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Changes in Myocardial *SERCA2a* Expression in Mouse Model of Chronic Alcohol Abuse

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Abstract

Chronic alcohol consumption remains a global health problem and has been implicated in many chronic and acute diseases. Alcohol consumption is a major risk factor for sudden cardiac death; chronic alcohol consumption contributes to collagen accumulation, myocardial fibrosis, and arrhythmias. However, the mechanism remains poorly understood. We hypothesized that chronic alcohol consumption causes an imbalance in the expression of MMPs and TIMPs, and also alters the regulation of proteins, including SERCA2a and CTGF, resulting in myocardial remodeling and cardiac dysfunction. After 6 weeks on diet, cardiac tissue isolated from ethanol-exposed mice and non-exposed mice were assayed for *Serca2a*, *Ctgf*, *Timp-1*, and *Mmp-3* expression by western blotting and QqRT-PCR. Ethanol exposure yielded a significant accumulation of the transcripts of *Ctgf* and *Mmp3*, without significantly affecting the levels of the corresponding proteins. *TIMP-1* mRNA and protein expression were not changed. Expression of the SERCA2a protein was significantly reduced in the ethanol group compared with the control group, despite the lack of significant changes in the accumulation of corresponding mRNA. Our findings on mRNA expression of the CTGF, MMP3, and TIMP1 encoding genes are consistent with previous reports on protein expression of these signal transducers, given that the data for the western blots did not reveal significant changes in the accumulation of these proteins. MMP/TIMP imbalance induced by extended alcohol exposure may significantly contribute to myocardial

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remodeling and cardiac dysfunction, which are responsible for sudden cardiac death in ethanol abusers.

Key words: Chronic alcoholism, Sudden death, Cardiac remodeling, MMPs, TIMPs

INTRODUCTION

The relationship between alcohol consumption and adverse effects on human health is not linear. Lower and moderate alcohol consumption (5–25 g/day) is associated with a significantly lower incidence of cardiovascular and all-cause mortality in patients with cardiovascular disease (1). However, some studies have reported a strong connection between alcohol consumption and premature mortality (2, 3). Despite the controversy on whether alcohol has a direct cardio-protective role, many studies have revealed that deaths from alcohol-related causes are increasing at an alarming rate. Increasing alcohol consumption is generally associated with alcoholic liver disease; however, the major cause of increased mortality in alcoholics is not alcoholic liver disease but cardiovascular disease (4).

Recently, sudden cardiac death has been reported to be an increasing issue in middle-aged people (5, 6), an observation that has drawn the attention of many health professionals and even the general public. Sudden cardiac death refers to unexpected natural death within a short interval (approximately 1 h) of the onset of cardiac symptoms in a person lacking a previously diagnosed severe cardiac condition. In the United States alone, sudden cardiac death annually accounts for 300,000–400,000 deaths, based on an assumption of 600,000 cardiovascular deaths per year in the mid-1970s (7, 8). These sudden deaths are attributed to cardiac arrhythmia that progresses into heart failure. Chronic alcohol consumption, which has been demonstrated in human and animal studies to result in collagen accumulation, myocardial fibrosis, and heart failure, is regarded as a major risk factor for sudden cardiac death (6, 9).

Chronic alcohol consumption remains a global health problem and has been implicated in many chronic and acute diseases. Alcohol has detrimental effects on the liver, where it is metabolized. The resulting metabolites are subsequently distributed to various parts of the body, including the central nervous system, cardiovascular system, kidneys, lungs, gastrointestinal tract, pancreas, and immune system (10, 11). The underlying mechanism of liver injury due to chronic alcohol consumption has been studied in depth. Although chronic consumption of ethanol leads to cell injury in nearly every tissue, to our knowledge, few studies have focused on the specific mechanism underlying the pathology in cardiac tissue. However, studies have reported that chronic alcohol consumption is a key factor in the etiology of heart disease, and several mechanisms have been proposed to contribute to alcohol-induced myocardial dysfunction, including oxidative stress, mitochondrial and sarcoplasmic reticulum abnormalities in myocytes, cardiomyocyte hypertrophy, and cardiac fibrosis (12, 13). Additionally, recent reports have suggested that genetic polymorphism is responsible for increased susceptibility to alcohol-induced heart damage. For example, the DD genotype of the gene

encoding angiotensin-converting enzyme has been shown to be associated with a 16-fold increase in susceptibility to the development of left-ventricle failure in consumers of alcohol (14).

