Objective: Association between chronic alcohol intake and cardiac abnormality is well known; however, the precise underlying molecular mediators involved in ethanol-induced heart abnormalities remain elusive. This study investigated the effect of chronic ethanol exposure on calcium/calmodulin-dependent protein kinase IIδ (CaMKIIδ) gene expression and monoamine oxidase (MAO) levels and histological changes in rat heart. It was also planned to find out whether Zingiber officinale (ginger) extract mitigated the abnormalities induced by ethanol in rat heart.

Methods: Male wistar rats were divided into three groups of eight animals each: control, ethanol, and ginger extract treated-ethanol (GETE) groups.

Results: After 6 weeks of treatment, the results revealed a significant increase in CaMKIIδ total and isoforms δ2 and δ3 of CaMKIIδ gene expression as well as a significant decrease in the MAO levels in the ethanol group compared to that in the control group. Moreover, compared to the control group, the ethanol group showed histological changes, such as fibrosis, heart muscle cells proliferation, myocyte hypertrophy, vacuolization, and focal lymphocytic infiltration. Consumption of ginger extract along with ethanol ameliorated CaMKIIδ total. In addition, compared to the ethanol group, isoforms gene expression changed and increased the reduced MAO levels and mitigated heart structural changes.

Conclusion: These findings indicate that ethanol-induced heart abnormalities may, in part, be associated with Ca2+ homeostasis changes mediated by overexpression of CaMKIIδ gene and the decrease of MAO levels and that these effects can be alleviated by using ginger extract as an antioxidant and anti-inflammatory agent.

Keywords: ethanol, heart, oxidative stress, CaMKIIδ, rat, fibrosis, ginger

Introduction

Recent studies have demonstrated that chronic ethanol exposure leads to a wide range of functional and structural abnormalities in the cardiovascular system (1-3). From the structural aspect, heart tissue fibrosis decreases the myocyte number, disrupts myofibrillar structure, and causes left ventricular hypertrophy and myocardial infarction. Moreover, even an increase in sudden death has been reported (1, 2). Large spectrums of functional alterations have also been reported as the result of chronic ethanol consumption. We have previously shown that ethanol consumption leads to an elevated systolic, diastolic, pulse, main arterial, and dicrotic pressure (4). In addition, reduced cardiac contractility, cardiac output, left ventricular ejection fraction, and abnormalities of the great vessels result from chronic ethanol exposure in animal and human models (5-7). Furthermore, alteration in Ca2+ transport, mitochondrial function, sarcoplasmic reticulum Ca2+ uptake/binding, and Ca2+ homeostasis have been demonstrated by several previous studies (3, 8, 9). Although different aspects of functional and structural cardiac alterations have been identified by early and recent studies, the precise mediating steps between exposure of heart muscle to ethanol and initiation of the cascade of responses leading to cardiac abnormality have not yet been completely clarified. Numerous mechanisms, such as oxidative stress, inflammatory reactions, toxicity of ethanol itself and its primary metabolite acetaldehyde, accumulation of fatty acid esters, and modification of lipoproteins, have been suggested to explain pathogenesis of chronic ethanol-induced abnormalities.

