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Mechanistic studies on ketamine-induced mitochondrial toxicity in zebrafish embryos

Bonnie L. Robinson, Melanie Dumas, Syed F. Ali, Merle G. Paule, Qiang Gu, and Jyotshna Kanungo*

Division of Neurotoxicology, National Center for Toxicological Research, U.S. Food and Drug Administration, 3900 NCTR Road, Jefferson, AR 72079, USA

Abstract

Ketamine, a phencyclidine derivative, is an antagonist of the Ca^{2+} -permeable *N*-methyl-D-aspartate (NMDA)-type glutamate receptors. It is a pediatric anesthetic and has been implicated in developmental neurotoxicity. Ketamine has also been shown to deplete ATP in mammalian cells. Our previous studies showed that acetyl L-carnitine (ALCAR) prevented ketamine-induced cardiotoxicity and neurotoxicity in zebrafish embryos. Based on our finding that ALCAR's protective effect was blunted by oligomycin A, an inhibitor of ATP synthase, we further investigated the effects of ketamine and ALCAR on ATP levels, mitochondria and ATP synthase in zebrafish embryos. The results demonstrated that ketamine reduced ATP levels in the embryos but not in the presence of ALCAR. Ketamine reduced total mitochondrial protein levels and mitochondrial potential, which were prevented with ALCAR co-treatment. To determine the cause of ketamine-induced ATP deficiency, we explored the status of ATP synthase. The results showed that a subunit of ATP synthase, *atp5a1*, was transcriptionally down-regulated by ketamine, but not in the presence of ALCAR, although ketamine caused a significant upregulation in another ATP synthase subunit, *atp5b* and total ATP synthase protein levels. Most of the ATP generated by heart mitochondria are utilized for its contraction and relaxation. Ketamine-treated embryos showed abnormal heart structure, which was abolished with ALCAR co-treatment. This study offers evidence for a potential mechanism by which ketamine could cause ATP deficiency mediated by mitochondrial dysfunction.

Keywords

Ketamine; Zebrafish; Acetyl L-carnitine; Mitochondria; Developmental toxicity

*Corresponding author. jyotshnabala.kanungo@fda.hhs.gov (J. Kanungo).

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The authors declare that there are no conflicts of interest.

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1. Introduction

The pediatric general anesthetic ketamine is a noncompetitive antagonist of the *N*-methyl-D-aspartic acid (NMDA) receptor (Kohrs and Durieux, 1998). Although recognized as a dissociative anesthetic (Corssen et al., 1968), ketamine also has analgesic and amnesic effects and possesses fast-acting anti-depressant properties (Aroni et al., 2009; Berman et al., 2000; Niesters and Dahan, 2012; Rowland, 2005). Ketamine is a Schedule III controlled substance in the USA (Morgan et al., 2010). Lately, several studies have shown ketamine's adverse effects on mitochondrial function. Ketamine decreased mitochondrial membrane potential in neural stem cells leading to apoptosis (Bai et al., 2013). In primary neurons, ketamine induced neurotoxicity by increased nitrotyrosine formation (Wang et al., 2008). In human lymphocytes and hepatocytes, ketamine caused mitochondrial dysfunction (Braun et al., 2010; Lee et al., 2009). Additionally, in various regions of rat brains, ketamine at subanesthetic doses altered mitochondrial respiratory chain activity (de Oliveira et al., 2011). Ketamine led to mitochondrial dysfunction in neurons derived from human induced pluripotent stem cell (Ito et al., 2015) and acute ketamine administration has been shown to impair mitochondrial function in rat brains (Venancio et al., 2015). Ketamine suppresses ATP biosynthesis in HepG2 cells (Chang et al., 2009). However, the molecular mechanism of how ketamine causes mitochondrial dysfunction and suppresses ATP production is not known.

