *Control* and *Alcohol* groups (Fig. 3F–3G). These results suggest that chronic alcohol feeding for 14 weeks did not cause cardiac dysfunction in the mice. However, both LV ESD and EDD significantly declined in the *Alcohol+Sil* group (Fig. 3F–3G).

Chronic Alcohol Consumption Increases Cardiac miR-214 Expression and Sildenafil Suppresses miR-214

To further investigate the epigenetic modification underlying the chronic alcohol feeding, we assessed miR-214 expression in the cardiac tissues. In addition, potential protein targets of miR-214 were predicted using the bioinformatics database miRBase by miRanda algorithm (http://www.mirbase.org) and also by miRWalk (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/), which retrieves validated microRNA target list. Both software analyses showed a highly conserved binding site for miR-214 at the 3'UTR of Bcl2 mRNA and more specifically at the 8 mer seed region with high miVScore. (Fig. 4A). Delta–delta Ct value analysis indicated a 2.4-fold increase in miR-214 level in the hearts of alcohol-treated mice. The increase of miR-214 expression was abolished by co-treatment of sildenafil in the liquid diet (Fig. 4B).

Chronic Alcohol Ingestion Enhances Cardiac Stress Marker P-ERK1/2 and Reduces Anti-Apoptotic Protein Bcl-2

Cardiac expression of phosphorylated ERK1/2 (P-ERK1/2; Thr-177 & 160 site), a putative stress marker, was significantly increased in *Alcohol* group (Fig. 5), suggesting excessive alcohol intake can impose stress to cardiac cells. Interestingly co-treatment with sildenafil did not show similar level of P-ERK1/2 induction implying that sildenafil partially attenuated the cellular stress caused by chronic alcohol intake (Fig. 5).

We also investigated the effects of chronic alcohol intake on Bcl-2, one of the protein targets of miR-214. Based on the microRNA target prediction, we identified that miR-214 can bind to the 3'UTR of Bcl-2 and may regulate its expression (Fig. 4A). Corresponding to an up-regulated miR-214, the protein level of Bcl-2 was markedly decreased in *Alcohol* group as compared with *Control* group (Fig. 6A). Similarly, the suppressive effects of sildenafil co-treatment on cardiac miR-214 restored Bcl-2 protein levels in the heart comparable to the *Control* group (Fig. 6A). Moreover, Bax expression in the same hearts was used to calculate Bcl-2/Bax ratio (Fig. 6A), which significantly decreased with sildenafil co-treatment as compared to alcohol treatment group (Fig. 6C). Furthermore, the level of cleaved PARP in the alcohol-fed mice was significantly higher than the control groups (Fig. 6E) and this increase was completely prevented in *Alcohol+Sil* group. Densitometric analysis of the intensity of cleaved PARP to total PARP ratio (Fig. 6F) or to actin ratio (Fig. 6G) also confirmed the observation.

Discussion

The salient findings of the present study are: 1) chronic alcohol consumption for 14 weeks significantly up-regulated miR-214 expression with concurrent down-regulation of its protein target Bcl-2 in the heart; 2) alcohol-induced cardiac stress was associated with increased P-ERK1/2 levels; 3) therapeutic intervention with co-administration of sildenafil prevented the dysregulated cardiac miR-214 and Bcl-2 expression and normalized the alcohol-enhanced P-ERK1/2 levels; and 4) LVEF and wall thickness in the mice following chronic alcohol treatment (with or without sildenafil co-administration) remained normal.

The effect of alcohol consumption on cardiovascular system is an area of intense research and there is a disparity in the prevalence of ACM among moderate and heavy drinkers [29, 30]. While the cardiac health benefits were observed at mild to moderate levels of alcohol consumption [31, 32], excessive alcohol intake can clearly induce many pathological changes including oxidative stress, organelle dysfunction [33], and compromised cardiac muscle contractility [34]. In addition, the role of aldehyde dehydrogenase (ALDH) [35] and ROS generation in alcohol-induced cardiac structural deformities and dysfunction has been suggested. Currently, there are no therapeutic approaches and strategies to treat ACM.

The mouse model of chronic alcohol ingestion used in the present report has many similar features observed in humans. Notably, animals fed with alcohol-enriched liquid diet showed similar addiction pattern as observed in alcoholics, which manifested from their increased volume of alcohol intake (Fig. 1A) and retarded gain in body weight (Fig. 1B). These harmful effects of addictive alcohol consumption on normal growth development may partially result from malnutrition and abnormalities in other organs such as liver. Regardless, PDE5 inhibitors, sildenafil and tadalafil (a long-acting PDE5 inhibitor) have been shown to reduce body weight in db/db and high fat diet induced obesity mouse models [36, 37].

