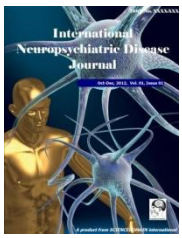

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MicroRNA Let-7f Mediates Mitochondrial Respiratory Deficit Induced by Repeated Ethanol Exposure and Withdrawal in HT22 Cells

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Authors' contributions

This work was carried out in collaboration between both authors. Author MEJ designed and conducted experiments and data analysis. Author DBM conducted experiments and data analysis. Both authors participated in writing and reviewing this manuscript.

Short Communication

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ABSTRACT

Aims: The expression of gene and gene product is typically inhibited by a small non-coding RNA (microRNA) or DNA methylation. The aim of this study is to investigate mechanisms involving microRNA let-7f by which the repeated cycles of ethanol exposure and withdrawal provoke mitochondrial respiratory damage.
Study Design: The rat or cell model of repeated withdrawal from a high dose of ethanol exposure was used to mimic human alcoholics who repeat the cycles of heavy drinking and unsuccessful attempts at abstaining.
Place and Duration of Study: Department of Pharmacology and Neuroscience University of North Texas Health Science Center at Fort Worth, between June 2011 and March 2014.
Methodology: Male adult rats received an ethanol program, consisting of two cycles of ethanol exposure (4 weeks) and withdrawal (2 weeks). At the end of the ethanol program, one hemisphere of each rat was used to measure the level of let-7f using TaqMan let-7f primers and qPCR. The other hemisphere was used to measure the methylation of cytosine in let-7f gene using bisulfite conversion and pyrosequencing. Separately, HT22 cells (mouse hippocampal cells) were exposed to an ethanol program, consisting of two cycles of ethanol exposure (20 hours) and withdrawal (4 hours). During the entire ethanol program, the cells were treated with let-7f antagomir (inhibitor) or a

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methylation-inducing methyl-donor. The role of let-7f in mitochondria was assessed by quantifying a mitochondrial enzyme, cytochrome c oxidase-IV (COX subunit IV) and real-time mitochondrial respiration using an immunoblot method and XF respirometry, respectively.

Results: The level of let-7f increased (2.4±0.5 fold increase), whereas the methylation of let-7f gene decreased in the brain of rats that underwent repeated ethanol exposure and withdrawal (called "repeated-ethanol/withdrawal"). The methyl-donor treatment completely abolished the increase in let-7f induced by repeated-ethanol/withdrawal. let-7f antagomir treatment also abolished the inhibiting effect of repeated-ethanol/withdrawal on COX-IV and mitochondrial respiration.

Conclusion: These data suggest that repeated-ethanol/withdrawal provokes the dysregulation of let-7f, thereby damaging brain mitochondria. Mitochondria-associated microRNA may be a potential research and drug target to manage alcoholism.

Keywords: Alcohol; repeated ethanol exposure and withdrawal; let-7f; microRNA; mitochondrial respiration.

1. INTRODUCTION

Long-term heavy drinking often results in alcoholism, of which symptoms include a craving for drinking, organ damage, bone loss [1], withdrawal syndromes, early brain aging [2,3], and even death [4]. Patients (alcoholics) with alcoholism often show poor psychomotor functions that are comparable to that of 10 years older non-alcoholics [2]. The neuroanatomical changes found in alcoholics are similar to those of older persons [5] and this phenomenon is also observed in young heavy drinkers [6]. Many alcoholics show functional brain damage such as alcoholic dementia [7] and ataxia (low limb malfunction) [8]. More evidence that alcohol adversely affects brain comes from ten to fourteen years of a longitudinal study where former alcoholics develop CNS disorders such as cerebrovascular disorders [9]. Our previous study has also demonstrated that treatment of hippocampal cells with a mitochondrial inhibitor mimics the stress of ethanol withdrawal [10]. These studies suggest that brain is one of the major targets of harmful drinking. However, how the drinking disorder adversely affects the brain is still under investigation. In the current study, we investigated the mechanism involving microRNA, by which repeated ethanol exposure and withdrawal (repeated-ethanol/withdrawal) perturbs brain mitochondria. Many human alcoholics repeat the cycles of heavy drinking and unsuccessful attempts at abstaining, exacerbating brain damage. MicroRNAs are a class of small, 18-23 nucleotide, non-coding RNAs that largely downregulate gene expression through base pairing the 3′ untranslated region of mRNA [11]. Emerging evidence indicates that alterations in microRNA levels mediate ethanol-induced tolerance, gastrointestinal damage, and neural stem cell proliferation [12]. Individual microRNAs have a capacity to control large numbers of different mRNAs. Therefore, ethanol-induced changes in microRNA expression can be readily amplified at the cellular and neuronal levels. let-7f (lethal-7f) is a prototype member of the let-7 microRNA family that consists of several isoforms. let-7 is conserved throughout vertebrate evolution, suggesting the physiological significance of this microRNA family. We focused on let-7f because it is associated with both ethanol and mitochondria. For instance, the increase in let-7f has been shown in the postmortem brain of human alcoholics [13]. let-7f is highly expressed in cells whose death depends on mitochondria compared to mitochondria-independent cells [14]. Bioinformatics predicts that let-7f directly inhibits the mRNA of COX subunit IV (COX-IV) (www.ebi.ac.uk/enright-srv/microcosm/cgi-bin/targets). Gene expression