There are growing concerns that long-term ketamine abuse harms the heart and increases the risk of sudden death (Weiner et al., 2000). Ketamine is known to induce serious side effects including cardiac deregulation (Craven, 2007) by altering the sympathomimetic effects (White and Ryan, 1996) through excessive production of catecholamines. Increased production of catecholamines results in oxidative damage to the heart (Haskova et al., 2011). Ketamine reduced heart rates in newborn human infants (Saarenmaa et al., 2001) as well as pregnant and infant non-human primates (Hotchkiss et al., 2007). Ketamine-induced cardiotoxicity in rats and its prevention by antioxidants (disulfiram, metyrosine and metoprolol) have been reported (Ahiskalioglu et al., 2015; Cetin et al., 2015) suggesting that these drugs may prove useful in clinical practice for any potential development of cardiotoxicity that may occur due to ketamine anesthesia. In both rats and rabbits, chronic ketamine treatment caused significant ventricular myocardial apoptosis and fibrosis that not only altered the electro-physiological properties of the heart but also increased its susceptibility to malignant arrhythmia leading to sudden cardiac death (Li et al., 2012). Metoprolol prevented such ketamine-induced cardiotoxicity, opening a potential new therapeutic avenue (Li et al., 2012).

Acetyl L-carnitine (ALCAR) is an L-lysine derivative and belongs to the family of carnitines, a group of naturally occurring compounds that is essential for β -oxidation of fatty acids in mitochondria during ATP generation (Bieber, 1988). It plays a major role in the transport of long chain fatty acids into mitochondria that enter the β -oxidation cycle (Bohles et al., 1994). ALCAR effectively prevents mitochondrial injury resulting from oxidative damage (Palacios et al., 2011). Carnitines have neuroprotective effects on conditions associated with mitochondrial dysfunction, oxidative stress and possibly in neurodegenerative disorders (Beals et al., 2003). During early developmental stages, co-

administration of ALCAR and ketamine significantly diminished reactive oxygen species (ROS) generation and provided significant protection of neurons from ketamine-induced neurodegeneration in rats (Liu et al., 2013). Additionally, ALCAR protected neurons from inhalation anesthetic-induced neurotoxicity in rat cortical neurons (Zou et al., 2008). In ischemic swine hearts perfused in the presence of free fatty acids, ALCAR preserved mechanical function (Liedtke and Nellis, 1979). In rats, ALCAR ameliorated ketamine-induced behavioral alterations and body weight deficits (Boctor et al., 2008).

The zebrafish (*Danio rerio*) embryo has become an important experimental model for studying various diseases, protein functions, drug toxicities, and various physiological responses (Kanungo et al., 2014; Vogel, 2000). Accordingly, the therapeutic and toxic responses for both cardiac and non-cardiac drugs can be assessed by simply immersing the embryos in water with the test drug (Kanungo et al., 2012; Langheinrich et al., 2003; Milan et al., 2003). We have previously shown in zebrafish embryos that consistent with its effects on the nervous system and heart function in mammals (Hotchkiss et al., 2007; Irifune et al., 1997; Irifune et al., 1991; Kim et al., 2006; Zausig et al., 2009), ketamine adversely affects the motor neurons (Kanungo et al., 2013), serotonergic and dopamine systems (Robinson et al., 2016; Robinson et al., 2015) as well as heart rate (Kanungo et al., 2012). Additionally, we have shown that ALCAR prevents ketamine's adverse effects on the heart rate (Guo et al., 2017; Kanungo et al., 2012; Robinson et al., 2016; Robinson et al., 2015). Our studies also suggested that ALCAR could function through modulating ATP synthase (Guo et al., 2017).

ATP synthase is a multi-subunit enzyme (Cross and Muller, 2004) and its dysregulation adversely affects mitochondrial respiration. Mitochondrial dysfunction leads to the development of cardiomyopathy (Walters et al., 2012). Since cardiac contraction and relaxation processes are dependent on energy in the form of ATP produced by the mitochondria, mitochondrial dysfunction results in developmental defects of the heart (Sinatra, 2009). Additionally, myocardial ATP depletion is a major factor in heart failure (Gupta et al., 2012; Sinatra, 2009). Ketamine has been shown to cause an undesirable strain on the myocardium of patients (Spotof et al., 1979) and we have previously reported on ketamine's adverse effects on heart rate in the absence of ALCAR (Kanungo et al., 2012). Here, as a follow up to our earlier studies, we investigated ketamine's effects on the components of the bioenergetic system and whether such effects could be prevented by ALCAR. This study was undertaken primarily to understand the molecular mechanism(s) of ketamine's effects on the heart and investigate potential preventive measures for unintended adverse effects during ketamine anesthesia or abuse.