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Accepted Date: 03.11.2017 Available Online Date: 28.12.2017
©Copyright 2018 by Turkish Society of Cardiology - Available online at www.anatoljcardiol.com DOI:10.14744/AnatolJCardiol.2017.8079
in the heart tissue (2, 4, 10, 11). However, studies have often sug-
gested that mechanisms such as oxidative stress and inflamma-
tory reactions explain alterations in heart structure and function
following ethanol exposure and thus have not provided precise
information concerning the specific molecules that could influ-
ence cardiac structure and functions molecular mediators after
ethanol exposure. Among dozens of molecular mediators related
to heart function, calcium/calmodulin-dependent protein kinase
II, particularly CaMKIIδ isoform, is documented as an important
mediator connecting pathological changes in sub-cellular envi-
ronments to alterations in cardiomyocyte Ca\(^{2+}\) handling. CaMKII
gene produces four isoforms (α, β, γ, and δ) with different tissue
distribution. The predominant form in the heart appears to be
CaMKIIδ (12). Although numerous cellular functions, such as cell
cycle, growth, and gene expression, are regulated by CaMKII in
the heart, CaMKIIδ has a significant involvement in the regula-
tion of Ca\(^{2+}\) homeostasis and cardiac contractility (13-16). Besides
physiological functions, numerous studies have shown that over-
expression of CaMKIIδ is a core mechanism for promoting heart
diseases, such as myocardial hypertrophy, arrhythmias, myocyte
apoptosis, defective ECG, Ca\(^{2+}\) homeostasis imbalances, and
transition from hypertrophy to heart failure (17, 18). Monoamine
oxidase (MAO) is another important mediator playing a prominent
role in cardiac function. MAO belongs to a class of flavoenzymes
located in the outer mitochondria membrane and is responsible
for the deamination of neurotransmitters and re-uptake of catech-
olamines (19). Because catecholamines released from the heart
sympathetic nervous system influence myocardium continuously,
their turnover and catabolism rate contribute to cardiac function
and structural alterations. Regarding the role of MAO in catech-
olamines turnover, previous studies have demonstrated that MAO
deletion or overproduction leads to cardiac abnormalities, such as
cardiomyocyte hypertrophy, left ventricular dilation, and heart
failure (20, 21). The fundamental role of CaMKIIδ and MAO in the
initiation and development of cardiac abnormalities and heart fail-
ure, as mentioned above, prompted us to examine the following
hypothesis: chronic ethanol consumption resulting in heart abnor-
malities is mediated, in part, by overexpression of CaMKIIδ related
genes and alteration of MAO levels in the heart tissue. In addition,
because of the well-documented oxidative and inflammatory
nature of ethanol, a second aim of this work was to determine
the possible protective effects of ginger extract against ethanol-
induced histopathological alteration, CaMKIIδ gene expression
changes. Among plants containing natural anti-oxidants, ginger
exhibits unique antioxidant and anti-inflammatory properties with
less unfavorable side effects (22). We also intended to investigate
MAO levels alteration in the heart of male rats.

**Methods**

**Animals and treatments**

All experimental procedures described herein were per-
formed in accordance with the Principles of Laboratory Animal
Care (NIH publication, no.85–23, revised 1985) and were approved
by the Urmia University of Medical Sciences Animal Care Com-
mittee. Overall, 24 male Wister rats with an initial body weight of
220±10 g were divided into three groups (n=8 in each group): con-
trol, ethanol, and ginger extract treated-ethanol (GETE) groups.
For the rats in the ethanol group, ethanol was saluted in tap wa-
ter (20% w/v) and gavaged intragastrically (4.5 g/kg) to rats in the
ethanol group, 6 days a week for 6 weeks. For the rats in the GETE
group, hydro-alcoholic extract of ginger was gavaged intra-
 gastrically (50 mg/kg) for 6 weeks. For the rats in the control group, tap
water was gavaged.

**Extract preparation**

Dried ginger rhizome (originally Chinese) was purchased from
a local market and coarsely powdered. Next, 3 kg of the powder
was mixed with 6 L of 70% ethanol in a suitable container at room
temperature for 3 days. After 3 days, it was filtrated through a filter
paper and concentrated using a rotary evaporator. The yield of the
extract was stored in a refrigerator at 4°C until use.

**Sample preparation**

After 6 weeks of treatment, the rats were anesthetized using
10% chloral hydrate (0.5 mL/100g body weight, IP). The anesthe-
sia depth was assessed by pinching a hind paw. At termination,
after weighing the animals, the thoracic cavity was opened and
the heart was removed. The excised heart was freed from adven-
titial tissues, fat, and blood clots and was subsequently washed
in ice-cold physiological saline and weighed. Next, the whole
left ventricular wall (with septum) was excised from the heart
and weighed. For total RNA isolation, 100 mg of ventricular tis-
sue was immersed in 1 mL RiboxEX (total RNA isolation solution)
(GeneALL, Seoul, Korea) and restored at −80°C until the time of
RNA isolation. For biochemical analysis, other parts of the vent-
ricles were washed with ice-cold physiological saline and dried
on filter papers. Subsequently, an ice-cold extraction buffer (10%
w/vol) containing a 50 mM phosphate buffer (pH 7.4) was added
and homogenized using Ultra Turrax (T10B, IKA, Germany). Next,
the homogenates were centrifuged at 10,000×g at 4°C for 20 min.
Finally, the supernatant sample was obtained and stored at −80°C
until the time of analysis. For analyzing histopathological changes,
a part of the ventricular was fixed in buffered formalin and embed-
ded in paraffin after standard dehydration steps were taken.