Previously, we showed that Janus kinases (JAKs) and the signal transducer and activator of transcription (STAT), proteins that play important roles in controlling myocardial cell death during cardiac failure and myocardial remodeling, are activated following repetitive alcohol consumption, and that activation of JAK/STAT may hasten progression to cardiac failure (15). We also observed significant increases in the serum concentrations of the cytokines interleukin (IL)-1α, IL-2, IL-3, IL-4, IL-5, IL-12 (p70), IL-13, monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, and tumor necrosis factor (TNF)-α (16, 17); these data suggested the existence of a cytokine-mediated inflammatory response in chronic alcoholic mice.

Although various signal transducers are believed to be involved in the instigation and progression of alcohol-induced cardiac dysfunction, the mechanism of this effect remains poorly understood. In the present study, we primarily focused on the role of chronic alcoholism in cardiac remodeling. During hepatic fibrosis, the expression and ratio of matrix metalloproteinases (MMPs) to tissue inhibitors of matrix metalloproteinases (TIMPs) are key factors in the remodeling of the extracellular matrix (ECM) in the liver (18). Similarly, cardiac fibroblasts modulate the expression and activity of MMPs and TIMPs in response to stressors, ultimately modifying collagen production and degradation (Truter et al., 2009). The expression of MMPs and TIMPs have been reported to be modified by alcohol in cardiac fibroblasts and the hearts of alcohol-exposed rats, leading to the accumulation of collagen (9). Connective-tissue growth factor (CTGF), a secreted matricellular protein, acts as a significant mediator during tissue remodeling and fibrosis; CTGF interacts with a variety of molecules, including cytokines, growth factors, receptors, and matrix proteins, resulting in alteration in signal transduction pathways and changes in cellular responses (19). The sarco/endoplasmic reticulum Ca^{2+} ATPase type 2a (SERCA2a) protein helps in maintaining a balanced Ca^{2+} concentration; this ion plays an essential role in the excitation and contraction functions of cardiac myocytes (20).

In the present study, the effect of chronic alcohol consumption on cardiac tissue was assessed by measuring the mRNA and protein expression levels of SERCA2a, CTGF, TIMP-1, and MMP-3 in a repeated-ethanol-exposed mouse model. These findings are expected to contribute to our understanding of the mediators of myocardial remodeling that are responsible for sudden cardiac death in ethanol abusers.

MATERIAL AND METHODS

1) Alcoholic mouse model

A chronic-alcohol-consuming mouse model was developed using 7-week-old male C57BL/6N mice; they were fed Lieber–DeCarli liquid diet (21) for 6 weeks. The study consisted of 31 mice which were divided into two groups: a control group (n = 7) fed the liquid diet without ethanol and an alcohol group (n = 24) fed the liquid diet supplemented with 4% (w/v) ethanol; animals were maintained on study for 41 days. Individual weight measurements were recorded weekly for each group. On the 42nd day, half of the mice in the alcohol group were selected for the withdrawal group (n = 10) and their diet was switched to the liquid diet without ethanol; the remaining members of the alcohol group were assigned as the ethanol group (n = 10) and continued to be fed the liquid diet supplemented with 4% ethanol. On the 43rd day, animals were fasted (deprived of access to the liquid diet) starting 1 h before termination of the mice. Group assignments and selection of experimental samples were designed in a manner to yield groups of similar mean body weights. At the end of the study, all of the fasted mice from the control, ethanol, and withdrawal groups were euthanized by decapitation, and the hearts were immediately excised.

2) Quantitative real-time reverse transcription-polymerase chain reaction (RT-qPCR)

A portion of each excised heart was immediately stored in RNAlater solution (Ambion, CA, USA). These samples then were homogenized using a Micro Smash® MS-100 (TOMY, Tokyo, Japan), and total RNA was extracted from cardiac tissue using an RNAeasy Mini Kit (Qiagen, Valencia, CA). The extracted RNA samples were used for the determination of mRNA expression of the genes encoding SERCA2a, CTGF, TIMP-1, and MMP-3 using a TaqMan® one-Step RT-qPCR Master Mix Reagents Kit. This assay performs RT and polymerase chain reaction amplification of specific target RNAs in a single-buffer system. The house-keeping gene encoding β-actin was used as an internal control for normalizations.

