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# Neurochemical mechanisms underlying acute and chronic ethanol-mediated responses in zebrafish: The role of mitochondrial bioenergetics

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ABSTRACT

Ethanol (EtOH) is a socially-accepted drug, whose consumption is a risk factor for non-intentional injuries, development of pathologies, and addiction. In the brain, EtOH affects redox signaling and increases reactive oxygen species (ROS) production after acute and chronic exposures. Here, using a high-resolution respirometry assay, we investigated whether changes in mitochondrial bioenergetics play a role in both acute and chronic EtOH-mediated neurochemical responses in zebrafish. For the first time, we showed that acute and chronic EtOH exposures differently affect brain mitochondrial function. Acutely, EtOH stimulated mitochondrial respiration through increased baseline state, CI-mediated OXPHOS, OXPHOS capacity, OXPHOS coupling efficiency, bioenergetic efficiency, and ROX/ETS ratio. Conversely, EtOH chronically decreased baseline respiration, complex I- and II-mediated ETS, as well as increased ROX state and ROX/ETS ratio, which are associated with ROS formation. Overall, we observed that changes in mitochondrial bioenergetics play a role, at least partially, in both acute and chronic effects of EtOH in the zebrafish brain. Moreover, our findings reinforce the face, predictive, and construct validities of zebrafish models to explore the neurochemical bases involved in alcohol abuse and alcoholism.

## 1. Introduction

Ethanol (EtOH) is one of the most socially-accepted addictive drug worldwide (Gneiting and Schmitz, 2016). Alcohol consumption is a risk factor for accidents, development of pathologies, as well as addiction and alcoholism (Rehm, 2011). Alcohol-related chronic disorders constitute a substantial health and economic burden due to the occurrence of different types of diseases, including neuropsychiatric conditions (Ridley et al., 2013). These disorders contribute to the alcoholism-related high morbidity and mortality (Shield et al., 2013).

Evidence shows that acute and chronic ethanol exposures affect redox signaling and increase free radicals production in the central nervous system (CNS), which impair proteins, carbohydrate, and fatty acid metabolism (Manzo-Avalos and Saavedra-Molina, 2010). Mitochondria play a key role in energy production via aerobic metabolism, and mitochondrial electron transport chain has been widely recognized as an endogenous source of reactive oxygen species (ROS) (Bolisetty and Jaimes, 2013). EtOH oxidation can affect mitochondria physiology, which culminates in the overproduction of ROS (Almansa et al., 2009). EtOH also impairs the membrane potential, decreases $Ca^{2+}$ intracellular levels (Goodlett and Horn, 2001), and affects the mitochondrial electron transport system, thereby reducing ATP production and triggering neuronal death (Bailey et al., 1999; Cunningham and Van Horn, 2003; Guo et al., 2013).

In translational neuroscience research, the zebrafish (*Danio rerio*) has been considered a suitable vertebrate for modeling human-related

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disorders (Fontana et al., 2018; Stewart et al., 2015). This species shares a high genomic conservation when compared to humans (Howe et al., 2013), and presents an evolutionarily conserved physiology (Holzschuh et al., 2001; Horzmann and Freeman, 2016; MacRae and Peterson, 2015). In zebrafish, EtOH-mediated effects on behavior are concentration- and time-dependent, and redox imbalances occur following acute and chronic exposures (Gerlai et al., 2000; Muller et al., 2017; Rosemberg et al., 2010, 2012). Because EtOH affects energy metabolism, as well as modulates redox signaling, which culminates in oxidative stress, we hypothesized that such responses could be related to changes in mitochondrial functionality in zebrafish. Thus, the goal of this study was to verify whether changes in mitochondrial bioenergetics play a role in EtOH-mediated effects on the CNS of zebrafish using a high-resolution respirometry assay.

## 2. Materials and methods

### 2.1. Animals

Subjects were 88 adult (4–6 months-old) short fin wild-type zebrafish (*Danio rerio*) of mixed genders (50:50 male:female ratio). Fish were obtained from a local supplier (Hobby Aquários, RS, Brazil) and acclimated in 40-L tanks for two weeks in a maximum density of four fish per liter. Tanks were filled with non-chlorinated water under constant mechanical, biological, and chemical filtration. Water temperature, pH, and conductivity were set at 28 ± 1 °C, 7.2 ± 0.5, and 400 ± 50 μS, respectively. Ammonia, nitrite, and nitrate values were kept lower than 0.2 ppm, 0.05 ppm, and 0.05 ppm, respectively. Animals were kept on a 14/10 light/dark photoperiod cycle (lights on at 7:00 a.m.), water dissolved oxygen equal or above 95% saturation and fed with a commercial flake fish food (Alcon BASIC®, Alcon, Brazil) twice daily. All protocols were approved by the Ethics Commission on Animal Use of the Federal University of Santa Maria (process number 026/2014).

### 2.2. Alcohol exposure protocols

We used two protocols to investigate whether EtOH modulates mitochondrial bioenergetics. To evaluate the acute effects of EtOH, 40 fish were individually exposed to non-chlorinated water (control) or 1.0% (v/v) EtOH (Merck, Darmstadt, Germany) for 1 h (20 animals per group). EtOH concentration used here is known to induce sedative/depressant-like behavior, as well as impairs oxidant processes in the zebrafish brain (Chatterjee and Gerlai, 2009; Rosemberg et al., 2010, 2012). Chronically, EtOH was administered as described previously, using the intermittent exposure protocol (Mathur and Guo, 2011; Muller et al., 2017). Briefly, 48 zebrafish were kept in housing tanks and exposed to non-chlorinated water (control) or 1.0% (v/v) EtOH for 8 consecutive days (20 min per day) and euthanized at 9th day (24 animals per group). Importantly, no physical abnormalities were observed during the exposure period. After euthanasia, the brains were dissected and samples were prepared to further biochemical analyses.