is also regulated by DNA methylation that largely silences gene products. DNA methylation mainly occurs in cytosine of the repeated sequence of CpG (cytosine-phosphatidyl-guanine) [15-17]. Although less frequent, it also occurs in non-CpG cytosine [18-21] or other nucleotide such as adenine [22]. It has been suggested that non-CpG cytosine methylation can affect transcription such that CpA methylation regulates gene enhancers that are required for olfactory receptor expression in the mouse brain [23]. The non-CpG methylation is enriched in the brain regions of low CpG density and represses transcription [24]. Folic acid that helps DNA methylation is associated with the expression of microRNA mir-10a under the condition of ethanol, suggesting that DNA methylation occurs in the microRNA genes [25]. In the current study, we report that repeated-ethanol/withdrawal increases the level of let-7f directly or indirectly through the hypomethylation of let-7f gene, in a manner that provokes mitochondrial damage.

2. MATERIALS AND METHODS

2.1 Chemicals

Major analytic reagents were purchased from Qiagen Inc. (Valencia, CA), Sigma Aldrich (St. Louis, MO), Santa Cruz Biotechnology (Santa Cruz, CA), and Mitosciences (Eugene, OR). Reagents for DNA methylation were purchased from Zymo Research (Orange, CA), Roche Diagnostic Corporation (Indianapolis, IN), and Bio-Rad Laboratories (Hercules, CA). Diet ingredients were obtained from Research Organics (Cleveland, OH) or MP Biomedicals (Irvine, CA). HT22 cells, a murine hippocampal cell line, were the generous gift of Dr. David Schubert (Salk Institute, San Diego, CA).

2.2 Animals

Male Sprague-Dawley rats, aged 3 months were housed individually at 22-25ºC and 55% humidity, with ad libitum access to water and a 12-hour light/dark cycle. All animal experimentation was conducted in accordance with the Guide to the Care and Use of Laboratory Animals [DHHS/NIH 85-23, 1996, Office of Science and Health Reports, DRR/NIH] and was approved by the University of North Texas Health Science Center Animal Care and Use Committee.

2.3 Ethanol Program

Rats (7rats/group) were assigned to either the ethanol group or the control group. Rats in the ethanol group received two cycles of a liquid diet. Each cycle consisted of ethanol diet containing 7.5% (v/v) ethanol for 4 weeks followed by withdrawal for 2 weeks. Rats in the control group were fed a liquid diet with dextrin isocalorically substituted for ethanol. Animals were fed chow pellets during withdrawal periods. An in vitro ethanol program was applied to HT22 cells (repeated-ethanol/withdrawal cells) [26]. We employed this cell line because it is derived from a brain area (hippocampus) vulnerable to ethanol or ethanol withdrawal stress. In addition, our in vitro model of HT22 cells has shown a consistent effect of ethanol/withdrawal on mitochondria as we previously reported [10,27]. HT22 cells were exposed to ethanol solution (0 or 100mM) for 20 hours and withdrawn for 4 hours in each cycle. This cycle was repeated once more. A non-toxic dose (0.1%v/v) of vehicle medium, DMSO, was used as non-ethanol control solution. All tests were done at the end of the ethanol program.