In the present study, the cardiac function and ventricular wall thickness evaluated by echocardiography did not exhibit LV dysfunction or hypertrophy (Fig. 3). Treatment with sildenafil decreased the heart rate (Fig. 3E) that was higher in alcohol group. Rapid heart rate with chronic alcohol consumption is a risk factor for the development of cardiac

problems such as atrial fibrillation (AF) and cardiac arrhythmias [38–40]. These results suggest that sildenafil treatment may protect the heart from future incidence of AF in alcohol subjects. A few previous studies using murine models of ACM reported involvement of autophagy and hypertrophy in the hearts exposed to chronic alcohol intake [35, 41]. In some mouse models of ACM, cardiac hypertrophy and contractile dysfunction were observed [35, 42, 43]. The Framingham Heart Study reported that alcohol consumption in humans was not associated with an increased risk of congestive heart failure even among heavy drinkers [44]. Nevertheless, excessive alcohol drinking was associated with an increased risk of heart failure with underlying myocardial ischemia [45]. We previously demonstrated that treatment with sildenafil or tadalafil decreased LV ESD and EDD induced by I/R injury [46, 47] similar to the present study. However, alcohol by itself did not increase the LV ESD and EDD values. Taken together, the present study seems to be in line with the human observations suggesting a preserved LVEF even after a prolonged period of alcohol consumption.

In the present study, we observed that chronic alcohol-feeding caused some profound molecular changes at epigenetic level, such as altered miR-214 expression, and protein levels including ERK, Bcl2, Bax, PARP in the heart (Fig. 4 to 6). The elevated miR-214 following alcohol consumption can regulate key proteins involved in cardiac cell death, which may eventually lead to ACM and heart failure. Interestingly, the prevalence of ACM with chronic alcohol consumption in patients is variable among asymptomatic (preclinical) or symptomatic (later stage) [2, 5, 48] and the severity of alcohol induced cardiac abnormalities largely depends on the amount as well as the duration of alcohol drinking habits [49]. A large population of alcoholics remains asymptomatic with normal cardiac function and they appear normal and healthy until challenged by ischemia, when they suffer increased susceptibility to heart failure and death. In fact, a recent study involving 10,824 adults concluded that increased alcohol consumption was associated with decreased LVEF and the heavy drinkers had ~1.5-fold higher risk of decreased LVEF [50]. However, cardiac remodeling due to alcohol injury is a dynamic process where many molecular changes occur much in advance of the actual onset of heart failure.

Recent developments in the field of microRNA and its role in alcohol toxicity [51, 52] have opened an interesting avenue to understand the molecular mechanisms of ACM. Non-coding RNAs including miRs add a new class of players for regulation of mRNA translation which influence protein stability. These non-protein coding small RNAs may play a key role in various cardiovascular disease conditions. However, little is known about the role of miRs in alcohol-induced cardiotoxicity. Several studies have focused on identification of miRs as useful biomarkers in alcohol-related diseases. Various miRs have been known to be aberrantly regulated in cardiac tissue such as miR-122 [53], miR-21 [54] and miR-214 [55, 56]. The role of miR-214 in cardiac hypertrophy was elucidated in a rat model of phenylephrine-induced cardiac hypertrophy. The upregulation of miR-214 by phenylephrine increased hypertrophy while its lentiviral knockdown prevented cardiac hypertrophy by regressing the expression of Enhancer of Zeste homology 2 (EZH2), a regulator of hypertrophy stimuli [13]. Interestingly ethanol-fed rats showed an increased expression of miR-214 in liver cells, which was associated with oxidative stress by targeting glutathione reductase and cytochrome P450 oxidoreductase [57]. Whether similar

mechanisms are involved in the pathogenesis of ACM remains uncertain. In relevance to our findings, genetic ablation of miR-214 prevented liver fibrosis and functions independent of TGF-β–Smad signaling pathway [58]. The upregulation of miR-214 observed in the present study after 14 weeks of alcohol ingestion may suggest an initiation of molecular signaling which could potentially lead to cardiac fibrosis.

Another interesting aspect of miR-214 is the relationship to ROS-induced cardiomyocyte injury. It is widely accepted that excessive alcohol drinking causes ROS overproduction that leads to organ damages [34, 43, 59–61]. It was suggested that miR-214 is involved in ROS generation [62, 63]. An increased tubular expression of miR-214 was also reported in mouse models of chronic kidney disease induced by ischemia/reperfusion [64]. The overexpression of miR-214 induced apoptosis and disrupted mitochondrial oxidative phosphorylation, which is similar to the findings reported in the present study. Similarly, an increased expression of miR-214 in monocytes was found in patients with chronic renal failure [65]. Another study using rat ventricular cardiomyocytes demonstrated a direct activation of miR-214 by H_2O_2, which was associated with increase in apoptosis via suppression of Phosphatase and Tensin (PTEN), another direct target of miR-214 [66]. Here we found that chronic alcohol consumption significantly enhanced miR-214 expression in the heart along with a concomitant decrease of its protein target Bcl-2, which is a key anti-apoptotic protein. Interestingly, previous studies demonstrated that miR-214 is one of the most robustly upregulated miRs in various heart diseases such as dilated cardiomyopathy, ischemic cardiomyopathy and aortic stenosis in human subjects [67]. A recent report suggested that miR-214 regulates cardiac hypertrophy through association with lncRNA Phospholipid Scramblase 4 (Plscr4) through regulation of mitofusin-2 (Mnf2) [68]. Plscr4 acted as an endogenous sponge of miR-214 and downregulated miR-214 expression to promote Mitofusin-2 (Mfn2) and attenuate hypertrophy.