**Isolation of total RNA, amplification primers, and real-time
polymerase chain reaction (RT-PCR)**

The total RNA was obtained from 100 mg of the left ventricular
frozen tissue using a kit (Gene all, South Korea, Cat no 305-101), in
accordance with the manufacturer’s instructions. RNA concen-
tration was verified by spectrophotometric measurement of the
absorbance at 260–280 nm and determined by mixture of Tris base,
acetic acid, and EDTA(TAE)-agarose gel electrophoresis.

Reverse transcription (RT) was performed using hyper-
scriptTM Reverse Transcriptase (Gene All, South Korea). RT-PCR
was performed using an amplification reagent kit (Ampliqon, Denmark) by the XP-Cycler instrument (TCXPD, Bieoe, USA) with CaMKII\textsubscript{total}, CaMKII\textsubscript{δ}, CaMKII\textsubscript{δ}, and the rat glyceraldehydes-3-phosphate dehydrogenase (GAPDH) primers. To amplify the cDNA, the 5' and 3' primer sequences (forward and reverse) of the CaMKII\textsubscript{total}, CaMKII\textsubscript{δ}, and CaMKII\textsubscript{δ}, designed via the Gene Bank (http://blast.ncbi.nlm.gov/Blast.cgi) revealed that the primers were gene specific. Furthermore, all the primers were verified using a Gene Runner software (Syngene, Cambridge, UK). Subsequently, the primers (forward and reverse) were synthesized to amplify the cDNA encoding GAPDH as a housekeeping gene; the sequences of related primers are presented in Table 1.

### Real-time quantification

Real-time quantification of the target genes was performed taking advantage of a Real-Time PCR Master Mix Green kit (Ampliqon, Denmark) in a total volume of 25 µL and in accordance with the manufacturer's instructions. Furthermore, the mentioned genes expressions were analyzed using an iQ5 RT-PCR detection system (Bio-Rad, CA, USA). Next, the reactions were prepared for 10 min at 95°C in a 96-well optimal plate followed by 40 cycles of 20 sec at 59°C. To confirm the specificity of the amplification reactions, a melting curve was recorded. Each sample was replicated three times. The value of the threshold cycle (Ct) was the same as that of the corresponding mean. The relative expression of each mRNA was calculated by employing the 2-ΔΔCt method, with Ct being the threshold cycle. Next, the calculated levels were normalized to GAPDH. They were then analyzed for statistical significance applying a one-way analysis of variance.

### MAO assay

MAO levels in the heart tissue was measured by the quantitative sandwich enzyme immunoassay method using a commercial rat MAO Elisa kit (ZellBio, Germany), in accordance with the manufacturer’s instructions.