2.4 Brain Tissue Extraction

At the end of the aforementioned ethanol program, rats were anesthetized using the mixture of xylazine (20mg/kg, ip) and ketamine (100mg/kg, ip) and the whole brain was harvested. For both ethanol and control groups, left and right hemisphere were selected from three and four rats, respectively.

2.5 Let-7f Quantification

The level of let-7f was measured to determine whether repeated-ethanol/withdrawal increases this microRNA. Total RNA was isolated from brain tissues or cells using Rneasy kit (QIAGEN, Valencia, CA) and quantified using Agilent 2100 bioanalyzer (Agilent Technologies, Inc. Santa Clara, CA). Total RNA was then reverse-transcribed to cDNA using TaqMan let-7f primers (forward-GAAAGAGATTGATGTTTATTTTAGAAG; reverse-AATTCACCTAAATTTATAATATCCTCT) and using the miScript reverse transcription kit (Qiagen, Valencia, CA). qPCR reactions were performed as follows: 50ºC for 2 minutes, 95ºC for 10 minutes, followed by 40 cycles of 95ºC for 15 seconds and 60ºC for 1 minute. Cycle threshold (C_t) values were calculated with SDS software v.2.3 (Applied Biosystems) using automatic baseline settings and a threshold of 0.2. The comparative C_t method was used to calculate the relative microRNA expression. The $C_t$value of an internal control gene (U6) was also measured and subtracted from the corresponding $C_t$value for let-7f gene to calculate the ΔC_tvalue.

2.6 DNA Methylation

This assay was conducted to determine whether cytosine methylation occurs in the gene of let-7f and if so, whether the methylation is altered by the stress of repeated-ethanol/withdrawal. This assay was done by the service of Nebraska university epigenetic core (Dr. David Klinkebiel) using the method of bisulfite conversion and pyrosequencing. Briefly, DNA was isolated from brain samples using DNA purification kits. DNA samples were then treated with bisulfite to deaminate unmethylated cytosine to uracil. Methylated cytosines were protected from the conversion and thus, remained intact. PCR reactions were then performed in bisulfite-modified DNA for 35 cycles using DNA polymerase, MgCl2 solution, dNTP's, sense primer (5'-GAAAGAGATTGATGTTTATTTTAGAAG-3'), and antisense primer (5'-AATTCACCTAAATTTATAATATCCTCT-3'). Bisulfite-modified DNA was then denatured at 95ºC for 30 seconds, annealed at 72ºC for 45 seconds, and extended at 72ºC for 1 minute. All PCR products were electrophoresed on 0.8% agarose gel, stained with ethidium bromide, and analyzed using Bio-Rad Gel-Doc UV illuminator. Methylation (%) of each C (cytosine) was determined using Qiagen pyrosequencer and sequencing primer (5'-TAGTAGATTGTATAGTTGTGG-3') according to manufacturer's recommendations.

2.7 Immunoblotting

This method was used to measure the protein level of COX-IV to determine whether let-7f mediates the inhibiting effect of repeated-ethanol/withdrawal on COX-IV. COX is composed of 13 subunits. We selected COX subunit IV (COX-IV) because this subunit is inhibited by ethanol or ethanol withdrawal [10]. Samples containing 30µg of protein were resolved by SDS-PAGE on 10% cross-linked gels and transferred onto polyvinylidene fluoride membranes. Nonspecific binding was blocked with 5% fat-free milk. Blots were washed in phosphate-buffered solution containing 0.05% Tween 20 and then probed overnight with

mouse monoclonal antibodies against COX-IV (1:1,000). Blots were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature, detected using Western blotting luminescence, and quantified by an image densitometer. β-actin was used as a loading control.