Taken together, the abnormally high expression of miR-214 following chronic alcohol treatment and the ability of sildenafil to prevent its induction reflected an alcohol-related epigenetic control of cardiac Bcl-2 expression. The decreased Bcl-2/Bax ratio indicated induction of apoptosis upon alcohol consumption which was prevented by sildenafil co-treatment. Downregulation of Bcl-2 protein was often associated with cardiac apoptosis by various pathological stimuli including ischemia/reperfusion and chemotherapeutic agent, doxorubicin [25, 69]. In addition, an increased expression of cleaved PARP observed in alcohol consuming animals suggests DNA damage and cell death, such as those observed in neurodegenerative disease condition [11]. These novel findings may have a broader significance in cardioprotection, since miR-214 upregulation has been reported in ischemia injury, liver fibrosis and HF [70, 71], clearly indicates the pathologic roles of miR-214.

In the present study, we observed increased levels of phosphorylated ERK1/2, which is a well-known stress marker in many disease conditions including alcohol intake. These results are compatible with those reported in similar studies on alcohol-induced liver disease [72, 73]. A consistent upregulation of ERK phosphorylation for a prolonged period of time may lead to cardiac fibrosis [74] and extracellular matrix deposition [73]. Interestingly, the alcohol-induced activation of ERK can be prevented by sildenafil co-treatment, indicating PDE5 inhibition alleviated cellular stress in the heart. These novel findings are conceptually

in agreement with the previous studies on resveratrol-induced cardioprotection via SIRT1-dependent inhibition of endoplasmic reticulum stress, caspase-12, and PDE activity [19].

In conclusion, as illustratively summarized in Figure 7, the present study demonstrated miR-214 as a potentially new therapeutic target of PDE5 inhibitor sildenafil in reducing cardiac stress induced by chronic alcohol consumption. Sildenafil may induce cardioprotective effect by downregulating miR-214 and restoration of its target anti-apoptotic protein Bcl-2 level in the heart. Future studies are needed to further understand molecular mechanisms underlying the protective effects of sildenafil against ACM.

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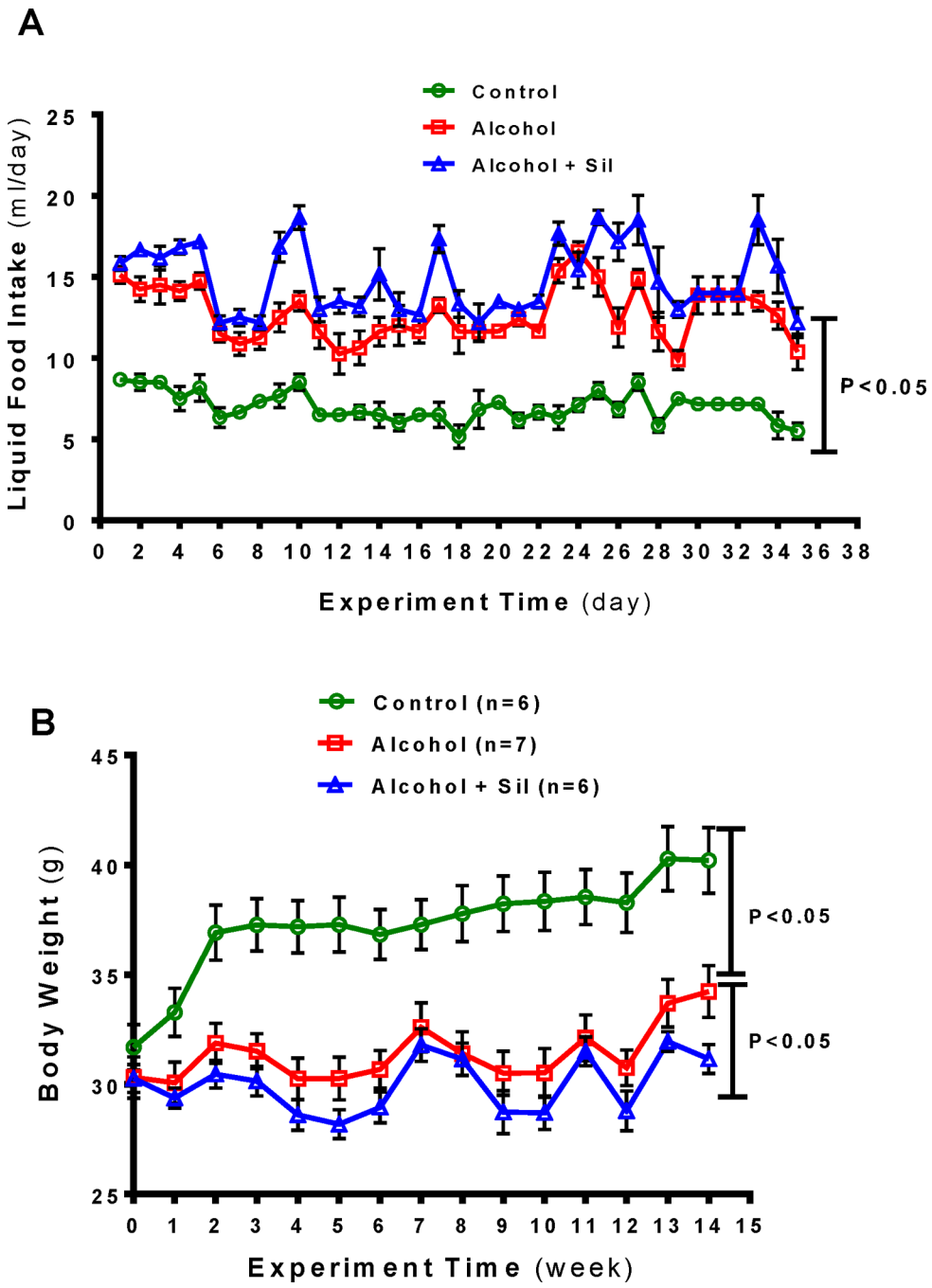