2. Materials and methods

2.1. Animals

Adult wild type (WT) zebrafish (*Danio rerio*, AB strain) were obtained from the Zebrafish International Resource Center (www.zirc.org) (Eugene, OR, USA). The fish were kept in fish tanks (Aquatic Habitats, FL, USA) at the NCTR/FDA zebrafish facility containing buffered water (pH 7.5) at 28.5 °C and were fed daily live brine shrimp and Zeigler dried flake food (Zeiglers, Gardeners, PA, USA). Each 3-liter tank housed eight adult males or

females. Handling and maintenance of zebrafish were in compliance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the NCTR/FDA IACUC. The day–night cycle was maintained at 14:10 h. For in-system breeding, crosses of males and females were set up the previous day in the tanks with partitions that were taken off the following morning at the time of light onset at 7:30 AM to stimulate spawning and fertilization. Fertilized eggs were collected from the bottom of the tank, placed in Petri dishes, washed thoroughly with buffered egg water [reverse osmosis water containing 60 mg sea salt (Crystal Sea®, Aquatic Eco-systems, Inc., Apopka, FL, USA) per liter of water (pH 7.5)] and then allowed to develop in an incubator at 28.5 °C for later use.

2.2. Reagents

Ketamine hydrochloride was purchased from Vedco, Inc. (St. Joseph, MO, USA). Acetyl L-carnitine (ALCAR) and other reagents were purchased from Sigma (St. Louis, MO, USA) unless stated otherwise. ALCAR stock (1 M) solutions were made fresh with buffered egg water.

2.3. Treatment of zebrafish embryos with ketamine and acetyl L-carnitine (ALCAR)

For treatment with ketamine and ALCAR, 28 h post-fertilization (hpf) embryos were used since our previous studies showed that these embryos as whole organisms respond to ketamine anesthesia (Cuevas et al., 2013; Kanungo et al., 2012) and ketamine exposure at the gastrula stage leads to non-specific effects displayed during later development (Robinson et al., 2017). For each experiment that analyzed all the endpoints, eggs laid at the same time from several females were pooled. For each treatment, 50 embryos were placed in individual 60 mm Petri dishes containing 35 ml buffered egg water and a total of 450 embryos (nine Petri dishes) were used for all the exposures in each experiment. The experiments were repeated three times. Ketamine (2.0 mM) treatment continued for 20 h (static exposures) in the presence or absence of ALCAR (1.0 mM). In our previous studies, these specific concentrations of ketamine and ALCAR for the specific time of exposure were found to be effective in inducing detectable toxicities and their amelioration, respectively (Kanungo et al., 2012; Robinson et al., 2017). Previously, we have shown that the amount of ketamine accumulation (~8 µM) in the zebrafish embryos/larvae treated with 2 mM is closer to the lower range of human anesthetic range (plasma concentration) (Trickler et al., 2013). Untreated control groups were examined in parallel.

2.4. Measurement of ATP concentration

Zebrafish embryo ATP content was determined using ATP colorimetric assay kit (Biovision, Milpitas, CA, USA). Briefly, for each sample, 10 embryos (out of the 50 embryos in each Petri dish) were homogenized in 200 µl ATP assay buffer and the homogenates were centrifuged at 15,000 ×g for 2 min to pellet insoluble materials. Supernatants were collected and 50 µl supernatant was added to a 96-well plate. ATP reaction mix (50 µl) was added to the samples in each well. After mixing by shaking gently, the 96-well plate was incubated at room temperature for 30 min, protected from light. The ATP levels were detected at 570 nm using a Synergy MX microplate reader (BioTek, Winooski, VT, USA). ATP levels in the embryo extracts were quantified using an ATP standard curve generated to calculate the amount of ATP and were normalized to the protein concentration of the samples. Protein

concentrations were determined using a bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Waltham, MA, USA). The statistical significance of the effects of the various treatments on ATP levels was determined by one-way ANOVA (Sigma Stat) using Holm–Sidak pairwise multiple comparison post-hoc analysis. Statistical significance (*) was based on $P < 0.05$.