2.8 Mitochondrial Respiration (Mitochondrial O_2Consumption Rate)

Mitochondrial O_2 consumption rate was measured as an indicator of mitochondrial respiration to determine whetherrepeated-ethanol/withdrawal suppresses mitochondrial respiration through let-7f. Briefly, 800 HT22 cells were seeded into each well of XFmicroplate provided by the manufacturer, cultured, and subjected to the aforementioned ethanol program [26]. The cell plate was then placed on an O_2 sensor cartridge and subsequently inserted to the XF respirometer. let-7f antagomir (anti-let-7f or let-7f inhibitor) (0 or 50nM) was applied to cells during the entire ethanol program. let-7f antagomir has been shown to counteract let-7f and thus used in numerous studies to inhibit let-7f. NaN3 (1mM, COX inhibitor) was acutely injected into the wells immediately after the basal O_2 consumption rate was read. Data were normalized based on mitochondrial contents or cell numbers in each well.

2.9 Statistical Analysis

The results of let-7f microRNA level were analyzed by Student t-test (Fig. 1) or one-way ANOVA (Fig. 2B). COX-IV levels were analyzed by one-way ANOVA (Fig. 3A). Methylation (Fig. 2A, ethanol x cytosine location) and mitochondrial respiration (Fig. 3B, ethanol x COX inhibitor) data were analyzed by two-way ANOVA. ANOVA was followed by a post hoc Tukey's test to identify a specific difference between groups. Values were expressed as mean±standard error of mean (SEM). The P value<.05 was used to indicate statistical significance.

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 The microrna level and gene methylation of let-7f

Rats (alcoholic rats) that underwent repeated-ethanol/withdrawal show a significant increase in the level of let-7f (P<.001, t=4.7, df=8) (Fig. 1). Recent studies have shown that the level of microRNA is regulated by DNA methylation that typically inhibits the expression of gene and gene products. DNA methylation largely occurs in cytosine of the repeated sequence of CpG [15,16,28] and requires methyl groups from methyl-donor S-adenosylmethionine (SAM) [29]. In the beginning of this experiment, we were not aware that let-7f gene sequence lacks the CpG domain. We thus expected that the methyl-donor treatment to cells of repeated-ethanol/withdrawal will induce the methylation of let-7f gene, thereby decreasing the level of let-7f. We indeed observed that the methyl-donor treatment completely abolished the increasing effect of repeated-ethanol/withdrawal on let-7f level (Fig. 2B) [F (3,28)=87.70, P<.0001 by a factor of treatment group]. The negligible level of let-7f (0.13±0.04 vs. 1 in untreated control cells) was detected in control cells treated with the methyl-donor. Based on these results, we speculated that let-7f gene contains the CpG domain that is normally methylated, and this methylation is inhibited by the stress of repeated-ethanol/withdrawal. We later found out that let-7f gene (Mirlet-7f1) lacks the CpG domain (National Center for

Biotechnology Information). Nevertheless, brains from control diet rats show a moderate but a statistically significant degree of methylation in non-CpG cytosine at 7 different sites. The methylation is decreased (*P*=.0132) in alcoholic rats such that a similar degree of cytosine methylation occurred only at 5 sites (Fig. 2A) [F(1,48)=4.8, *P*=.0132 by a factor of diet; F (6, 48) = 6.7, *P*<.001 by a factor of a methylation site]. Therefore, it is plausible that the level of let-7f is normally limited by the methylation of non-CpG area in let-7f gene, and this regulation is weakened by the stress of EW, resulting in an overexpression of let-7f.

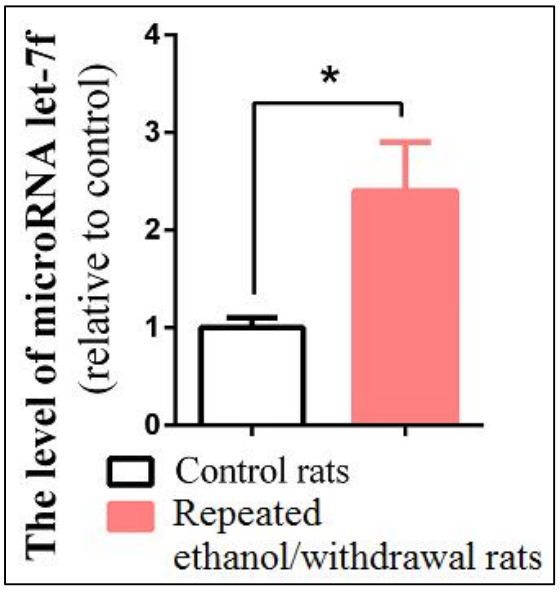

Fig. 1. The level of let-7f

*Rats (alcoholic) received two cycles of an ethanol diet (0 or 7.5% ethanol, 4 weeks) and withdrawal (2 weeks). Total rna was then isolated from one hemisphere and reverse-transcribed to cdna using taqman let-7f primers and the miscript reverse transcription kit. Qpcr reactions were performed to quantify the level of let-7f. Alcoholic rats show an increase in let-7f (*p<.001). Data are expressed as mean±s.e.m for 7 rats/group*