Figure 1.

(A) Mean volume (ml/day) of liquid diet consumed by the mice during a 35-day experimental period. *Symbols*: open circle – *Control* group (n=6); open square – *Alcohol* group (n=7); open triangle – *Alcohol+Sil* group (n=6). (B) Time course of weekly measurement of body weight of the animals received *Control* diet (green), *Alcohol* (red), or *Alcohol + Sil* (blue) for 14 weeks. Data are presented as Mean ± Standard Error.

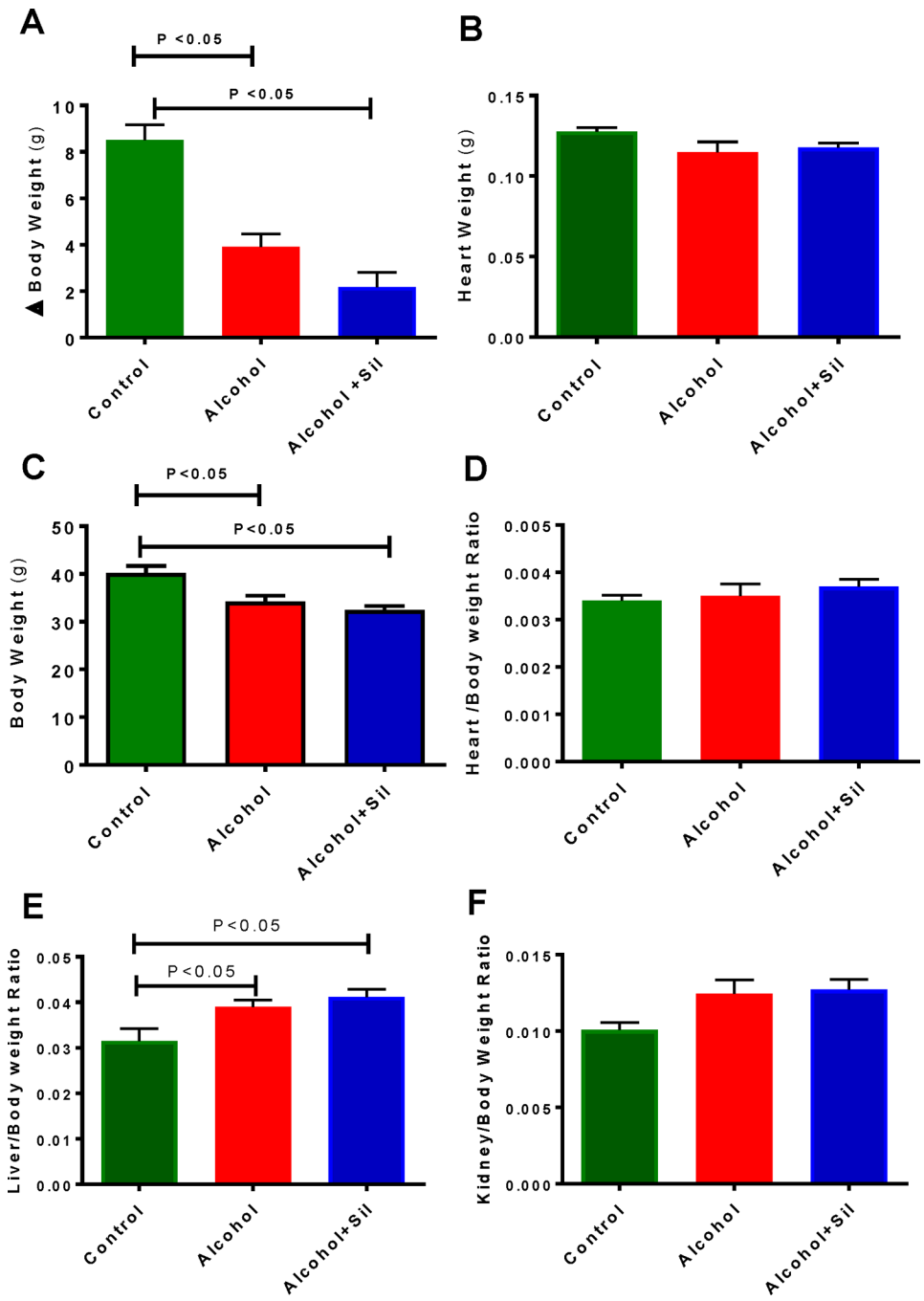

Figure 2.