3) Tissue lysis for protein extraction

Another portion of each excised heart was immediately frozen and stored at −80°C to prevent proteolytic degradation. These samples then were weighed and homogenized with zirconia balls in homogenization tubes using Micro Smash® MS-100 (TOMY). Pierce® RIPA buffer (Thermo Scientific, Waltham, USA) supplemented with protease and phosphatase inhibitors [Halt® Protease Inhibitor Cocktail (Thermo Scientific)] was used as lysis buffer. Mechanical homogenization was performed until the tissues were completely lysed. Next, the lysates were transferred to centrifuge tubes and centrifuged at 15,000 g for 10 min at 4°C. The supernatants were transferred to new tubes and the BCA assay [Pierce® BCA Protein Assay Kit (Thermo Scientific)] was performed to determine the concentration of protein in the lysates.

4) Western blotting

Western blot analysis of protein expression in cardiac tissue lysates was performed using

Invitrogen's Mini Gel Tank and Blot Module set. After determination of total protein concentration by BCA assay, equal amounts of reduced and denatured protein samples were loaded on a Bolt 4%–12% bis-Tris Plus gradient gel, and electrophoresis was conducted in Bolt MES-SDS running buffer. Separated proteins were transferred to PVDF membrane by the semi-wet method using the Blot module. The membrane was blocked using 5% skim milk for 1 h. Expression of proteins in tissue lysates was assayed with the primary antibodies against the following targets: SERCA2a, CTGF, TIMP-1, and MMP-3 (Abcam plc, Cambridge, UK). Antibody against beta-actin (Abcam) was used to confirm equal loading of protein from each sample. Images were developed in a LAS 400 mini imager using the ECL Prime western blotting detection reagent (Amersham, Buckinghamshire, UK). Protein bands were quantified using Multigauge software and normalized by β-actin expression.

5) Histology

FIHC was performed with SERCA2a as the primary antibody in accordance with IHC and immunofluorescence (IF) protocols (Abcam). Rabbit anti-mouse SERCA2a and donkey anti-rabbit IgG Alexa Fluor® 488 conjugated secondary antibodies were purchased from Abcam. The paraffinized specimens were sectioned at a thickness of 5 μm using a microtome. After heat-mediated antigen retrieval with Histofine® Antigen Retrieval Reagent (pH = 9.0), sections were treated with a block solution (5% skim milk in PBS 150 mL) for 1 h at room temperature. The sections were incubated in a primary antibody solution (SERCA2a in block solution, 1:100) overnight at 4°C. Next, samples were incubated in a secondary antibody solution (α-rabbit 488 in block solution, 1:1000 for antibodies) for 1 h at RT. We used a BZ-9000 (Keyence, Osaka, Japan) for microscopic observations.

6) Statistical analysis

Data are expressed as mean ± standard error of mean (SEM). Statistical analysis was performed via a two-tailed unpaired Student's *t*-test (for comparisons between two groups) or by a two-tailed one-way analysis of variance followed by Tukey's honestly significant difference test where significance was indicated (for comparisons between more than two groups). A p-value of <0.05 was considered statistically significant.

RESULTS

We compared the transcript accumulation of the genes encoding SERCA2a, CTGF, TIMP-1, and MMP-3 among the three groups to determine whether ethanol had any effect on the expression of these genes in our mouse model. We also investigated the protein expression of these factors in cardiac tissue.

1) Ethanol exposure upregulates *Ctgf* mRNA levels in cardiac tissue

CTGF is a significant mediator of tissue remodeling and fibrosis; we therefore assessed *Ctgf* expression. We observed a significant increase in the transcript levels of *Ctgf* in the ethanol group compared with those in the control group in the 6-week model ($p < 0.05$, Figure 1b). In contrast, no significant difference in *Ctgf* mRNA accumulation was found between the withdrawal and control groups.