2.5. Measurement of total mitochondrial protein

Crude mitochondrial and cytoplasmic fractions were isolated using a Mitoiso 1 isolation kit (Sigma, St. Louis, MO, USA). All steps were carried out at 4 °C with ice cold buffers. Briefly, 30 embryos/sample (a total of three samples taken from three Petri dishes) were homogenized in the supplied extraction buffer. Homogenates were spun at 600 ×g for 5 min at 4 °C and then the supernatants were spun at 11,000 ×g for 10 min at 4 °C. The resulting pellet was re-suspended in extraction buffer and the centrifugation steps were repeated. The final pellets representing the mitochondrial fraction were re-suspended in the supplied storage buffer. Protein concentration was determined using BCA assay (Thermo Fisher Scientific, Waltham, MA, USA). The statistical significance of the effects of the various treatments on total mitochondrial protein levels was determined by one-way ANOVA (Sigma Stat) using Holm–Sidak pairwise multiple comparison post-hoc analysis. Statistical significance (*) is based on $P < 0.05$.

2.6. Evaluation of mitochondrial inner membrane potential

The mitochondrial inner membrane potential was evaluated using the Mitoiso 1 isolation kit (Cat# MITOISO1, Sigma, St. Louis, MO, USA). Briefly, 30 embryos/sample (a total of three samples taken from three Petri dishes) were homogenized in the supplied extraction buffer. Homogenates were spun at 600 ×g for 5 min at 4 °C and then the supernatants were spun at 11,000 ×g for 10 min at 4 °C. The resulting pellet was re-suspended in extraction buffer and the centrifugation steps were repeated. The final pellets representing the mitochondrial fraction were re-suspended in the supplied storage buffer. The integrity of the mitochondrial inner membrane potential was evaluated by measuring the uptake of the fluorescent dye 5, 5', 6', 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1) provided in the kit. Mitochondrial fractions were incubated with the JC-1 in JC-1 assay buffer for 10 min at room temperature following the manufacturer's instruction. Fluorescence was detected using a Synergy MX microplate reader (BioTek, Winooski, VT, USA) with settings of excitation wavelength at 490 nm; and emission wavelength at 590 nm. The fluorescence produced in the original mitochondria suspension per mg mitochondrial protein (FLU/mg protein) was calculated. The statistical significance of the effects of the various treatments on mitochondrial inner membrane potential was determined by one-way ANOVA (Sigma Stat) using Holm–Sidak pairwise multiple comparison post-hoc analysis. Statistical significance (*) was based on $P < 0.05$.

2.7. RNA extraction and cDNA synthesis

Total RNA (50 embryos/sample) was extracted from whole embryos using the Trizol reagent (Invitrogen, Carlsbad, CA, USA). For each treatment, a total of three samples (50 embryos each from three Petri dishes) were used. An aliquot of each RNA sample was used to spectrophotometrically (using a NanoDrop ND-1000 instrument; NanoDrop Technology,

Wilmington, DE, USA) determine RNA quality ($A_{260}/A_{280} > 2.0$) and concentration. First-strand cDNA was synthesized from total RNA (2 µg; 20 µl final reaction volume) with oligo(dT) priming using SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions.

2.8. Primers

Zebrafish gene-specific primers (Table 1) were used for the quantitative real-time polymerase chain reaction (RT-qPCR) assays to quantify glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), cardiac muscle ATP synthase, H^+ transporting mitochondrial F1 complex alpha subunit 1 (*atp5a1*) (GenBank entry # NM_001077355.1), and cardiac muscle ATP synthase, H^+ transporting mitochondrial F1 complex beta subunit (*atp5b*) (GenBank entry # NM_001024429.2).