### Histopathological examinations

For histopathological staining, 5-µm thick histological sections from paraffin-embedded heart tissue were used. Proliferating cells were implemented, in accordance with our published protocol, by performing immunohistochemistry using an antibody against the proliferation cell nuclear antigen (PCNA) (10). Briefly, after taking tissue processing steps, such as deparaffinization, rehydration, and gradual ethanol passage, sections of the heart tissue (thickness, 5-µm) were stained using the Monoclonal Mouse anti-PCNA antibody (Dako, Catalog no: M0879, Copenhagen, Denmark). Optimal results were achieved using the EnVision™ visualization system. Furthermore, Hematoxylin was used as a counterstain. The assessment included proper negative controls. Moreover, all the slides were inspected by two expert pathologists independently. PCNA-positive indices were considered as indicators of heart cell proliferation. To assess PCNA-positive indices percentage, four non-overlapping fields of view per section from 2–3 sections per animal were analyzed. The number of positively stained cells and the total number of cells were counted for each field of view. In addition, for each animal, the number of positively stained cells was then presented as a percentage of the total number of counted cells. The criteria applied in scoring the quality of PCNA-positive indices were as follows: normal (i.e., PCNA-positive indices present in less than 5% of the heart cells), mild (i.e., PCNA-positive indices present in less than 25% of the heart cells), mild to moderate (i.e., PCNA-positive indices present in 25%–50% of the heart cells), moderate to severe (i.e., PCNA-positive indices present in 50%–75% of the heart cells), and severe (i.e., PCNA-positive indices present in 75%–100% of the heart cells). To evaluate the heart tissue fibrosis, 5-µm heart tissue sections were stained using Masson Trichrome, in accordance with the manufacturer’s instructions (Asiapajohesh, Amol, Iran). The severity of tissue fibrosis was estimated maintaining a semi-quantitative method explained by Ashcroft et al. (23) and our published protocol. A score ranging from zero (normal heart) to eight (total fibrosis) was set. The criteria appointed in scoring heart fibrosis were as follows: grade 0=normal heart; grade 1=minimal fibrosis thickening of heart tissue, grade 2 and 3=moderate thickening of heart tissue without obvious damage to the structure of heart tissue; grade 4 and 5=increased fibrosis with definite damage to architecture of the heart and formation of fibrosis bands or small fibrosis masses; grade 6 and 7=severe distortion of structure and large fibrosis areas; and grade 8=total fibrotic obliteration (23).

### Table 1. Sequences of primers used to evaluate expression of GAPDH, CaMKII\textsubscript{total}, and CaMKII\textsubscript{δ}, CaMKII\textsubscript{δ}, CaMKII\textsubscript{δ},

<table>
<thead>
<tr>
<th>Product size</th>
<th>Primer sequence</th>
<th>Target Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>199</td>
<td>‘TGG CAA ACT AAA GAG GGA GC-3’</td>
<td>CaMKII\textsubscript{total} (forward)</td>
</tr>
<tr>
<td></td>
<td>‘5’-CCA AAA TCC CAA TGA GAA GCC C-3’</td>
<td>CaMKII\textsubscript{total} (reverse)</td>
</tr>
<tr>
<td>230</td>
<td>‘5’-AAC CCG ATG GGG TAA AGG AG-3’</td>
<td>CaMKII\textsubscript{total} (forward)</td>
</tr>
<tr>
<td></td>
<td>‘CAA TGC TTC GGG TTC AAA GG-3’</td>
<td>CaMKII\textsubscript{total} (reverse)</td>
</tr>
<tr>
<td>164</td>
<td>‘CGG ATG GGG TAA AGA AAA GG-3’</td>
<td>CaMKII\textsubscript{δ} (forward)</td>
</tr>
<tr>
<td></td>
<td>‘CTC GAA GTC CCC ATTT GTT GA-3’</td>
<td>CaMKII\textsubscript{δ} (reverse)</td>
</tr>
<tr>
<td>207</td>
<td>‘AGA CAG CCG CAT CTT CTG AT-3’</td>
<td>GAPDH (forward)</td>
</tr>
<tr>
<td></td>
<td>‘5’-CTT GCC GTG GGT AGA GTC AT-3’</td>
<td>GAPDH (reverse)</td>
</tr>
</tbody>
</table>
In addition, to assess general histological changes of heart tissue, paraffin-embedded sections of the heart tissue were stained with hematoxylin and All histological measurements in inter-intra observer variability were conducted by at least two independent expert examiners in a blinded manner and expressed in comparison to controls.

Statistical analyses
Normal distribution of data within each group was verified using Kolmogorov–Smirnov test. Statistical analyses were performed using the computer software SPSS 16.0 for Windows (SPSS, IBM, Chicago, USA). The statistical differences between the groups were tested using one-way ANOVA and then Tukey’s post hoc test. The data obtained from each test are presented as the mean±SD, and p<0.05 is considered as statistically significant.