3.1.2 let-7f and COX-IV

We determined whether let-7f contributes to the inhibition of COX and mitochondrial respiration induced by repeated-ethanol/withdrawal. The cells of repeated-ethanol/withdrawal show a decrease in COX-IV protein content (*P*<.005) (Fig. 3A) in a manner that is reversed by let-7f antagomir treatment (*P*<.005) [F (3, 28) = 196.2, *P*<.0001 by a factor of treatment group]. The cells of repeated-ethanol/withdrawal also show a decrease in mitochondrial respiration (*P*<.01) (Fig. 3B) and further so in the presence of COX inhibitor treatment (*P*<.05) [F (3, 56) =236.7, *P*=.0132 by a factor of repeated-ethanol/withdrawal in the presence or absence of let-7f antagomir; F (1, 56)=886.3, *P*<.001 by a factor of NaN3]. Let-7f antagomir treatment virtually abolished the inhibiting effect of repeated-ethanol/withdrawal on mitochondrial respiration (*P*<.01). Treatment of control cells with let-7f antagomir moderately increased the level of COX-IV protein (118±10% of control) or mitochondrial respiration (121±12% of control). These results suggest that let-7f directly or indirectly mediates mitochondrial respiratory suppression through COX-IV inhibition under the condition of repeated-ethanol/withdrawal.

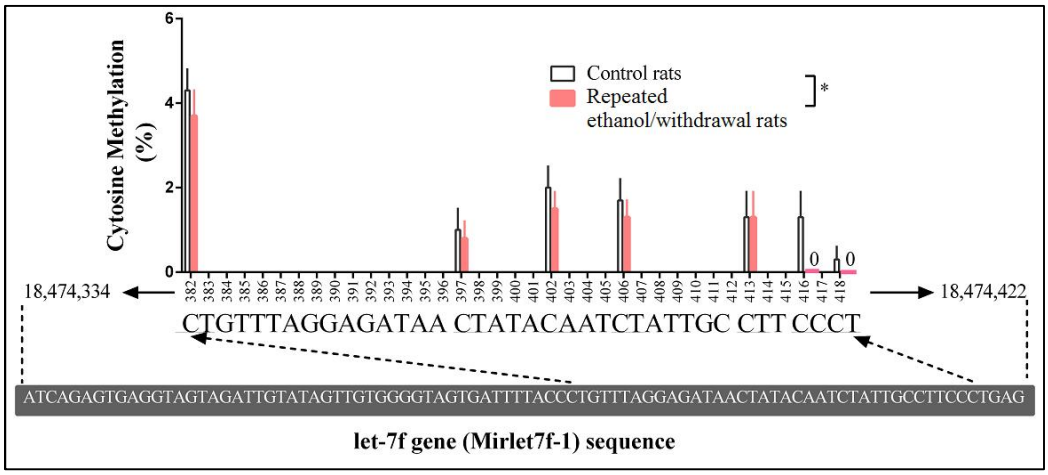

Fig. 2A. Let-7f gene methylation

*Rats (alcoholic) received an ethanol program as described in figure 1 legend. DNA was isolated from one hemisphere and treated with bisulfite. PCR reactions were then performed using DNA polymerase, a sense primer, and an antisense primer. Cytosine (c) methylation (%) was determined using a pyrosequencer and a sequencing primer. "0" indicates that no methylation occurred in the particular site of cytosine in the rat brain of repeated ethanol/withdrawal. Alcoholic rats show an overall decrease (*p=.0132) in the methylation of let-7f gene. The gene (mirlet-7f1) of let-7f is located in the chromosome 17 with the coordinate of 18,474,334-18,474,422 as indicated in xaxis. n=7 rats/group*