2.9. Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay for gene expression analysis

Real-time qPCR was performed using a CFX96 C1000 (Bio-Rad, Hercules, CA, USA) detection system with SYBR green fluorescent label (Bio-Rad). Samples (25 µl final vol) contained the following: 1× SYBR green master mix (Bio-Rad), 5 pmol of each primer and 0.25 ml of the reverse transcriptase (RT) reaction mixture. Samples were run in triplicate in optically clear 96-well plates. Cycling parameters were as follows: 50 °C × 2 min, 95 °C × 10 min, then 40 cycles of 95 °C × 15 s, 60 °C × 1 min. A melting temperature-determining dissociation step was performed at 95 °C × 15 s, 60 °C × 15 s and 95 °C × 15 s at the end of the amplification phase. The 2^{-C_t} method was used to determine the relative gene expression (Livak and Schmittgen, 2001). The *GAPDH* gene was the internal control for all qRT-PCR experiments. Data from each group ($n = 3$) were averaged and shown as normalized gene expression ± SD. One-way ANOVA and Holm–Sidak pair-wise multiple comparison post-hocs (Sigma Stat) were performed. Statistical significance (*) was based on $P < 0.05$.

2.10. Estimation of relative ATP synthase levels

ATP synthase levels were measured in 48 hpf zebrafish embryos homogenates using the.

ATP synthase Microplate Assay Kit (Cat # ab109716; Abcam, Cambridge, MA, USA) following the manufacturer's protocol. Briefly, for each assay sample, 25 embryos (each collected from three Petri dishes) from a treatment group were homogenized in 200 µl of homogenization buffer and the homogenates were frozen in dry ice/ethanol slurry. After thawing and centrifugation of the samples, 50 µl of each solubilized fraction with a protein concentration of 5.5 µg/µl was loaded into the wells of a microplate pre-coated with monoclonal anti-ATP synthase antibody. ATP synthase is immunocaptured within the wells. The relative quantity was measured following the manufacturer's protocol.

using an ELISA plate reader. One-way ANOVA and Holm–Sidak pair-wise multiple comparison post-hocs (Sigma Stat) were performed on the optical density (OD) values. Statistical significance (*) was based on $P < 0.05$.

3. Results

3.1. Ketamine reduces ATP in zebrafish embryos in the absence of ALCAR

Zebrafish embryos at 28 hpf were exposed to 2 mM ketamine in the presence or absence of 1.0 mM ALCAR (Fig. 1). The static exposure duration was 20 h. Post-exposure, the 48 hpf embryos were analyzed for total ATP content. The ATP levels were estimated in these groups by normalization against total protein content of the embryos. There were statistical differences between treatment groups [$F(2, 26) = 15.962$, $P < 0.05$]. Ketamine-treated embryos had significantly reduced ATP levels compared to those in the control and ketamine/ALCAR-treated embryos ($P < 0.05$). However, there was no significant difference between the control and ketamine/ALCAR co-treated embryos ($P = 0.057$) (Fig. 2). These results indicate beneficial effects of ALCAR on ketamine-induced toxicities to the bioenergetic system.

3.2. Ketamine reduces total mitochondrial protein in the absence of ALCAR

Since ATP is produced by the mitochondria, we sought to determine whether ketamine also altered mitochondrial biology in the embryos treated with 2 mM ketamine in the presence or absence of 1.0 mM ALCAR. The indices to determine mitochondrial function in this study were total mitochondrial protein amount and mitochondrial potential as reflected by the mitochondrial inner membrane integrity. The results revealed significant changes in total mitochondrial protein levels in the treatment groups [$F(2, 8) = 176.577$, $P < 0.001$]. The mitochondrial protein amount was significantly reduced in the ketamine-treated embryos compared to control ($P < 0.001$) (Fig. 3A) indicating that ketamine adversely affected mitochondria health. Co-treatment with ALCAR, on the other hand, prevented this specific effect of ketamine, and even resulted in a significant increase in the mitochondrial total protein levels compared to both the control ($P < 0.001$) and ketamine-treated group ($P < 0.001$) (Fig. 3A). These data suggest that ketamine's adverse effects on the mitochondria health are prevented by ALCAR.