Results
Biochemical and gene expression
The effects of ethanol consumption and treatment with ginger extract on the heart tissue MAO levels, gene expression of molecular markers of pathological cardiac hypertrophy, and left ventricular weight/body weight (LVW/BW) are shown in Table 2. Chronic ethanol administration, as an indicator of left ventricular hypertrophy, significantly increased the ratio of LVW/BW compared to that of the control group (p=0.05). Ginger extract administration along with ethanol reduced the ratio of LVW/BW significantly compared to that in the ethanol group (p>0.002), and no significant differences were found between the GETE and control groups. MAO levels in heart tissue was lower in the ethanol rats (p=0.05) than in the control group. Even though ginger extract administration along with ethanol increased the MAO levels in heart tissue, it was not significantly increased compared to that in the ethanol group. MAO levels was still significantly lower in the GETE group than in the control group (p=0.05). Chronic ethanol consumption significantly increased the expression of CaMKII\(\delta\)\text{total} and isoforms \(\delta_1\), \(\delta_2\), and \(\delta_3\) of CaMKII\(\delta\) related genes (mRNA) in the left ventricular of the ethanol group when compared with the control group (p>0.004). Although ginger extract administration along with ethanol reduced the CaMKII\(\delta\) isoform related genes expressions significantly compared to the ethanol group (p<0.004), they were still significantly higher than those in the control group (p=0.05).

Histopathological alterations
General histological changes
Results from the heart tissue histopathological examination are given in Figures 1–3. Compared to the control group, several histopathological changes, such as myocardial hypertrophy with enlarged nuclei and some areas of irregularity arrangement, were observed in the ethanol-treated group. Scattered cytoplasmic vacuoles, infiltrated polymorphonuclear leukocytes (PMN), and focal lymphocytic infiltration were also observed in the ethanol-treated group. There were no significant differences in terms of heart tissue structure between the GETE and control groups (Fig. 1).

Heart cells proliferation
Heart cells proliferation, as detected by the percentage of cells stained positive for the nuclear antigen (PCNA) are given in Figure 2 and Table 2. The PCNA-positive indices were dramati...
cally increased (mild to moderate) in the ethanol-treated group compared to that in the control group (p=0.001). Ginger extract administration along ethanol reduced PCNA-positive indices significantly compared to the ethanol group, and there were no significant differences between the GETE and the control group.

Heart tissue fibrosis

Figure 3 shows microscopic fibrosis scores in heart tissue obtained from different study groups. There were no lesion scores in heart tissue of the control group (grade 0). The microscopic lesion score in the heart tissue from ethanol-treated group was 4–5, which indicates increased fibrosis with definite damage to the heart architecture and formation of fibrosis bands or small fibrosis masses. Moreover, there were no significant differences found between the GETE and the control group.

Discussion

Although several studies have reported that chronic ethanol ingestion is an identifiable cause of heart tissue disease and heart function abnormalities, none have focused on the precise molecular mediating steps between exposure of heart muscle to ethanol and initiation of the cascade of responses leading to cardiac abnormalities (6-9). A large amount of data shows that the CaMKIIδ pathway is one of the hallmarks of molecular alteration that promotes myocardial hypertrophy and heart failure. Alterations in the CaMKIIδ expression, particularly two major splices of CaMKIIδ in heart tissue including CaMKIIδ2 and CaMKIIδ3, and its associations with shifts in cardiac function have been reported in some pathologic conditions, such as dilated cardiomyopathy, myocardial infarction, early after depolarization (EAD), arrhythmia, and heart failure upon injuries, such as pressure overload and ischemia-reperfusion (24-26). Similarly, the protective effect of CaMKIIδ gene knockout mice against cardiac dysfunction and interstitial fibrosis after pressure overload and β-adrenergic stimulation provide strong evidence for CaMKIIδ maladaptive functions in cardiac pathogenesis (27). The overexpression of CaMKIIδ induces heart abnormalities through multiple processes. Overexpression and activation of CaMKIIδ due to reactive oxygen species shift I⁢Na availability and enhance accumulation of Na channels in the intermediated state; leading to intracellular Na and Ca overloading (27). Intracellular overloaded Ca is then transmitted to the nucleus and activates the nuclear localized isoform of CaMKII, which plays a predominant role in Ca-mediated transcriptional genes associated with cardiac hypertrophy (28). In addition, recently it has been reported that activation of CaMKII by redox signaling induces AngII stimulation that causes cardiomyocyte mitogen activated protein kinase (MAPK) activation and apoptosis during heart failure (29). Further research is needed to understand the molecular basis of these findings and to develop strategies to prevent or treat these cardiac abnormalities.
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DOI:10.14744/AnatolJCardiol.2017.8079