Body weight and organ to body weight ratio among animals at the end of the protocol. (A) Body weight changes (Δ Body Weight) between the pre-treatment baseline and after 14 week of treatment with control diet (n=6), alcohol diet (n=7), and alcohol + sildenafil (n=6); (B) Heart weight, (C) Body weight, (D) Heart to body weight ratio, (E) Liver to body weight ratio, (F) kidney to body weight ratio from the various treatment groups, i.e. *Control* (n=6); *Alcohol* (n=7), and *Alcohol + Sil* (n=6). Data in the bar graphs are Mean ± Standard Error.

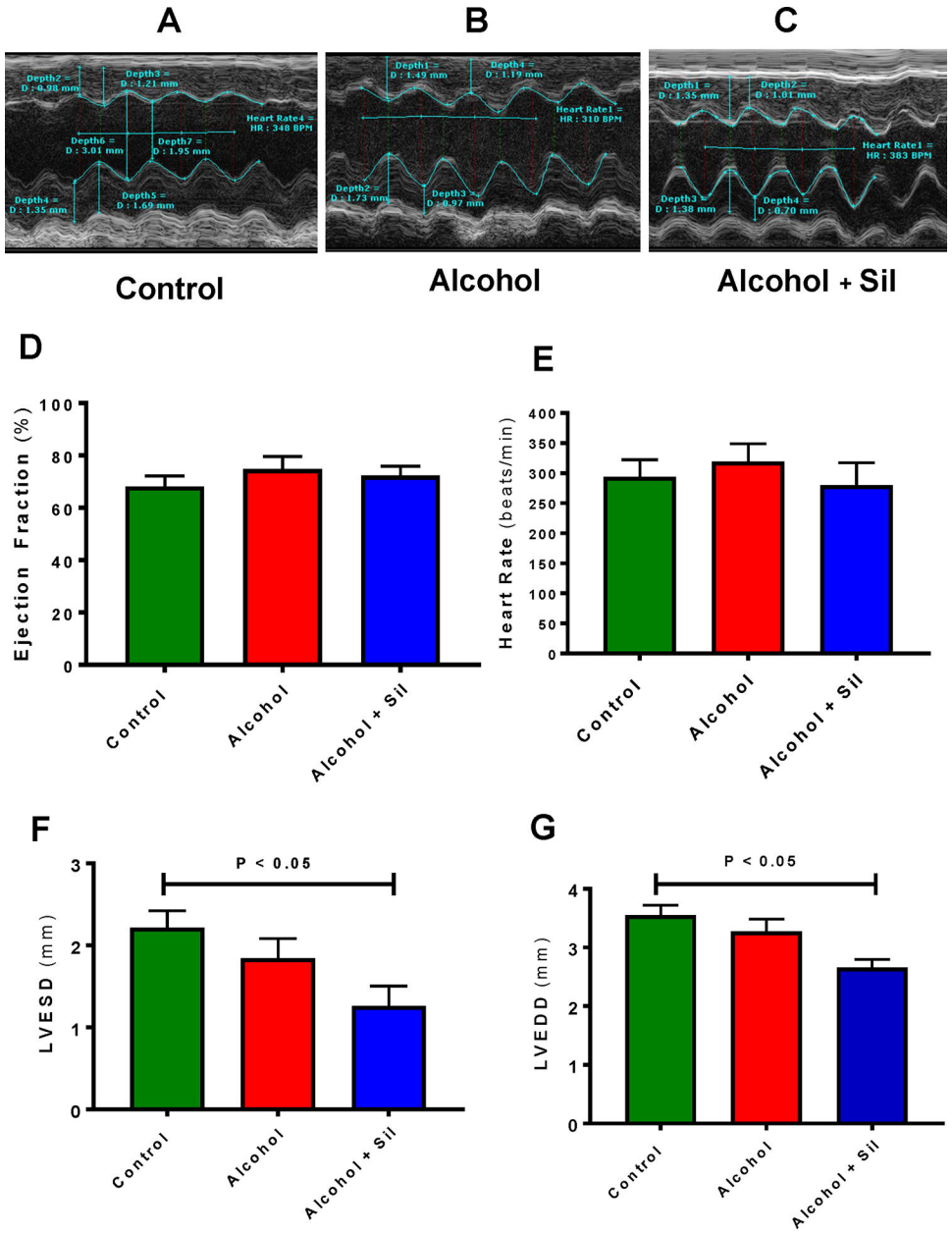

Figure 3.

Representative images of echocardiography analysis of cardiac function in the mice after received (A) *Control*, (B) *Alcohol*, or (C) *Alcohol + Sil* liquid diet for 14 weeks. The results from quantitative analyses are shown in (D) left ventricular ejection fraction; (E) heart rate; (F) LV end-systolic diameter - ESD; and (G) LV end-diastolic diameter - EDD. Data in the bar graphs are Mean ± Standard Error.