Significant difference was observed for *Ctgf* (b) and *Mmp3* (d). Expression of *Serca2a* (a) and *Timp1* (c) showed no significant difference among the three groups. Data are shown as means ± SEM (standard error of mean), (n = 6, control; n = 9, ethanol; and n = 9, withdrawal). GAPDH or β-actin was used as the house-keeping gene. Statistical significance was determined by Tukey's honestly significant difference test. *$p < 0.05$

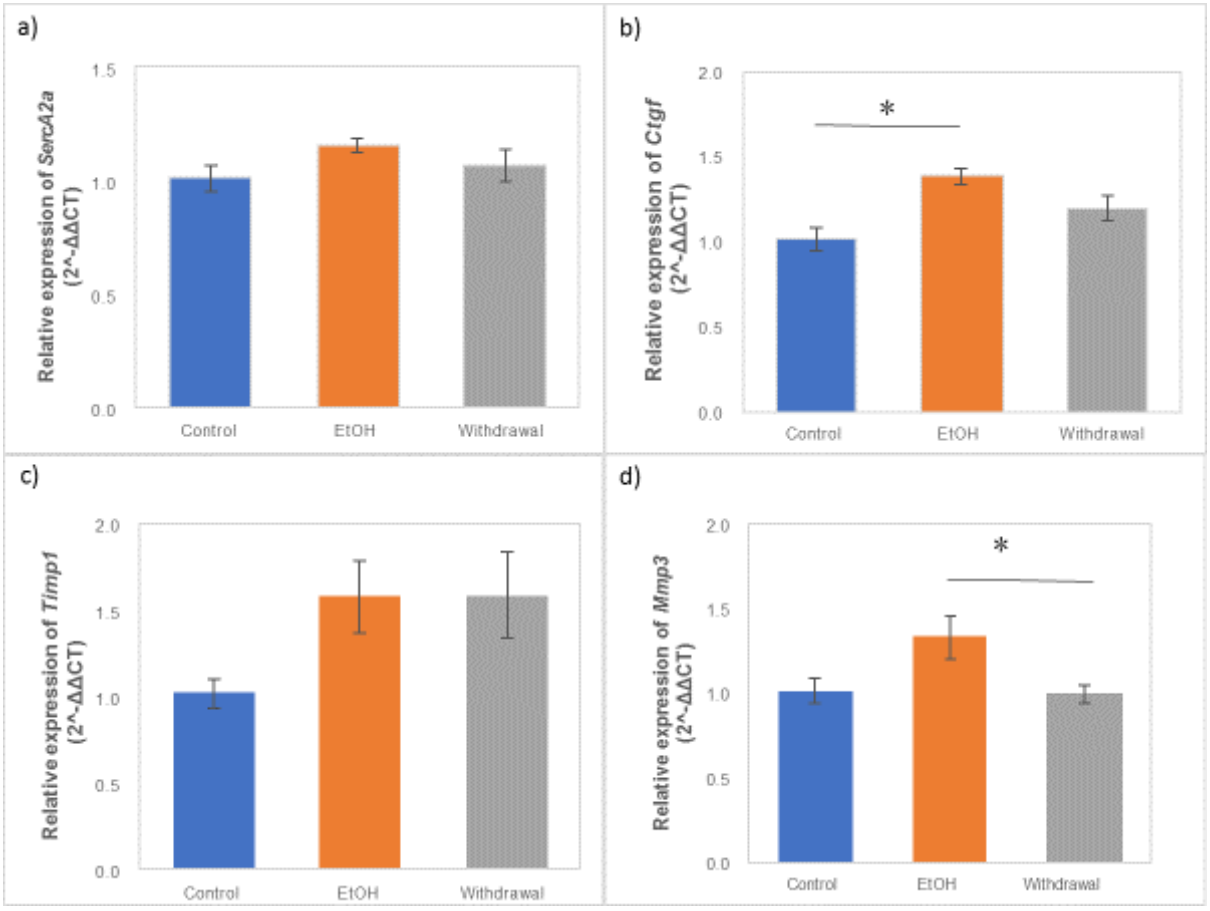


Figure 1 Gene expression of messenger RNAs (mRNAs) in the ethanol (EtOH), withdrawal, and control groups.