3.3. ALCAR prevents ketamine's adverse effects on the mitochondrial inner membrane potential

Normal regulation of mitochondrial membrane potential is instrumental for electron transport and ATP generation. Since ketamine reduced total mitochondrial protein in the zebrafish embryos (Fig. 3B), the status of mitochondrial membrane potential was assessed using a lipophilic-cationic dye JC-1, which accumulates within the mitochondria depending on mitochondrial membrane potential. The results revealed significant changes in the mitochondrial inner membrane potential among the treatment groups [$F(2, 8) = 207.099$, $P < 0.001$]. The data indicated that ketamine significantly reduced mitochondrial inner membrane potential in the zebrafish embryos ($P < 0.001$) and co-treatment with ALCAR not only prevented the adverse effects of ketamine but also significantly increased the mitochondrial inner membrane potential over that seen in the control embryos ($P < 0.001$) (Fig. 3B). These data showed that ketamine's adverse effects on the mitochondrial function can be prevented by ALCAR.

3.4. Ketamine alters ATP synthase gene expression, ATP synthase level and heart development in the absence of ALCAR

Based on our earlier report that ALCAR's protection of zebrafish embryos from ketamine toxicity could involve ATP synthase, we explored whether ketamine and ALCAR differentially affected ATP synthase gene expression. Data from qRT-PCR analyses revealed a significant changes in *atp5a1* mRNA expression among the treatment groups [F (2, 8) = 109.673, $P < 0.001$]. A significant reduction in *atp5a1* mRNA expression in the ketamine-treated embryos compared to control was evident ($P < 0.001$) (Fig. 4A). Co-treatment with significantly increased *atp5a1* mRNA expression compared to the ketamine-treated group ($P < 0.001$). However, compared to the control, co-treatment with ketamine and ALCAR did not cause any significant change ($P = 0.105$) in *atp5a1* mRNA expression (Fig. 4A).

Additionally, the treatment groups showed significant changes in *atp5b* mRNA expression [F (2, 8) = 15.962, $P < 0.05$]. While ketamine induced a significant increase in *atp5b* mRNA expression compared to control ($P < 0.004$) (Fig. 4B), ALCAR co-treatment significantly reduced *atp5b* mRNA expression compared to the ketamine-treated group ($P = 0.033$) (Fig. 4B). There was no statistical difference between the control and ketamine/ALCAR co-treated embryos ($P = 0.057$). These results indicated that ketamine differentially altered the expression of the two different subunits of ATP synthase, but not in the presence of ALCAR.

There was also statistically significant difference in ATP synthase protein levels among the treatment groups [F (2, 8) = 11.982, $P < 0.05$]. Interestingly, the ATP synthase protein levels were significantly higher in the ketamine-treated embryos compared to the control ($P < 0.011$) and ketamine/ALCAR-cotreated embryos ($P < 0.021$) (Fig. 5). There was no significant difference in the ATP synthase protein levels between the control and ketamine/ALCAR co-treated embryos ($P = 0.369$). These results suggest that despite a reduced expression of *atp5a1*, an increased *atp5b* mRNA expression in the ketamine-treated embryos may have contributed to an overall increase in the ATP synthase protein levels.

Alteration in specific ATP synthase subunits, especially ATP synthase α has been shown to affect cardiac function (Wang et al., 2011). Since ketamine down regulated *atp5a1* expression, we imaged the hearts of the live embryos to pinpoint any structural changes (Fig. 6). In our previous studies (Guo et al., 2017; Kanungo et al., 2012), we reported that ALCAR ameliorated ketamine-induced adverse effects on heart rate. The current results show in the ketamine-treated embryos, an expanded (sac-like), membranous auricle with no looping of the heart (Fig. 6B, E) as observed in controls (Fig. 6A, D) and ketamine/ALCAR co-treated embryos (Fig. 6C, F). While the ketamine-treated embryo heart appeared as a continuous chamber (Fig. 6B, E) with no clear demarcation between the auricle and ventricle (in 100% of the embryos), the control (Fig. 6A, D) and ketamine/ALCAR co-treated (Fig. 6C, F) hearts clearly showed two separated chambers, the ventricle (arrow and v) and auricle (arrowhead and a) (in 100% of the embryos). These results suggest that ketamine's adverse effects on the heart structure/development are ameliorated by ALCAR.