### 2.3. Mitochondrial respiration assays

Mitochondrial activity was measured by high-resolution respirometry using an Oxygraph-2k (O2k, Oroboros Instruments, Innsbruck, Austria). For each independent preparation, four brains were pooled (~24 mg of tissue) and homogenized in 240 μL of medium containing 5 mM Tris-HCl (pH 7.4), 250 mM sucrose, and 2 mM EGTA. Samples were homogenized gently with a pestle and 100 μL of homogenate was further transferred to 2 mL respiration buffer (115 mM KCl, 10 mM $KH_2PO_4$, 2 mM $MgCl_2$), 3 mM HEPES, 1 mM EGTA, essentially fatty acid-free BSA (0.2%, pH 7.2). All experiments were performed in duplicate at 28 °C using DatLab 4.0 software (Oroboros Inc., Austria), with continuous stirring at 750 rpm (de Carvalho et al., 2017).

Using titration protocols based on previous reports (Carvalho et al., 2013; Gnaiger, 2009; Pesta and Gnaiger, 2012), we assessed the influence of various substrates and inhibitors in mitochondrial function as reflected in different respiration states. Glutamate + pyruvate + malate and succinate were used as oxidizable substrates. We determined the changes in mitochondrial respiratory chain complexes, respiratory rates, and the production of oxidative oxygen species.

After signal stabilization, the baseline respiration supported by endogenous substrates was measured. The complex I (CI)-mediated leak (LEAK; L(n)) respiration was determined using 5 mM pyruvate, 5 mM glutamate and 1 mM malate. CI-mediated oxidative phosphorylation (OXPHOS) was tested using 2.5 mM ADP. The convergent electron flow during the maximal OXPHOS respiration (CI + $CII_{OXPHOS}$) was determined with substrates of CI and CII (10 mM succinate). To induce LEAK state, we added 2 μg/mL oligomycin, an inhibitor of ATP synthase by blocking its proton channel. The electron transport system (ETS) respiration represents the uncoupled respiration, which was measured using carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) as uncoupler (optimum concentration reached between 0.5 and 1.5 μM); CI + CII-mediated ETS respiration (CI + $CII_{ETS}$) was determined in the presence of FCCP, while CII-mediated ETS respiration ($CII_{ETS}$) was measured in the presence of 0.5 μM rotenone. The addition of 2.5 μM antimycin A was performed to inhibit complex III activity, which abolished mitochondrial respiration. Then, the residual oxygen consumption (ROX) with small contributions from electron leak in the uncoupled state was measured. We also determined the magnitude of residual oxygen consumption relative to the maximum oxygen consumption (expressed as fold change of ROX/ETS ratio), ETS/OXPHOS ratio, OXPHOS capacity, and OXPHOS coupling efficiency, which is based on the ratio of free to total OXPHOS capacity (1-L/P). Mitochondrial bioenergetics capacity was quantified by subtracting the ADP-induced $CI_{OXPHOS}$ values from the $CI_{LEAK}$. Moreover, the respiratory control rates (RCR) were measured as indicators of the mitochondrial coupling state (RCR = $CI_{OXPHOS}/CI_{LEAK}$ ratio), as well as the succinate control factor ($CI_P/CI + CII_O$, fold change). Substrate control ratio (SCR) ($CI_{OXPHOS}/CII_{ETS}$ ratio) was quantified to evaluate the effects of EtOH on mitochondrial respiratory control. Using the high-resolution respirometry protocol measured by Oxygraph-2k, the limit of detection of respiratory flux was 1 $\rho mol \cdot s^{-1} \cdot cm^{-3}$ (0.001 $\mu M\, s^{-1}$) and the limit of detection of oxygen concentration extends to 0.005 μM $O_2$. Low intra- and inter-assay CV values (ranging from 4.4–9.2% and 7.7–11.8%, respectively) were observed for each endpoint measured (Table 1).

### 2.4. Statistics

Normality of data and homogeneity of variances were analyzed by Kolmogorov-Smirnov and Bartlett's tests, respectively. Because results were normally distributed and homoscedastic, data were expressed as means ± standard error of the mean (S.E.M.) and the effects on mitochondrial activity were analyzed by unpaired Student's *t*-test, considering $p \leq 0.05$ as significant. Statistical analyses were performed using the GraphPad Prism software (version 7.0 for Macintosh OS X,

**Table 1**
Coefficient of variation (CV) obtained from each endpoint measured.

| Endpoints | Coefficient of variation (%) | |
|---|---|---|
| | Intra-assay | Inter-assay |
| Basal | 5.32 | 10.04 |
| $Cl_{LEAK}$ | 4.46 | 7.79 |
| $Cl_{OXPHOS}$ | 6.75 | 10.84 |
| Cl + $Cll_{OXPHOS}$ | 5.20 | 11.16 |
| Cl + $Cll_{ETS}$ | 4.46 | 9.30 |
| $Cll_{ETS}$ | 9.22 | 7.92 |
| ROX | 4.35 | 11.86 |

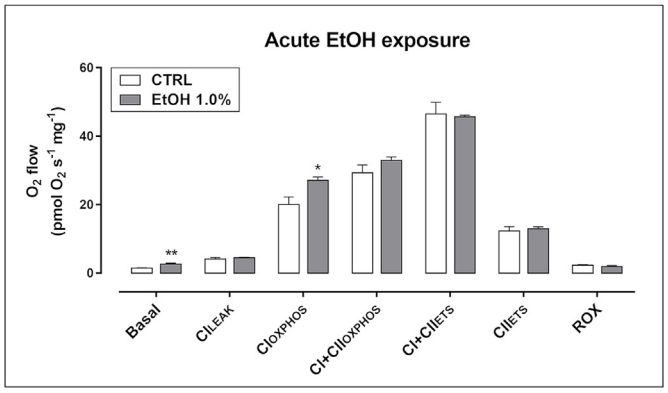


**Fig. 1.** Mitochondrial $O_2$ consumption in the zebrafish brain following acute 1.0% (v/v) EtOH exposure. Mitochondrial functions are presented with the abbreviation(s) of the complex(es) involved followed by the state of respiration measured in the presence of endogenous substrates (baseline), pyruvate + malate + glutamate ($CI_{LEAK}$), + ADP ($CI_{OXPHOS}$), + succinate (CI + $CII_{OXPHOS}$), + FCCP (CI + $CII_{ETS}$), + rotenone ($CII_{ETS}$), + antimycin A (Ama) used to correct for residual $O_2$ consumption (ROX). Data were expressed as mean ± SEM and analyzed by unpaired Student's *t*-test (*$p < 0.05$, $n = 5$ independent preparations per group).