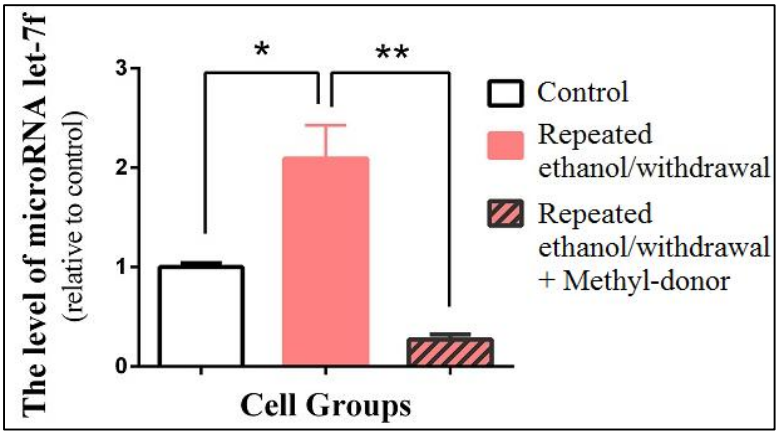

Fig. 2B. The effect of amethyl-donor treatment on let-7f

*The level of let-7f was determined in HT22 cells that received two cycles of ethanol exposure (0 or 100mm, 20 hours) and withdrawal (4 hours). The methyl-donor [s-adenosylmethionine 0 or 5μm] was applied to cells during the entire ethanol program. The level of let-7f was then measured using the method described in figure 1 legend and in the method section. Repeated-ethanol/withdrawal cells treated with the methyl-donor show a much lower level of let-7f than vehicle-treated cells. *p<.001, **p<.0001 vs. Control cell value at 1. N=8 wells/group*