In addition, deletion of MAO activity in mice caused elevation of norepinephrine in the heart, leading to cardiac abnormalities, such as cardiomyocyte hypertrophy, left ventricular dilation, and a lower left ventricular contractility (20, 40). Moreover, elevated norepinephrine reportedly results in cardiomyocyte apoptosis, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation, intracellular Ca-overload, myocardial cell damage, and ventricular arrhythmia (37). Furthermore, previous studies demonstrated that ethanol increases urinary excretion of catecholamines and raises plasma concentration of catecholamines (41-43). Studies have also shown that ethanol inhibits noradrenaline release from peripheral nerves (44, 45). Another study indicated that ethanol consumption resulted in decreased circulatory clearance of noradrenaline, suggesting that increased noradrenaline level in plasma is due to the inhibitory action on uptake and/or metabolism of noradrenaline rather than from an increase of sympathetic nervous outflow (42).

As mentioned above, the deleterious effects of low MAO activity and a high norepinephrine level in heart function and structure and ethanol-induced heart structural abnormalities along with reduced heart tissue MAO levels prompted us to form a hypothesis that ethanol may, at least in part, induce its hazardous effects on the heart by reducing MAO activity in the heart tissue.

The second issue addressed in the present study was the mitigating effect of ginger extract against CaMKIIδ isofoms, gene expression transition, MAO levels reduction, and heart structural alteration induced by ethanol exposure in the heart tissue. Antioxidant and anti-inflammatory properties of ginger supplementation are well established in previous studies (46).

Our recent works have demonstrated that ginger supplementation mitigates oxidative DNA damage and NADPH oxidase. In addition, it increases the total antioxidant capacity and reduces lipid and protein oxidation as two main ROS generator sources in diabetics and other oxidative stress conditions, such as ethanol exposure (46-48). In addition, it has been shown that ginger supplementation inhibits inflammation process by suppressing pro-inflammatory cytokine expressions, such as tumor necrosis factor alpha (TNF-α), arachidonic acid cascade, interleukin-1β (IL-1β), and macrophage chemoattractant protein-1(MCP-1), and inhibits prostaglandin and leukotriene biosynthesis via suppression of 5-lipoxygenase synthetize activities (49). Chronic ethanol consumption reportedly induces some functional and structural abnormalities in different organs, such as the heart, brain, and kidney, through oxidative stress and inflammation (2, 47). Moreover, recent studies have revealed that conditions such as oxidative stress predispose heart failure by damaging membrane, proteins, and DNA and by redox signaling or even activating physiological signaling pathways (50). Reactive oxygen species activate CaMKII by oxidation dependent pathways, and kinase activated by oxidative stress affects cardiac function through increasing AngII and MAPK activation and apoptosis during transition to HF (29). Accordingly, if ethanol induces
functional and structural abnormalities through oxidative stress, mediated CaMKII overexpression, and also reduced MAO levels, the effect of ginger supplementation on rescue abnormalities will be due to its antioxidant and anti-inflammatory properties.

**Study limitations**

Our study had a few limitations. First, as a molecular underlying for heart failure, along with CaMKIIα gene expression, the protein levels of this key enzyme was not analyzed in the present study. We did not study alterations of calcium ion homeostasis or norepinephrine, which are important hallmarks of molecular alteration in heart failure. Second, we did not assess acute phase inflammatory protein changes, such as alpha and beta globulins, in plasma of the rats after the treatment.

**Conclusions**

In conclusion, according to results of the present study, we conclude that ethanol exerts its deleterious effect on the heart, at least in part, by CaMKIIα and splicing genes overexpression and lowering the heart tissue MAO levels mediated by oxidative stress. However, further research is still required to elucidate the comprehensive details of the mechanisms through which ethanol consumption exerts its deleterious effects on heart causing abnormalities. Furthermore, whether ethanol exposure induces heart failure via overexpression of CaMKII genes needs to be discovered by studies using knockout of CaMKII gene expression analysis to elucidate the underlying molecular mechanism of the subjects.

**Acknowledgment:** This work derived from a Master of Science thesis in Nutrition by Urmia University of Medical Sciences, Urmia, Iran.

**Funding:** None declared.

**Conflict of interest:** None declared.

**Peer-review:** Externally peer-reviewed.


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