2) Differential modulation of *Mmp3* expression in cardiac tissue under ethanol exposure

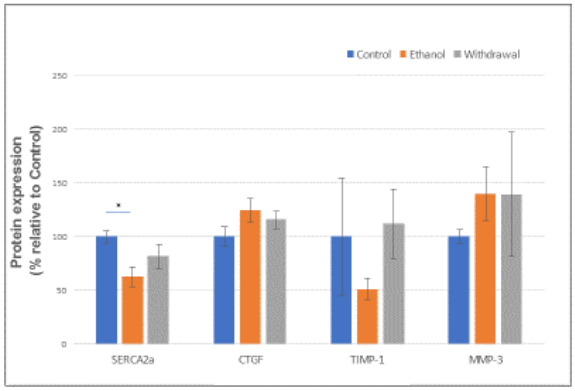
We compared the expression of *MMP3* among the control, ethanol, and withdrawal groups to determine whether ethanol has any effect on the expression of this gene in our mouse model. We observed that the cardiac *Mmp3* transcript levels were significantly elevated in the ethanol group compared with those in the withdrawal group ($p < 0.05$, Figure 1d).

3) *Timp1* and *Serca2a* mRNA levels in the cardiac tissue of ethanol-exposed mice

Next, we examined the transcript levels of the genes encoding TIMP-1 (an inhibitor of gelatinases and stromelysin) and SERCA2a in ethanol-exposed mice. For both genes, the mRNA accumulation was not significantly different in the cardiac tissue of the ethanol and withdrawal groups compared with that of the control group (Figure 1c and 1a, respectively).

4) SERCA2a protein levels in the cardiac tissue of ethanol-exposed mice

Although *Serca2a* mRNA levels did not significantly differ among the three groups, western blotting revealed that the level of SERCA2a protein (normalized to β-actin and then expressed as percentage relative to control) was significantly decreased in the ethanol group compared with that in the control group ($p < 0.05$, Figures 2a and b and 3). In contrast, the levels of CTGF, TIMP-1, and MMP-3 proteins did not differ among the various groups, despite the significant changes observed in the accumulation of *Ctgf* and *Mmp3* transcripts in the various groups.



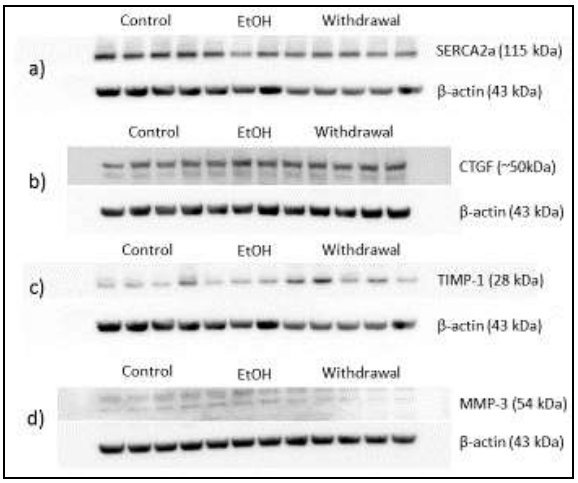


Figure 2 Cardiac protein expression in the control, ethanol, and withdrawal groups

Graphical representation (a): Graph showing protein expression in cardiac tissues of the control, ethanol, and withdrawal groups. Significant reduction of SERCA2a in the ethanol group compared with the control group ($p < 0.05$) was found. No significant differences were observed for CTGF, TIMP-1, and MMP-3. Densitometry was normalized to β-actin and then expressed as percentage relative to control mean. (*$p < 0.05$)

Representative blots (b): Cardiac tissue homogenates were assessed for Serca2a, Ctgf, Timp-1, and Mmp-3 expression by western blot. Figure shows the representative blots of these proteins and β-actin as the loading control.

model. Specifically, we analyzed mRNA and protein levels of SERCA2a, CTGF, TIMP-1, and MMP-3 in cardiac tissue; all of these proteins are possible mediators of cardiac damage. We showed significant differences in the levels of *Ctgf* and *Mmp*3 transcripts, but not in those of *TIMP1* and