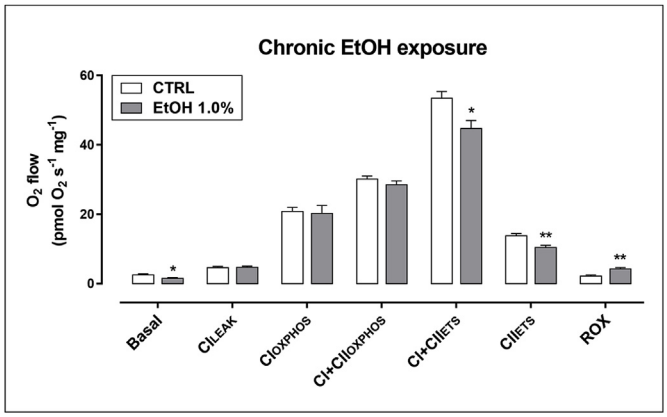


**Fig. 3.** EtOH (1.0%, v/v) chronically alters mitochondrial $O_2$ flow in the zebrafish brain. Mitochondrial functions are presented with the abbreviation(s) of the complex(es) involved followed by the state of respiration measured in the presence of endogenous substrates (baseline), pyruvate + malate + glutamate ($CI_{LEAK}$), + ADP ($CI_{OXPHOS}$), + succinate (CI + $CII_{OXPHOS}$), + FCCP (CI + $CII_{ETS}$), + rotenone ($CII_{ETS}$), + antimycin A (Ama) used to correct for residual $O_2$ consumption (ROX). Data were expressed as mean ± SEM and analyzed by unpaired Student's *t*-test. Data were expressed as mean ± SEM and analyzed by unpaired Student's *t*-test (*$p < 0.05$, **$p < 0.01$, $n = 6$ independent preparations per group).

San Diego, CA).

## 3. Results

### 3.1. EtOH acutely stimulates mitochondrial $O_2$ consumption

Fig. 1 depicts the acute effects of 1.0% EtOH exposure on the mitochondrial bioenergetics. EtOH-exposed group showed higher baseline respiration ($t_{(0.05;\ 8)} = 3.991$, $p = 0.004$) and complex I-induced oxidative phosphorylation ($CI_{OPHOX}$) ($t_{(0.05;\ 8)} = 3.265$, $p = 0.0114$) than control. However, $CI_{LEAK}$ respiration, and complex I- and II-induced oxidative phosphorylation (CI + $CII_{OPHOX}$) did not change between groups. When the respiration was uncoupled by FCCP (CI + $CII_{ETS}$), no differences were observed. Moreover, the $C_{IIETS}$ respiration and ROX values did not show significant differences between groups. EtOH exposure also increased ROX/ETS ratio ($t_{(0.05;\ 8)} = 3.639$, $p = 0.0066$) (Fig. 2A) and decreased ETS/OXPHOS ($t_{(0.05;\ 8)} = 6.088$, $p = 0.0006$) (Fig. 2B). The OXPHOS capacity ($t_{(0.05;\ 8)} = 2.391$, $p = 0.0438$) (Fig. 2C), OXPHOS coupling efficiency ($t_{(0.05;\ 8)} = 3.017$, $p = 0.0116$) (Fig. 2D), bioenergetic efficiency ($t_{(0.05;\ 8)} = 2.695$, $p = 0.0273$) (Fig. 2E), RCR ($t_{(0.05;\ 8)} = 2.791$, $p = 0.0235$) (Fig. 2F) increased after EtOH exposure. Succinate control factor was lower ($t_{(0.05;\ 8)} = 2.528$, $p = 0.0353$) (Fig. 2G) in EtOH group, while SCR did not change following acute EtOH regimen (Fig. 2H).

### 3.2. EtOH chronically impairs mitochondrial respiration

Fig. 3 shows the effects of 1.0% chronic EtOH exposure on mitochondrial respiration. EtOH decreased the baseline state ($t_{(0.05;\ 10)} = 2.783$, $p = 0.0193$), while no changes in $CI_{LEAK}$ respiration, as