A

mmu-miR-214/Bcl2 Alignment

```
    3' ugacggaCAGACACGGACGACa 5' mmu-miR-214
               | || | |||||||
637:5' ccacugaGACU-UCCCUGCUGa 3' Bcl2
```

mirSVR score: -0.1102
PhastCons score: 0.6955

B

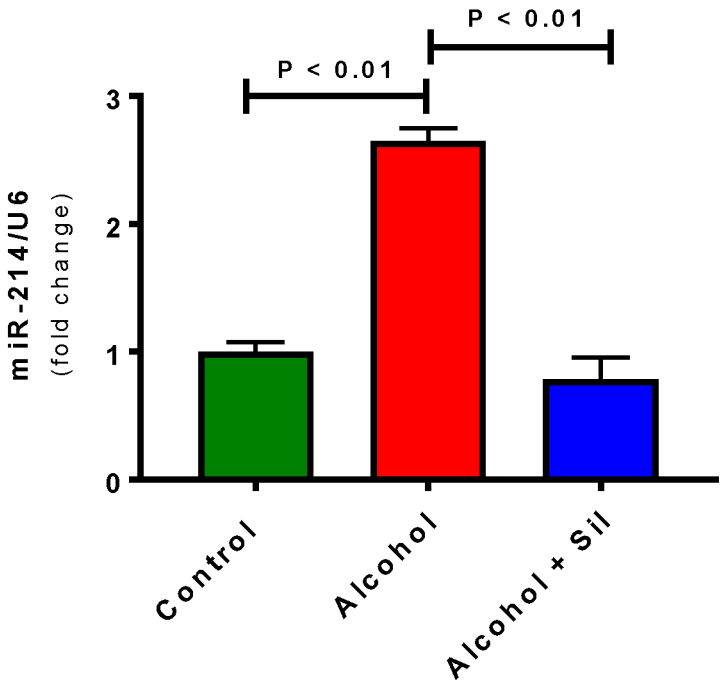

Figure 4.

(A) Computational analysis of miR-214 sequence alignment with Bcl-2 mRNA target using miRANDA prediction algorithm. (B) Real-Time PCR analysis of miR-214 expression in the cardiac tissues collected from the treatment groups of *Control* (n=6), *Alcohol* (n=7), and *Alcohol + Sil* (n=6). Data in the bar graphs are Mean ± Standard Error.