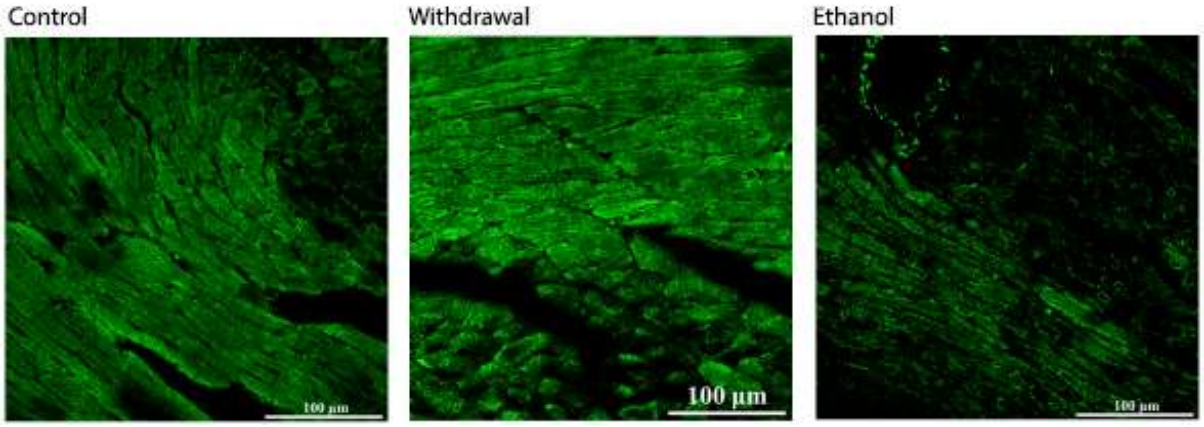


Figure 3 FIHC with α-SERCA2a

DISCUSSION

Cytokines and chemokines play important roles in alcohol-induced cardiac dysfunction and remodeling, as first proposed by Ferrari et al. (1999) (22) in his review of the data regarding the role of cytokines in general, and TNF-α in particular, in cardiovascular disease. In a previous study performed on mouse serum, we observed significant increases in the concentrations of multiple cytokines [including IL-1α, IL-2, IL-3, IL-4, IL-5, IL-12 (p70), IL-13, MCP-1, MIP-1α, and TNF-α], suggesting a cytokine-mediated inflammatory response in chronic alcohol-exposed mice (17). In the present study, we investigated the mechanisms underlying heart damage associated with heavy alcoholism using a murine

SERCA2, among the three experimental groups with distinct alcohol exposure. In contrast, western blot analysis revealed a significant difference in the level of SERCA2a protein, but not in those of CTGF, MMP-3, or TIMP-1, between the ethanol group and the control group.

CTGF is a cysteine-rich protein induced in connective-tissue cells by transforming growth factor beta (TGF-β). Different cellular processes such as the proliferation, adhesion, migration of cells and the synthesis of ECM, which are the underlying causes of fibrosis, can be triggered by CTGF. Upregulation of *Ctgf* expression coincides with an increase in the production of fibronectin, collagen type I, and plasminogen activator inhibitor-1, an effect that has been shown to cause

fibrotic diseases in various human and animal studies (Chenn MM et al., 2000). In the present work, we observed significant upregulation of *Ctgf* transcription in the ethanol group, suggesting activation of proliferative and fibrogenic processes following extended periods of alcohol consumption. This excess *Ctgf* expression is consistent with the corresponding protein's active role in the processes of myofibroblast activation and differentiation, leading to increases in ECM deposition, cell motility, and adhesion, and finally resulting in tissue remodeling.