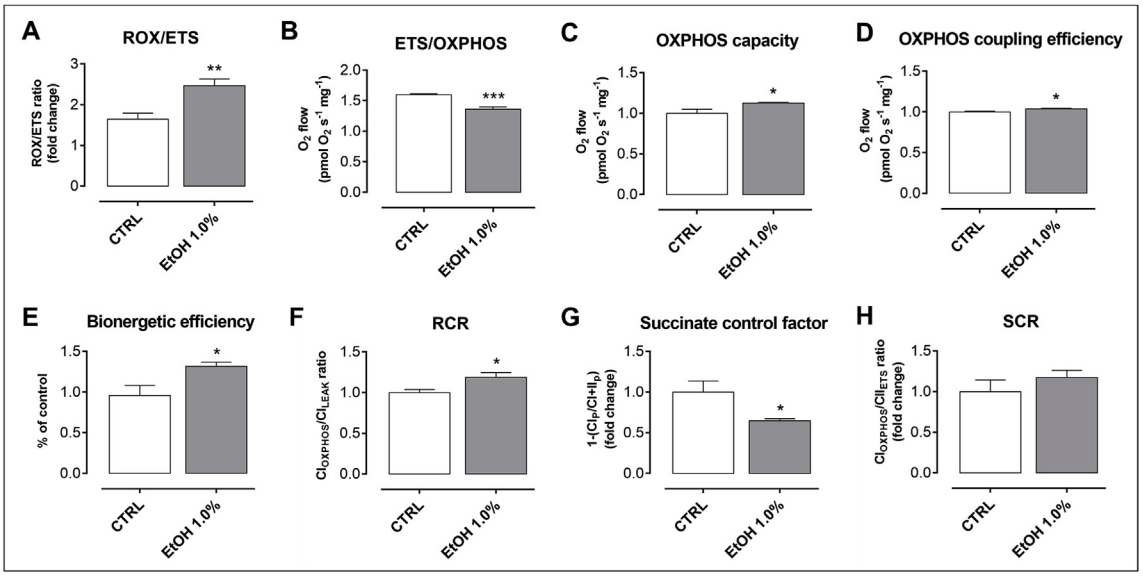


**Fig. 2.** Acute effects of 1.0% (v/v) EtOH on residual oxygen consumption (ROX/ETS ratio, fold change) **(A)**, ETS/OXPHOS **(B)**, OXPHOX capacity **(C)**, OXPHOS coupling efficiency **(D)**, bioenergetic efficiency (by subtracting the ADP-induced $CI_{OXPHOS}$ values from the $CI_{LEAK}$) **(E)**, respiratory control rate (RCR = $CI_{OXPHOS}$/$CI_{LEAK}$ ratio) **(F)**, succinate control ratio ($CI_P$/CI + $CII_O$, fold change) **(G)**, and substrate control ratio (SCR) ($CI_{OXPHOS}$/$CII_{ETS}$ ratio, fold change) **(H)**. Data were expressed as mean ± SEM and analyzed by unpaired Student's *t*-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, $n = 5$ independent preparations per group).

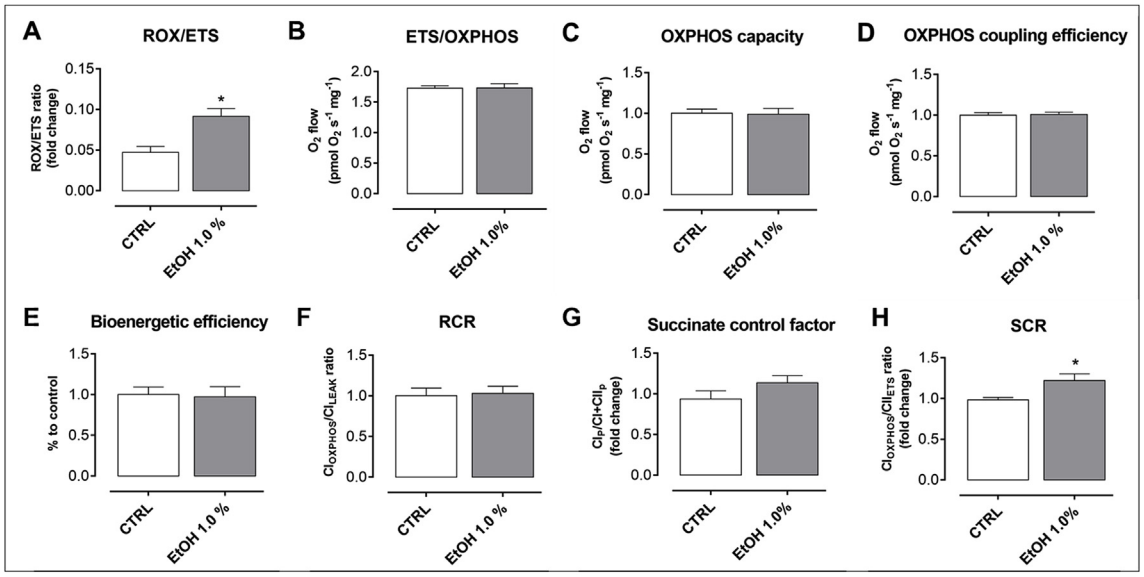


**Fig. 4.** Effects of 1.0% (v/v) chronic EtOH exposure on residual oxygen consumption (ROX/ETS ratio, fold change) **(A)**, ETS/OXPHOS **(B)**, OXPHOX capacity **(C)**, OXPHOS coupling efficiency **(D)**, bioenergetic efficiency (by subtracting the ADP-induced $CI_{OXPHOS}$ values from the $CI_{LEAK}$) **(E)**, respiratory control rate (RCR = $CI_{OXPHOS}/CI_{LEAK}$ ratio) **(F)**, succinate control ratio ($CI_P/CI + CII_O$, fold change) **(G)**, and substrate control ratio (SCR) ($CI_{OXPHOS}/CII_{ETS}$ ratio, fold change) **(H)**. Data were expressed as mean ± SEM and analyzed by unpaired Student's *t*-test (*$p < 0.05$, $n = 6$ independent preparations per group).