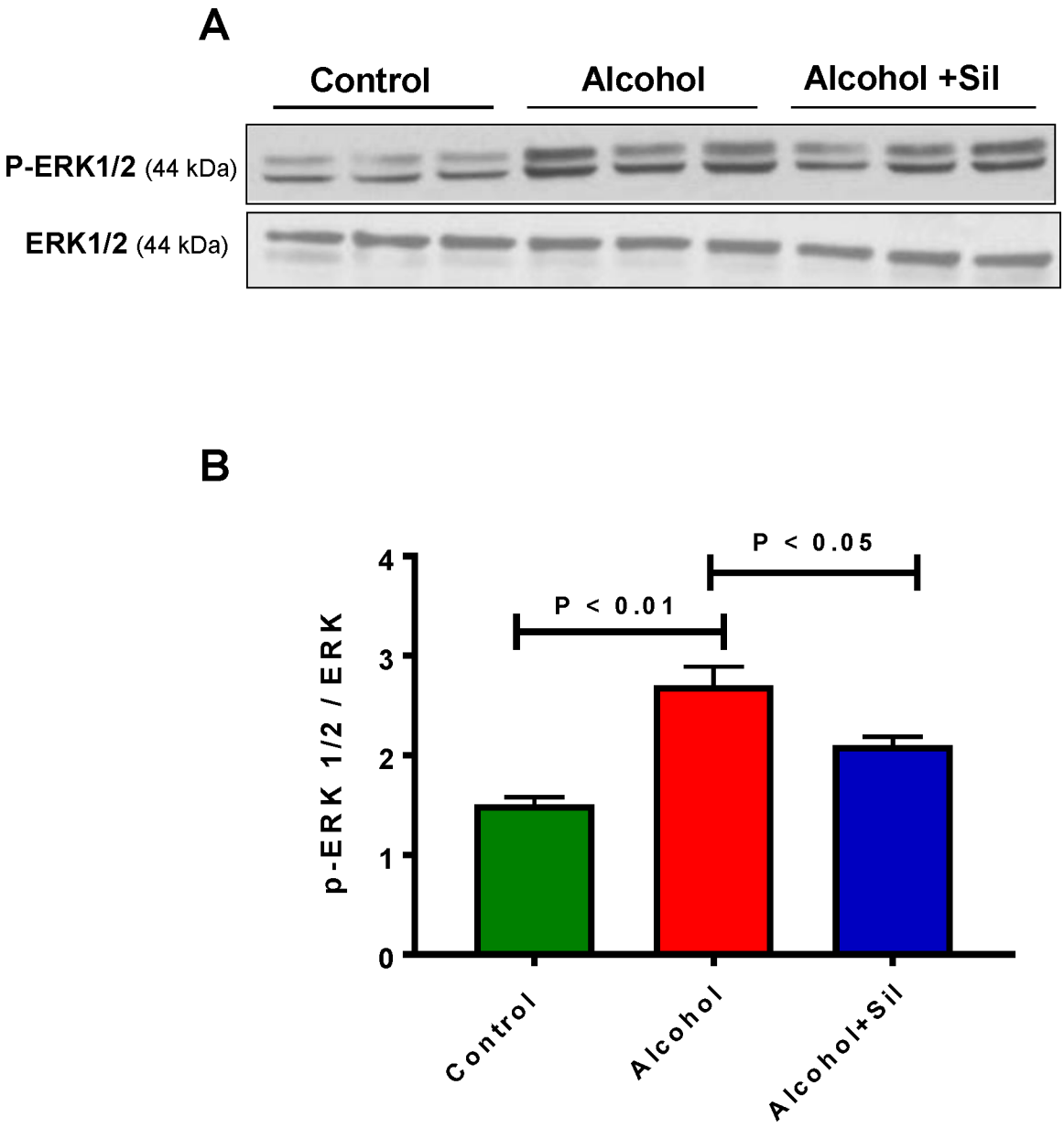

Figure 5.

(A) Expression of phospho-ERK1/2 (Thr-177/160) and total ERK1/2 protein levels in the heart showing stress level induced by alcohol treatment and prevention by co-treatment with sildenafil. (B) Densitometry quantification of phospho-ERK1/2 bands normalized to total ERK. Data in the bar graphs are Mean ± Standard Error (n=3/group).

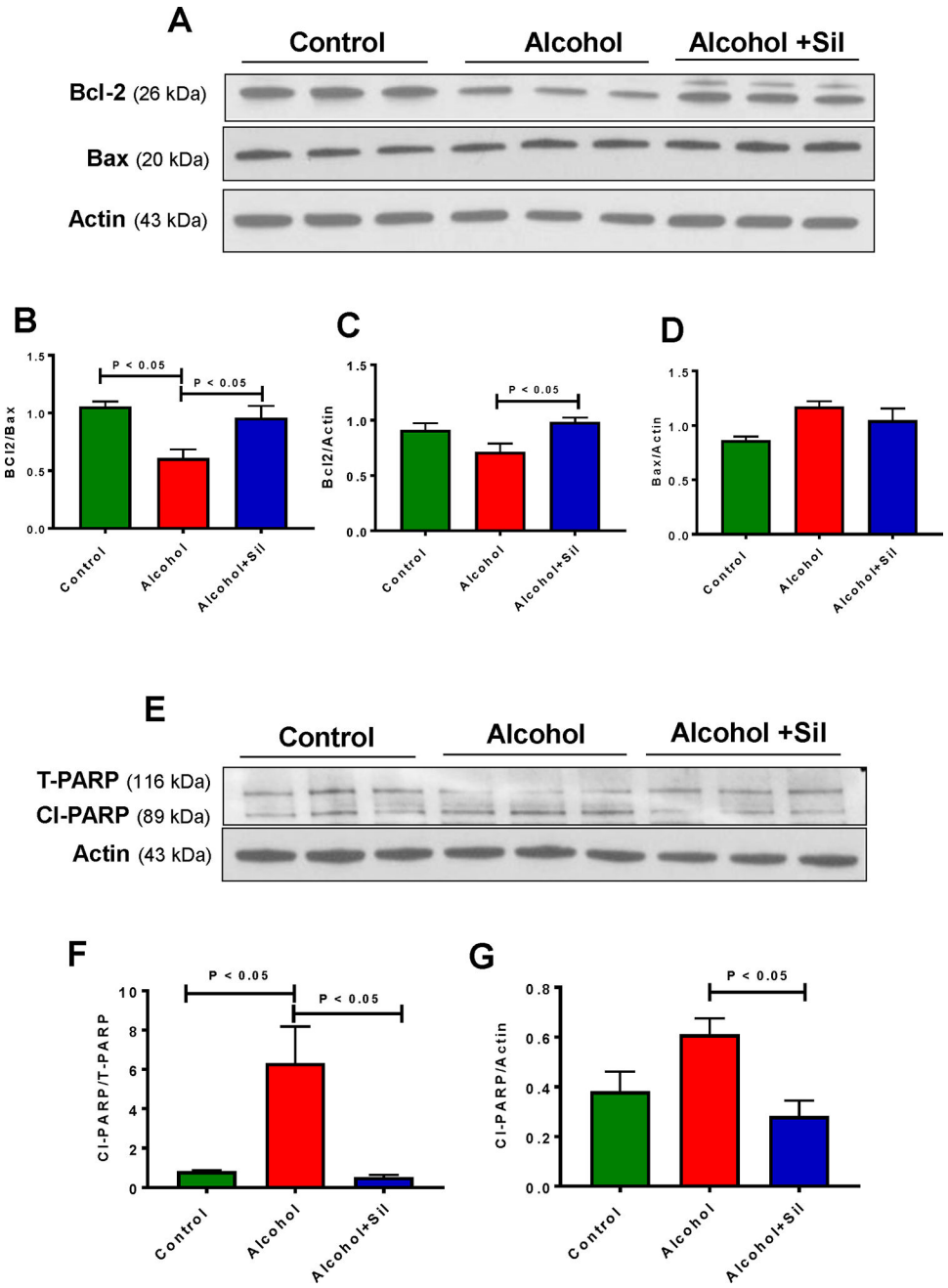

Figure 6.