The MMPs are a large and diverse family of extracellular, zinc-dependent endopeptidases required for the degradation of ECM components during normal embryo development, morphogenesis, and tissue remodeling. Members of this family have been implicated in the pathophysiology of many diseases. TIMPs are a family of small extracellular proteins known to inhibit the enzymatic activity of MMPs. A balance between MMP and TIMP activities is required to maintain tissue homeostasis; disruption of the balance between the MMPs and TIMPs results in the dysregulation of MMP activity. Elevated MMP/TIMP expression ratios are associated with many central nervous system diseases, cardiovascular diseases, and liver fibrosis (9, 18, 23). In our experiment using a chronic-alcohol-consuming mouse model, we observed differential transcription of *Mmp3* (which encodes a protein responsible for the activation of procollagenase) in the cardiac tissue of the ethanol group. The significant upregulation of *Mmp3* transcription is consistent with previous reports of the accumulation of collagen in the hearts of the ethanol-exposed animals. Other researchers have observed dramatic increases in the expression of *TIMP-1* in response to a variety of injuries and inflammatory insults (24), and the alcohol-dependent increase in *TIMP-1* expression has been shown to contribute to the progression of liver fibrosis (18). TIMP-1 inhibits gelatinases A and B (MMP-2 and MMP-9, respectively), exhibiting a high affinity for MMP-9. Structural analysis indicates that TIMP-1 presents five different chain regions along the face of the protein capable of occupying the entire length of the active site cleft of MMP-3 (25). Although TIMP-1 is best known for its endogenous regulation of MMP activities, the C-terminal domain of TIMP-1 has also been found to mediate intracellular signaling via receptor binding, revealing that TIMP-1 can exhibit direct cellular action independent of its ability to regulate MMP activities (26). A study by El Hajj et al. (9) reported an increase in TIMP-1 protein levels in the cardiac tissue of ethanol-exposed rats. In contrast, we nominally observed (although not significantly) decreased levels of TIMP-1 protein in the cardiac tissue of mice in the ethanol group. These apparent inconsistencies may reflect differences in the models used, including differences in the species (rat vs. mouse), the dose of ethanol, route of administration, and exposure duration. We suspect that the reduced levels of TIMP-1 protein expression in the heart contribute to acute heart muscle injury during ethanol

exposure. However, the mRNA expression of *Timp1* was nominally (though not significantly) upregulated in both the ethanol and withdrawal groups. Although the effect was not significant, we infer that accumulation of *Timp1* transcript could be a response to the upregulation of MMP-encoding genes. Alternatively, changes in *Timp1* expression may reflect the MMP-independent function of TIMP. Nevertheless, changes in the balance between TIMP-1 and MMP activities may contribute to the reduced contractility and derangement of myofibrillar architecture observed during prolonged ethanol intake.

The Ca^{2+} ion has an important role in the excitation and contraction functions of cardiac myocytes. SERCA2a helps in maintaining a balanced concentration of Ca^{2+} during the cardiac cycle (20). Decreased SERCA2a has been frequently observed in studies of heart failure in humans and animals. In an experiment performed on Serc2a-knockout (KO) mice, heart failure developed between 4 and 7 weeks after gene deletion, and was associated with an elevation of Na^{+} ion levels and intracellular acidosis (27). The authors of that study suggested that Na^{+} accumulation in the Serc2a-KO results from the upregulation of the NCX $Na^{+}/Ca2^{+}$ exchanger; the resulting intracellular acidosis is considered to initiate a vicious cycle involving a mismatch between ATP demand and supply; an increasingly compromised metabolism; decreased pH; and, finally, an even greater Na^{+} elevation, which potentially plays a role in the development of heart failure. Our data on protein expression indicated that SERCA2a protein levels are significantly downregulated in animals suffering from chronic ethanol exposure, suggesting an impairment in $Ca2^{+}$ homeostasis. In alcohol abusers, the decreased levels of SERCA2a may result in the contractile defects noted in individuals with heart failure. This loss of $Ca2^{+}$ homeostasis imposed by SERCA2a depletion, coupled with myocardial ECM remodeling due to an imbalance of MMPs and TIMPs, may be a major cause of sudden cardiac death in alcohol abusers.

CONCLUSIONS

Several studies have investigated the role of MMPs and TIMPs in the modulation of alcohol-induced fibrosis. Our study also emphasized an imbalance between MMPs (specifically MMP-3) and TIMP-1 in the cardiac tissue of a mouse model of chronic alcohol consumption. Our data on the depletion of SERCA2a protein and accumulation of *Ctgf* and *Mmp3* transcripts in the ethanol group suggested that multiple mechanisms are involved in the induction of heart failure associated with alcohol abuse. Our results also indicated that the pattern of alcohol intake has distinct impacts on the cardiac tissue of alcohol consumers, as demonstrated by differences between the ethanol and withdrawal groups in our study. Our research was limited to examining the RNA and protein expression of SERCA2a, CTGF, TIMP-1, and MMP-3. Explorations of MMPs and TIMPs other than MMP-3 and TIMP-1, as well as other mediators, will be necessary to further

elaborate the mechanism of cardiac remodeling induced by chronic ethanol intake.

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Conflict of Interest Disclosure: The authors declare that they have no conflict of interest.

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