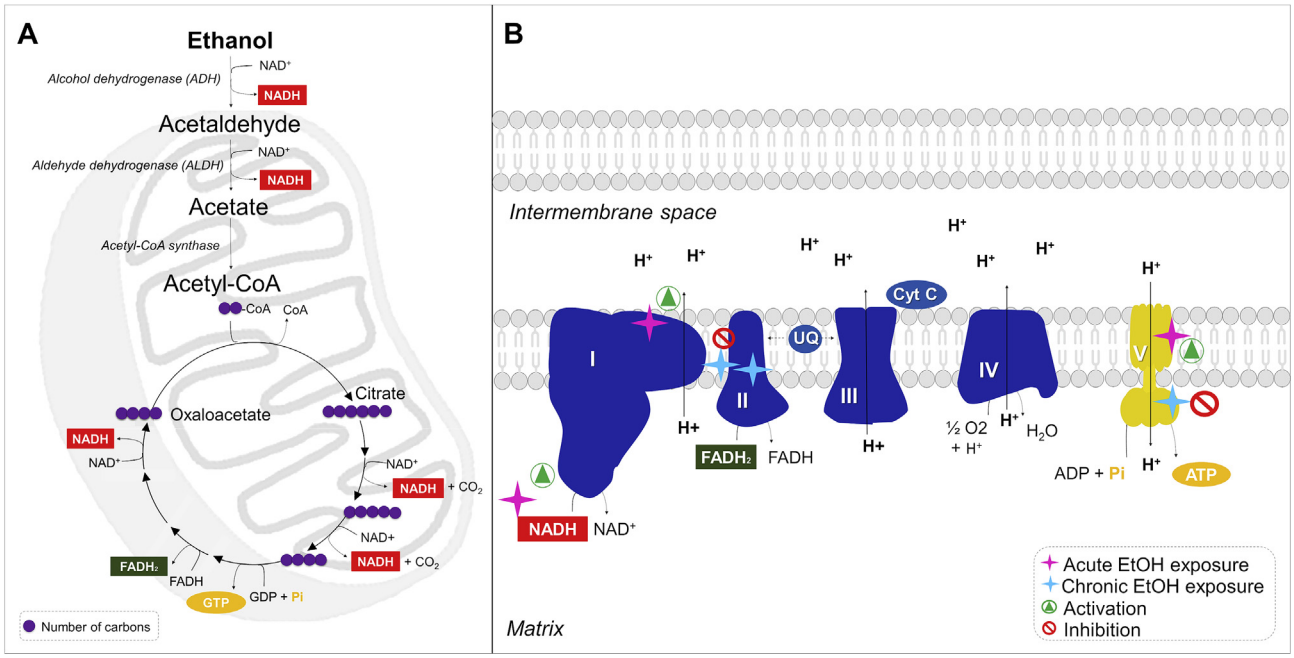


**Fig. 5.** Schematic representation of the production of reduced equivalents via EtOH catabolism **(A)** and mechanisms underlying the effects of acute and chronic EtOH exposure in the zebrafish brain mitochondria **(B)**. EtOH acutely stimulates CI-mediated OXPHOS, while the chronic exposure decreases complex I- and II-mediated ETS.

well as in complex I- and II-induced oxidative phosphorylation ($CI_{OPHOX}$, CI + $CII_{OPHOX}$) were verified. EtOH-exposed fish showed decreased CI + $CII_{ETS}$ ($t_{(0.05;\ 10)} = 2.817$, $p = 0.0183$) and $C_{IIETS}$ ($t_{(0.05;\ 10)} = 4.048$, $p = 0.0023$) and higher ROX values ($t_{(0.05;\ 10)} = 3.84$, $p = 0.0033$) and ROX/ETS ratio ($t_{(0.05;\ 10)} = 3.696$, $p = 0.0031$) than control (Fig. 4A). Although the ETS/OXPHOS (Fig. 4B), OXPHOS capacity (Fig. 4C), OXPHOS coupling efficiency (Fig. 4D), bioenergetic efficiency (Fig. 4E), RCR (Fig. 4F), and succinate control factor (Fig. 4G) did not change, SCR was increased ($t_{(0.05;\ 8)} = 2.593$, $p = 0.0268$) after EtOH exposure (Fig. 4H). Fig. 5 shows a schematic representation of the energy metabolism of acetate from EtOH catabolism (Fig. 5A) and the main effects of acute and chronic EtOH exposures on mitochondrial bioenergetics described (Fig. 5B).

## 4. Discussion

Evidence has shown that EtOH can modulate redox signaling and induce oxidative stress in the zebrafish brain (Muller et al., 2017; Rosemberg et al., 2010). Oxidative stress is one of the main mechanisms associated with the harmful effects of EtOH on the CNS (Augustyniak et al., 2005; Pereira et al., 2015; Sun et al., 2001; Sun and Sun, 2001), and mounting data support a crucial role of mitochondrial dysfunction

in alcohol-related neurotoxicity in various animal models (Pereira et al., 2015; Wu and Cederbaum, 2003; Yang and Luo, 2015; Zimatkin et al., 2006). To date, there are no data reporting whether redox alterations in the CNS occur due to changes in mitochondrial respiration in zebrafish. Here, we observed that acute EtOH exposure overstimulated mitochondrial $O_2$ consumption, while EtOH chronically decreased mitochondrial respiration by negatively modulating the ETS activity. Therefore, our novel findings demonstrate that both acute and chronic EtOH exposures affect, at least in part, the mitochondrial function by different mechanisms depending on the administration protocol.

EtOH acutely stimulated mitochondrial respiration through increased baseline respiration and $CI_{OXPHOS}$. OXPHOS capacity (directly related to CI electron flux), coupling efficiency. Furthermore, bioenergetics efficiency increased after acute EtOH exposure, reinforcing the EtOH stimulatory effect on mitochondrial $O_2$ consumption. EtOH acutely also increased RCR, which is related to the mitochondrial functionality and state of mitochondrial coupling, suggesting an enhancement of OXPHOS process. The enhanced baseline respiration and $CI_{OXPHOS}$ may be related with EtOH metabolism pathway in the brain, which increases NADH levels during the oxidation process (Deitrich et al., 2006; Hipolito et al., 2007). The acetate from EtOH metabolism can be incorporated into acetyl-coenzyme A (acetyl-CoA), a substrate of the Krebs cycle, which increases the formation of reducing equivalents (Deng and Deitrich, 2008; Lieber, 2005). NADH plays a role in ATP generation during the OXPHOS, facilitating ATP production. However, excessive NADH formation may overstimulate CI complex, thereby generating the leak of electrons (Vinogradov and Grivennikova, 2016). This phenomenon may reflect higher mitochondrial $O_2$ consumption, which facilitates ROS formation (*e.g.*, $O_2^{\cdot}$, $H_2O_2$) (Bailey and Cunningham, 2002; Bailey et al., 1999; Hoek et al., 2002). Importantly, the reduction of $NAD^+$/NADH ratio as a consequence of EtOH metabolism can disrupt fatty acid oxidation, inducing ketogenesis, lactic acidosis, and hypoglycemia (Cunningham and Bailey, 2001; Haorah et al., 2013; Lieber, 2005; McGuire et al., 2006). Based on our findings showing a decreased succinate control factor, the increased OXPHOS following acute EtOH exposure does not result from changes in complex II activity. Although EtOH can acutely decrease ATP production (Budd and Nicholls, 1996; Liu et al., 2014), the mitochondrial overstimulation could facilitate ROS formation in the CNS (Hoek et al., 2002), corroborating the higher ROX/ETS ratio observed here. These results support a role of mitochondria in mediating oxidative stress in zebrafish, which showed impaired brain antioxidant enzyme activities and increased lipid peroxidation in our previous report (Rosemberg et al., 2010).