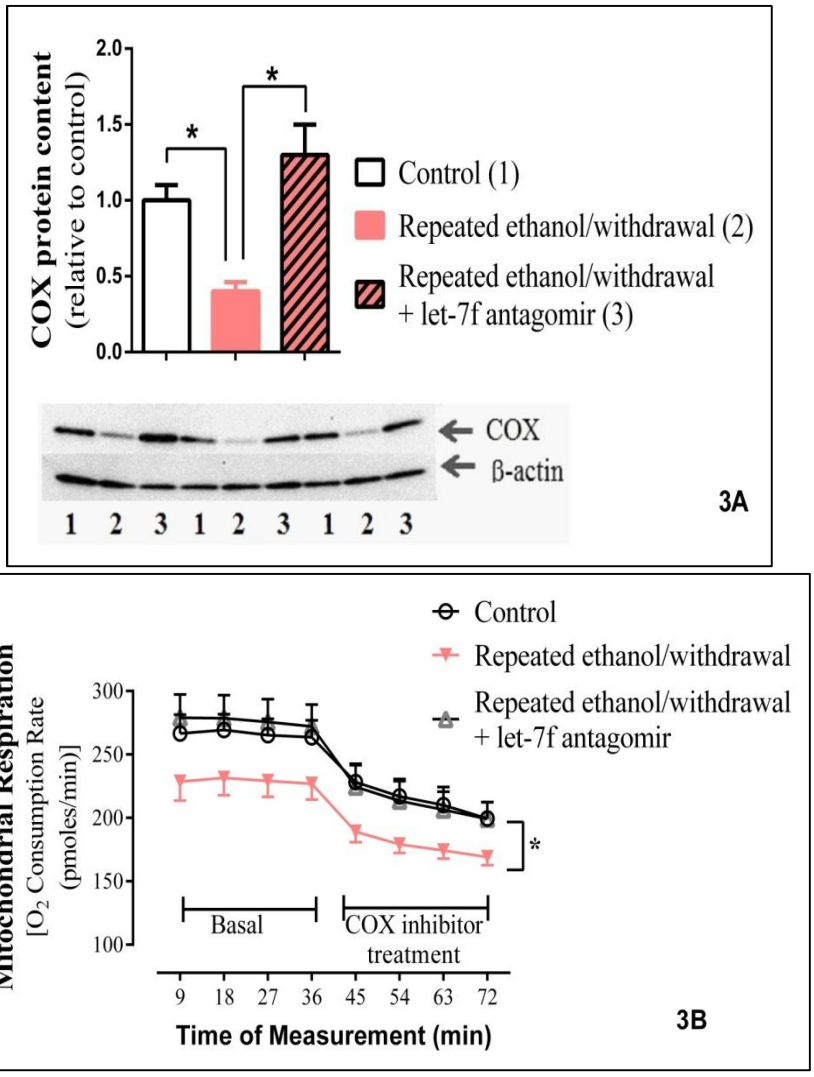

Fig. 3. The effect of let-7f on mitochondria

*Ht22 cells were subjected to an ethanol program with or without let-7f antagomir (0 or 50nm) treatment (see figure 2b legend). COX-IV protein (figure 3a) was then measured using an immunoblot method and mouse monoclonal antibodies against COX subunit IV. B-actin was used as a loading control. COX-IV content is lower in cells of repeated-ethanol/withdrawal than control cells (*p<.005) in a manner that is reversed by let-7f antagomir treatment (*p<.005). N=8 wells/group. For mitochondrial respiration (figure 3b), HT22 cells were plated in XF microplate and received an ethanol program with or without let-7f antagomir (0 or 50 nm) treatment. Mitochondrial respiration was then assessed by measuring O_2 consumption rate using XF respirometer. After basal mitochondrial respiration was measured, a COX inhibitor (NAN3, 1mm) was injected to cells. Repeated-ethanol/withdrawal suppresses mitochondrial respiration (*p<.01) and further so in the presence of cox inhibitor (p<.05). These effects of ethanol are abolished by let-7f antagomir (0 or 50nm) treatment (*p<.01). N=8 wells/group*

3.2 Discussion

We have demonstrated that the level of microRNA let-7f increases in the brain of rats that underwent repeated-ethanol/withdrawal. We have also demonstrated that non-CpG methylation occurs in let-7f gene in control diet rats, and this methylation is decreased by repeated-ethanol/withdrawal stress. Moreover, let-7f antagomir treatment attenuates the inhibiting effect of repeated-ethanol/withdrawal on COX-IV and mitochondrial respiration. These findings suggest that repeated-ethanol/withdrawal stress provokes the dysregulation of this microRNA, contributing to mitochondrial damage.

We employed the ethanol program of repeated ethanol exposure and withdrawal, which models a drinking pattern of many human alcoholics [30]. Using this model, we observed that the brain obtained from alcoholic rats show an increase in the level of let-7f (Fig. 1). In agreement, Lewohl et al. have shown an increase in let-7f in the postmortem brain of human alcoholics [13]. Controversially, the level of let-7f does not differ between ethanol-exposed and control cells in a study done by Guo et al. [31]. The discrepancy is an open question as different studies used different experimental parameters. For instance, Guo et al. used primary cortical neuronal cells whereas we used rat brain or mouse hippocampal cells. In addition, the ethanol administration regimes differ between our and their studies. It is possible that the expression of this microRNA is sensitive to sample types, an ethanol regimen, or the length of ethanol administration.

DNA methylation is critical to maintain the silenced state of gene expression for a tight control of gene product. Any disturbance of this regulation can result in aberrant cellular pathways, potentially responsible for adverse consequences to brain function. Ethanol inhibits the methylation of fetal DNA [32], sperm DNA, and methyltransferase (methylating enzyme) [33]. Reductions in DNA methylation are associated with the aging of cells and tissues [34]. DNA methylation largely occurs in CpG domains (e.g. CGCGCG) [15,16,28]. Although less frequent, non-CpG methylation also occurs, meaning that cytosine outside the CpG domain is methylated [18-21]. Such non-CpG methylation is relatively prevalent in brain tissues [35] or neurons [36] compared to other organs or non-neuronal cells. Despite that let-7f gene (Mirlet-7f1) does not contain CpG sequence, we found that a moderate level of methylation occurs in cytosines at 7 different sites of the gene sequence in control diet rats (Fig. 2A). In alcoholic rats, the methylation is missing, slightly decreased, or unchanged, giving a statistically significant decrease in the degree of methylation. These results raise a possibility that normally, let-7f is tightly regulated through the methylation of its gene but this regulation is perturbed by repeated-ethanol/withdrawal, resulting in an increase in let-7f. This view is supported by our experiment using a methyl-donor that is required for DNA methylation [37]. The methyl-donor treatment significantly decreased the level of let-7f in cells of repeated-ethanol/withdrawal (Fig. 2B). A study done by Brueckner et al. has shown that cytosine in CpG domain of let-7a3 gene is methylated, affecting the expression of the gene product (microRNA let-7a) [38]. Although the methylation found in that study occurred in CpG domain, it supports the idea that the level of this microRNA family (let-7) can be altered by the methylation of the microRNA gene. Heberlein et al have measured DNA methylation in the blood of human alcoholics 7 days after ethanol withdrawal. Similar to our finding, they observed a decrease in the methylation of CpG in the nerve growth factor gene [16]. In that study, the difference in the methylation (%) between alcoholic and normal individuals was even smaller (≈ 0.3% difference) than the one in our study. These results raise two important points: 1) DNA methylation can persist even after abstinence and 2) even a small degree of disturbance in DNA methylation can perturb neurobiology. Such