(A) Western blot pictures showing protein expression of Bcl-2, a target of miR-214 and β-actin as loading control; (B) Densitometry of Bcl-2 normalized to β-actin. (C) Western blot analysis of cleaved PARP to total PARP ratio as marker of apoptosis in control; alcohol and alcohol+Sil groups. (D) Bar graph representation of cleaved PARP to total PARP ratio. Data in the bar graphs are Mean ± Standard Error (n=3/group).

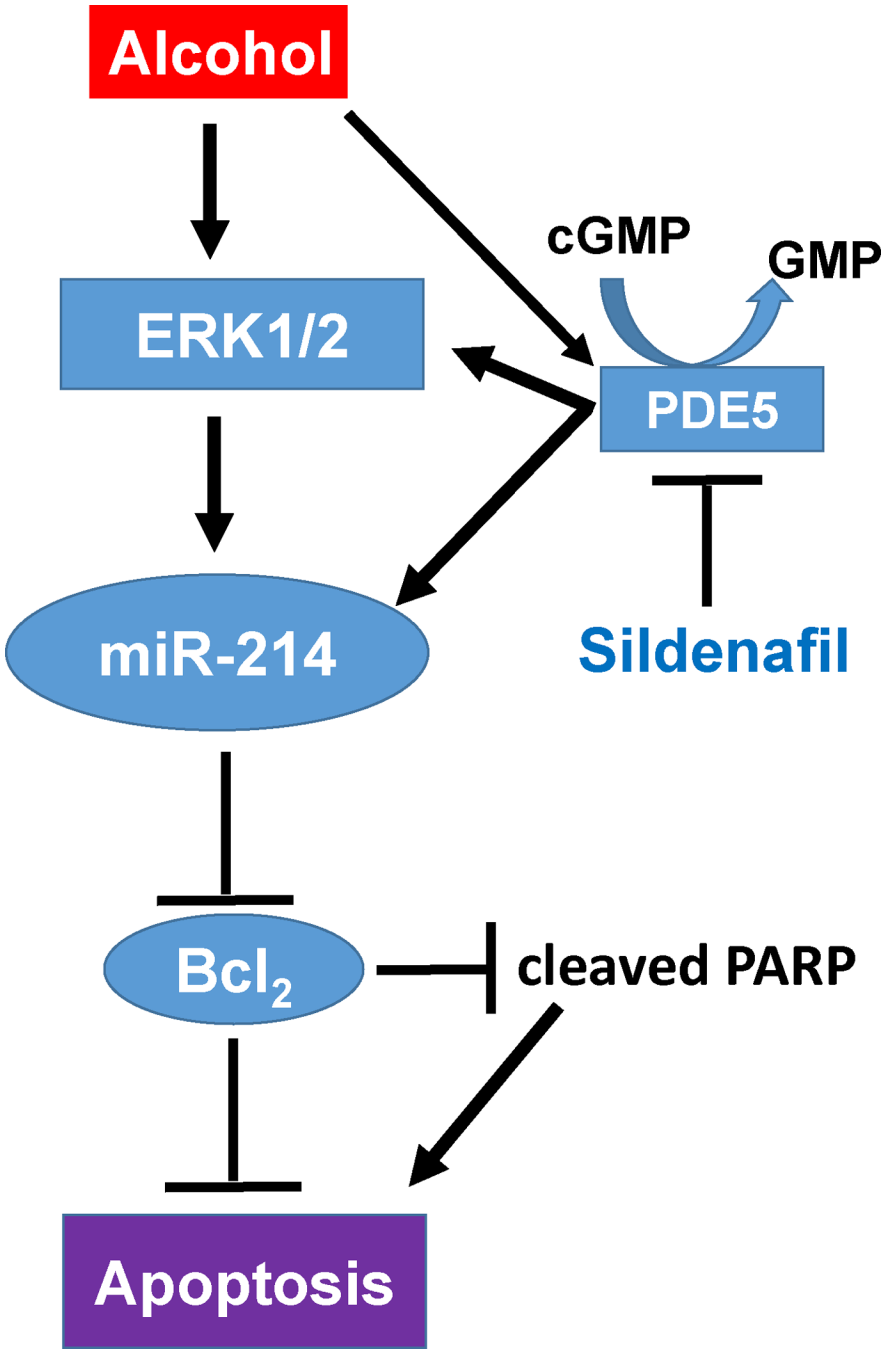

Figure 7.
An illustrative summary of cytoprotective pathways by which PDE5 inhibition with sildenafil protects against alcohol cardiomyopathy and its pathological signaling involving miR-214, Bcl2, ERK1/2 and PARP.