In addition to the acute exposure protocol, we explored the chronic effects of EtOH in zebrafish. Chronically, EtOH-exposed group showed a reduced baseline respiration as well as an impaired ETS, reflected by the lower CI + CII- and CII-mediated ETS. A dysfunction of CII-mediated respiration may overload other mitochondrial complexes, thereby affecting ETS and accentuating endogenous ROS formation. Importantly, the increased SCR suggest a main involvement of complex I in ETS. Moreover, the higher ROX state and ROX/ETS ratio corroborate with the increased ROS levels and pro-oxidant effects in the zebrafish brain described elsewhere (Muller et al., 2017). Thus, we suggest that part of the $O_2$ is not being consumed by mitochondria, but rather by other EtOH detoxification pathways (*e.g.*, catalase and CYP450 enzymes), which are directly involved in EtOH metabolism (Moghe et al., 2011). Oxidative damage after chronic EtOH exposure can alter the fluidity of the mitochondrial membrane (Kowaltowski et al., 2009; Tapia-Rojas, 2018), disrupt the mitochondrial membrane potential (Karadayian et al., 2015), and reduce the mitochondrial complexes I, III, and IV activities, which are necessary for ATP formation (Bustamante et al., 2012; Karadayian et al., 2015).

The use of the high-resolution respirometry assay can be a promising strategy to assess mitochondrial bioenergetics in zebrafish models. However, to perform such analysis, the zebrafish presents some limitations. For example, the small size of brain tissue requires more than two brains per independent sample to perform replicate experiments. Furthermore, the use of other oxidizable substrates and inhibitors, as well as the investigation of enzyme activities related to the Krebs cycle could provide a more detailed response involving the mechanistic bases of EtOH in brain energy metabolism of zebrafish. Although we show distinct effects of alcohol depending on the exposure period, we cannot affirm whether such responses are mediated by EtOH alone and/or by its metabolite, acetaldehyde. Because acetaldehyde can mediate deleterious effects of EtOH in the CNS (*e.g.*, lipid peroxidation, ROS formation, DNA damage) (Balino et al., 2019; Pereira et al., 2015; Quertemont et al., 2005), further studies are needed to investigate a putative involvement of this metabolite on the biochemical responses measured here.

In conclusion, our novel findings show that EtOH affects the mitochondrial respiration in the zebrafish brain. These effects on bioenergetic in the zebrafish CNS could be related to multifactorial mechanisms (*e.g.*, pro-oxidant properties of EtOH concomitant with its toxic metabolite acetaldehyde, ROS generation, and OXPHOS dysfunction), playing a central role in EtOH-mediated neurotoxicity. Due to the similarity of zebrafish CNS physiology with those of rodents and humans, this species can provide robust and translational data regarding the neurobiological bases of alcohol abuse and addiction, contributing to unravel novel therapeutic strategies.

## Declaration of competing interest

The authors declare no conflict of interest.

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## References

Howe, K., Clark, M.D., Torroja, C.F., Torrance, J., Berthelot, C., Muffato, M., Collins, J.E., Humphray, S., McLaren, K., Matthews, L., McLaren, S., Sealy, I., Caccamo, M., Churcher, C., Scott, C., Barrett, J.C., Koch, R., Rauch, G.J., White, S., Chow, W., Kilian, B., Quintais, L.T., Guerra-Assuncao, J.A., Zhou, Y., Gu, Y., Yen, J., Vogel, J.H., Eyre, T., Redmond, S., Banerjee, R., Chi, J., Fu, B., Langley, E., Maguire, S.F., Laird, G.K., Lloyd, D., Kenyon, E., Donaldson, S., Sehra, H., Almeida-King, J., Loveland, J., Trevanion, S., Jones, M., Quail, M., Willey, D., Hunt, A., Burton, J., Sims, S., McLay, K., Plumb, B., Davis, J., Clee, C., Oliver, K., Clark, R., Riddle, C., Elliot, D., Threadgold, G., Harden, G., Ware, D., Begum, S., Mortimore, B., Kerry, G., Heath, P., Phillimore, B., Tracey, A., Corby, N., Dunn, M., Johnson, C., Wood, J., Clark, S., Pelan, S., Griffiths, G., Smith, M., Glithero, R., Howden, P., Barker, N., Lloyd, C., Stevens, C., Harley, J., Holt, K., Panagiotidis, G., Lovell, J., Beasley, H., Henderson, C., Gordon, D., Auger, K., Wright, D., Collins, J., Raisen, C., Dyer, L., Leung, K., Robertson, L., Ambridge, K., Leongamornlert, D., McGuire, S., Gilderthorp, R., Griffiths, C., Manthravadi, D., Nichol, S., Barker, G., Whitehead, S., Kay, M., Brown, J., Murnane, C., Gray, E., Humphries, M., Sycamore, N., Barker, D., Saunders, D., Wallis, J., Babbage, A, Hammond, S., Mashreghi-Mohammadi, M., Barr, L., Martin, S., Wray, P., Ellington, A., Matthews, N., Ellwood, M., Woodmansey, R., Clark, G., Cooper, J., Tromans, A., Grafham, D., Skuce, C., Pandian, R., Andrews, R., Harrison, E., Kimberley, A., Garnett, J., Fosker, N., Hall, R., Garner, P., Kelly, D., Bird, C., Palmer, S., Gehring, I., Berger, A., Dooley, C.M., Ersan-Urun, Z., Eser, C., Geiger, H., Geisler, M., Karotki, L., Kirn, A., Konantz, J., Konantz, M., Oberlander, M., Rudolph-Geiger, S., Teucke, M., Lanz, C., Raddatz, G., Osoegawa, K., Zhu, B., Rapp, A., Widaa, S., Langford, C., Yang, F., Schuster, S.C., Carter, N.P., Harrow, J., Ning, Z., Herrero, J., Searle, S.M., Enright, A., Geisler, R., Plasterk, R.H., Lee, C., Westerfield, M., de Jong, P.J., Zon, L.I., Postlethwait, J.H., Nusslein-Volhard, C., Hubbard, T.J., Roest Crollius, H., Rogers, J., Stemple, D.L., 2013. The zebrafish reference genome