results argue that ethanol-induced molecular cascade [14,32] might involve the dysregulation of DNA methylation, as a potential mediator of alcoholic brain disorders.

We next examined the physiological significance of the increase in let-7f at the mitochondrial level. New emphasis has been made to the role of microRNAs in mitochondrial functions [39]. Mitochondria provide the majority of cellular energy through transferring electrons across the four electron transfer enzyme complexes. COX is the terminal enzyme complex adjacent to ATP synthase, plays a pivotal role by consuming the most mitochondrial O_2, and thereby helping ATP synthesis. Chen et al. [40] have demonstrated that microRNA miR-210 directly targets the mRNA of COX assembly protein (COX10). Bioinformatics (www.ebi.ac.uk/enright-srv/microcosm/cgi-bin/targets) predicts that mRNA of COX-IV is a direct target of let-7f. This information led us to the question of whether let-7f plays a role in ethanol-induced COX-IV damage. Cells of repeated-ethanol/withdrawal show a decrease in the protein level of COX-IV in a manner that is completely reversed by let-7f antagomir treatment (Fig. 3A). These results argue that COX-IV deficit induced by repeated-ethanol/withdrawal is at least partly attributed to an increase in let-7f. Since COX is critical of mitochondrial function, we further determined whether ethanol-induced mitochondrial respiratory suppression is attributed to let-7f increase. let-7f antagomir treatment virtually abolished the inhibiting effect of ethanol on mitochondrial respiration and improved mitochondrial respiration of control cells. The enhancement of mitochondrial respiration by let-7f antagomir suggests that let-7f inhibits COX-IV, contributing to mitochondrial respiratory suppression under the condition of repeated-ethanol/withdrawal. Relevant to these results, microRNA miR-210 which targets COX assembly protein, downregulates mitochondrial respiration in cancer cells [40]. MicroRNAmiR-338 inhibits the mRNA of COX-IV, resulting in the reduction of ATP levels [41]. Given this, one can argue that the dysregulation of microRNAs including let-7f perturbs the integrity of mitochondria, promoting a variety of cellular and neuronal damage. It should be mentioned that we used 100mM of ethanol because we have previously observed that withdrawal from this dose of ethanol suppresses mitochondrial respiration. Since this dose is a relatively high dose, and ethanol suppresses cell viability at a lower dose than 100mM [27], a future study will need to determine the dose effect of ethanol/withdrawal on the parameters measured in the current study.

4. CONCLUSION

We are aware of the limitation of this study such that rat and cell models were not from the same species and same brain area. In addition, we did not assess a direct binding of let-7f to the mRNA of COX-IV due to the technical limitation of our laboratory. Therefore, it is entirely possible that the inhibiting effect of let-7f on COX-IV is a secondary consequence of other mechanisms that we did not determine in the current study. In addition, we do not know at the current moment whether the level of let-7f microRNA and let-7f gene methylation differ depending upon the side of hemisphere or the area of brain. Nevertheless, our findings provide empirical evidence that repeated-ethanol/withdrawal perturbs the methylation of let-7f gene. This may subsequently permit the increase in let-7f, directly or indirectly inhibiting COX-IV and mitochondrial respiration. These findings may provide a new mechanistic insight into mitochondria-associated microRNAs as a potential target of alcoholic brain damage.

CONSENT

Not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

ACKNOWLEDGEMENTS

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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