sequence and its relationship to the human genome. Nature 496, 498–503.
Almansa, I., Fernandez, A., Garcia-Ruiz, C., Muriach, M., Barcia, J.M., Miranda, M., Fernandez-Checa, J.C., Romero, F.J., 2009. Brain mitochondrial alterations after chronic alcohol consumption. J. Physiol. Biochem. 65, 305–312.
Augustyniak, A., Michalak, K., Skrzydlewska, E., 2005. [The action of oxidative stress induced by ethanol on the central nervous system (CNS)]. Postepy Hig. Med. Dosw. 59, 464–471.
Bailey, S.M., Cunningham, C.C., 2002. Contribution of mitochondria to oxidative stress associated with alcoholic liver disease. Free Radic. Biol. Med. 32, 11–16.
Bailey, S.M., Pietsch, E.C., Cunningham, C.C., 1999. Ethanol stimulates the production of reactive oxygen species at mitochondrial complexes I and III. Free Radic. Biol. Med. 27, 891–900.
Balino, P., Romero-Cano, R., Sanchez-Andres, J.V., Valls, V., Aragon, C.G., Muriach, M., 2019. Effects of acute ethanol administration on brain oxidative status: the role of acetaldehyde. Alcohol Clin. Exp. Res. 43, 1672–1681.
Bolisetty, S., Jaimes, E.A., 2013. Mitochondria and reactive oxygen species: physiology and pathophysiology. Int. J. Mol. Sci. 14, 6306–6344.
Budd, S.L., Nicholls, D.G., 1996. A reevaluation of the role of mitochondria in neuronal Ca2+ homeostasis. J. Neurochem. 66, 403–411.
Bustamante, J., Karadayian, A.G., Lores-Arnaiz, S., Cutrera, R.A., 2012. Alterations of motor performance and brain cortex mitochondrial function during ethanol hangover. Alcohol 46, 473–479.
Carvalho, N.R., da Rosa, E.F., da Silva, M.H., Tassi, C.C., Dalla Corte, C.L., Carbajo-Pescador, S., Mauriz, J.L., Gonzalez-Gallego, J., Soares, F.A., 2013. New therapeutic approach: diphenyl diselenide reduces mitochondrial dysfunction in acetaminophen-induced acute liver failure. PLoS One 8, e81961.
de Carvalho, N.R., Rodrigues, N.R., Macedo, G.E., Bristot, I.J., Boligon, A.A., de Campos, M.M., Cunha, F.A.B., Coutinho, H.D., Klamt, F., Merritt, T.J.S., Posser, T., Franco, J.L., 2017. Eugenia uniflora leaf essential oil promotes mitochondrial dysfunction in *Drosophila melanogaster* through the inhibition of oxidative phosphorylation. Toxicol. Res. 6, 526–534.
Chatterjee, D., Gerlai, R., 2009. High precision liquid chromatography analysis of dopaminergic and serotoninergic responses to acute alcohol exposure in zebrafish. Behav. Brain Res. 200, 208–213.
Cunningham, C.C., Bailey, S.M., 2001. Ethanol consumption and liver mitochondria function. Biol. Signals Recept. 10, 271–282.
Cunningham, C.C., Van Horn, C.G., 2003. Energy availability and alcohol-related liver pathology. Alcohol Res. Health 27, 291–299.
Deitrich, R., Zimatkin, S., Pronko, S., 2006. Oxidation of ethanol in the brain and its consequences. Alcohol Res. Health 29, 266–273.
Deng, X.S., Deitrich, R.A., 2008. Putative role of brain acetaldehyde in ethanol addiction. Curr. Drug Abuse Rev. 1, 3–8.
Fontana, B.D., Mezzomo, N.J., Kalueff, A.V., Rosemberg, D.B., 2018. The developing utility of zebrafish models of neurological and neuropsychiatric disorders: a critical review. Exp. Neurol. 299, 157–171.
Gerlai, R., Lahav, M., Guo, S., Rosenthal, A., 2000. Drinks like a fish: zebra fish (*Danio rerio*) as a behavior genetic model to study alcohol effects. Pharmacol. Biochem. Behav. 67, 773–782.
Gnaiger, E., 2009. Capacity of oxidative phosphorylation in human skeletal muscle: new perspectives of mitochondrial physiology. Int. J. Biochem. Cell Biol. 41, 1837–1845.
Gneiting, U., Schmitz, H.P., 2016. Comparing global alcohol and tobacco control efforts: network formation and evolution in international health governance. Health Policy Plan. 31 (Suppl. 1), i98–109.
Goodlett, C.R., Horn, K.H., 2001. Mechanisms of alcohol-induced damage to the developing nervous system. Alcohol Res. Health 25, 175–184.
Guo, C., Sun, L., Chen, X., Zhang, D., 2013. Oxidative stress, mitochondrial damage and neurodegenerative diseases. Neural Regen Res. 8, 2003–2014.
Haorah, J., Rump, T.J., Xiong, H., 2013. Reduction of brain mitochondrial beta-oxidation impairs complex I and V in chronic alcohol intake: the underlying mechanism for neurodegeneration. PLoS One 8, e70833.
Hipolito, L., Sanchez, M.J., Polache, A., Granero, L., 2007. Brain metabolism of ethanol and alcoholism: an update. Curr. Drug Metabol. 8, 716–727.
Hoek, J.B., Cahill, A., Pastorino, J.G., 2002. Alcohol and mitochondria: a dysfunctional relationship. Gastroenterology 122, 2049–2063.
Holzschuh, J., Ryu, S., Aberger, F., Driever, W., 2001. Dopamine transporter expression distinguishes dopaminergic neurons from other catecholaminergic neurons in the developing zebrafish embryo. Mech. Dev. 101, 237–243.
Horzmann, K.A., Freeman, J.L., 2016. Zebrafish get connected: investigating neurotransmission targets and alterations in chemical toxicity. Toxics 4.
Karadayian, A.G., Bustamante, J., Czerniczyniec, A., Lombardi, P., Cutrera, R.A., Lores-Arnaiz, S., 2015. Alcohol hangover induces mitochondrial dysfunction and free radical production in mouse cerebellum. Neuroscience 304, 47–59.
Kowaltowski, A.J., de Souza-Pinto, N.C., Castilho, R.F., Vercesi, A.E., 2009. Mitochondria and reactive oxygen species. Free Radic. Biol. Med. 47, 333–343.
Lieber, C.S., 2005. Metabolism of alcohol. Clin. Liver Dis. 9, 1–35.
Liu, Z., Liu, Y., Gao, R., Li, H., Dunn, T., Wu, P., Smith, R.G., Sarkar, P.S., Fang, X., 2014. Ethanol suppresses PGC-1alpha expression by interfering with the cAMP-CREB pathway in neuronal cells. PLoS One 9, e104247.
MacRae, C.A., Peterson, R.T., 2015. Zebrafish as tools for drug discovery. Nat. Rev. Drug Discov. 14, 721–731.
Manzo-Avalos, S., Saavedra-Molina, A., 2010. Cellular and mitochondrial effects of alcohol consumption. Int. J. Environ. Res. Public Health 7, 4281–4304.
Mathur, P., Guo, S., 2011. Differences of acute versus chronic ethanol exposure on anxiety-like behavioral responses in zebrafish. Behav. Brain Res. 219, 234–239.
McGuire, L.C., Cruickshank, A.M., Munro, P.T., 2006. Alcoholic ketoacidosis. Emerg. Med. J. 23, 417–420.
Moghe, A., Joshi-Barve, S., Ghare, S., Gobejishvili, L., Kirpich, I., McClain, C.J., Barve, S., 2011. Histone modifications and alcohol-induced liver disease: are altered nutrients the missing link? World J. Gastroenterol. 17, 2465–2472.
Muller, T.E., Nunes, S.Z., Silveira, A., Loro, V.L., Rosemberg, D.B., 2017. Repeated ethanol exposure alters social behavior and oxidative stress parameters of zebrafish. Prog. Neuropsychopharmacol Biol. Psychiatr. 79, 105–111.
Pereira, R.B., Andrade, P.B., Valentao, P., 2015. A comprehensive view of the neurotoxicity mechanisms of cocaine and ethanol. Neurotox. Res. 28, 253–267.
Pesta, D., Gnaiger, E., 2012. High-resolution respirometry: OXPHOS protocols for human cells and permeabilized fibers from small biopsies of human muscle. Methods Mol. Biol. 810, 25–58.
Quertemont, E., Tambour, S., Tirelli, E., 2005. The role of acetaldehyde in the neurobehavioral effects of ethanol: a comprehensive review of animal studies. Prog. Neurobiol. 75, 247–274.
Rehm, J., 2011. The risks associated with alcohol use and alcoholism. Alcohol Res. Health 34, 135–143.
Ridley, N.J., Draper, B., Withall, A., 2013. Alcohol-related dementia: an update of the evidence. Alzheimer's Res. Ther. 5, 3.
Rosemberg, D.B., da Rocha, R.F., Rico, E.P., Zanotto-Filho, A., Dias, R.D., Bogo, M.R., Bonan, C.D., Moreira, J.C., Klamt, F., Souza, D.O., 2010. Taurine prevents enhancement of acetylcholinesterase activity induced by acute ethanol exposure and decreases the level of markers of oxidative stress in zebrafish brain. Neuroscience 171, 683–692.
Rosemberg, D.B., Braga, M.M., Rico, E.P., Loss, C.M., Cordova, S.D., Mussulini, B.H., Blaser, R.E., Leite, C.E., Campos, M.M., Dias, R.D., Calcagnotto, M.E., de Oliveira, D.L., Souza, D.O., 2012. Behavioral effects of taurine pretreatment in zebrafish acutely exposed to ethanol. Neuropharmacology 63, 613–623.
Shield, K.D., Parry, C., Rehm, J., 2013. Chronic diseases and conditions related to alcohol use. Alcohol Res. 35, 155–173.
Stewart, A.M., Ullmann, J.F., Norton, W.H., Parker, M.O., Brennan, C.H., Gerlai, R., Kalueff, A.V., 2015. Molecular psychiatry of zebrafish. Mol. Psychiatry 20, 2–17.
Sun, A.Y., Sun, G.Y., 2001. Ethanol and oxidative mechanisms in the brain. J. Biomed. Sci. 8, 37–43.
Sun, A.Y., Ingelman-Sundberg, M., Neve, E., Matsumoto, H., Nishitani, Y., Minowa, Y., Fukui, Y., Bailey, S.M., Patel, V.B., Cunningham, C.C., Zima, T., Fialova, L., Mikulikova, L., Popov, P., Malbohan, I., Janebova, M., Nespor, K., Sun, G.Y., 2001. Ethanol and oxidative stress. Alcohol Clin. Exp. Res. 25, 237S–243S.
Tapia-Rojas, C.P., Pérez, M.J., Jara, C., Vergara, E.W., Quintanilla, R.A., 2018. Ethanol consumption affects neuronal function: role of the mitochondria. In: Open, I. (Ed.), InTech Open, pp. 361–384.
Vinogradov, A.D., Grivennikova, V.G., 2016. Oxidation of NADH and ROS production by respiratory complex I. Biochim. Biophys. Acta 1857, 863–871.
Wu, D., Cederbaum, A.I., 2003. Alcohol, oxidative stress, and free radical damage. Alcohol Res. Health 27, 277–284.
Yang, F., Luo, J., 2015. Endoplasmic reticulum stress and ethanol neurotoxicity. Biomolecules 5, 2538–2553.
Zimatkin, S.M., Pronko, S.P., Vasiliou, V., Gonzalez, F.J., Deitrich, R.A., 2006. Enzymatic mechanisms of ethanol oxidation in the brain. Alcohol Clin. Exp. Res. 30, 1500–1505.