4. Discussion

The results from this study showed that ketamine affects the bioenergetic pathway in the zebrafish embryos. Ketamine reduced ATP levels along with a reduction in total

mitochondrial proteins and mitochondrial membrane potential in zebrafish embryos. Similar effects of ketamine have been reported in human cells (Ito et al., 2015). At 100 μ M, ketamine has been shown to cause a reduction in the ATP levels in human induced pluripotent stem cells (hiPSC) (Ito et al., 2015). Also in human urothelial cells, ketamine (3 mmol/L) caused significant ATP deficiency thought to be mediated through mitochondrial dysfunction (Baker et al., 2016).

In the heart, > 95% of ATP is generated in the mitochondria and the heart utilizes the majority (60–70%) of generated ATP to regulate myocardial contraction (Gibbs, 1978; Suga, 1990). Ketamine-induced cardiac dysfunction has been reported in human fetus (Saarenmaa et al., 2001), infant non-human primates (Hotchkiss et al., 2007), *Xenopus* (Guo et al., 2016) and zebrafish (Guo et al., 2017; Kanungo et al., 2012; Robinson et al., 2017). Ketamine can affect the right ventricular function and pulmonary artery pressure since it increases pulmonary vascular resistance (Craven, 2007). Complications due to compromised myocardial functions are also observed during ketamine anesthesia (Reich and Silvay, 1989). To fully understand the effects of anesthetics on the mammalian heart, use of lower vertebrate models, such as zebrafish, is advantageous especially in delineating the mechanism(s) by which toxic effects occur. Our current study shows that ketamine induced adverse effects on the heart development.

In the cells, mitochondria perform a multitude of functions, the major one being ATP generation through oxidative phosphorylation (Green and Kroemer, 2004; McBride et al., 2006). In the mitochondria, ATP production occurs by via the electron transport chain and involves ATP synthase. Movement of electrons through the electron transport chain creates an electrochemical gradient, which is called the mitochondrial membrane potential. Pumping of protons from the mitochondrial intermembrane space to its matrix occurs via ATP synthase producing ATP from ADP (McBride et al., 2006). ATP is produced when the mitochondrial membrane potential couples proton pumping from the mitochondrial inter membrane space to the mitochondrial matrix by complex IV, followed by ATP synthesis by ATP synthase or complex V (Ramzan et al., 2010; Roy et al., 2008). Therefore, a change in mitochondrial membrane potential would certainly affect ATP generation. Compared to the control, ketamine at 500 μ M, with exposure for 6 or 24 h, significantly decreased the mitochondrial membrane potential in both the human iPSC-derived neurons and a cell line derived from cortical neurons of a 14-week-old human fetal brain (Ito et al., 2015). Data from the current study suggest that ketamine-induced reduction in ATP levels resulted from decreased mitochondrial membrane potential and potentially compromised ATP synthase function. In our previous study, we have shown that ALCAR's beneficial effects to ameliorate ketamine toxicity in the zebrafish involved functional ATP synthase (Guo et al., 2017). Besides having a major role in ATP production, the mitochondrial ATP synthase also reverses ATP hydrolysis activity and thus helps maintain the mitochondrial membrane potential (Appleby et al., 1999). Additionally, ATP synthase participates in mitochondrial protein import (Martin et al., 1991). It has been shown in rats that mitochondrial ATP synthase inhibition adversely impacts mitochondrial membrane potential (Wang et al., 2012).

Mitochondrial calcium is a major player in the process of oxidative phosphorylation that allosterically activates various enzymes, including ATP synthase (Balaban, 2002). Ketamine, by antagonizing calcium-permeable NMDA receptors, can affect cellular calcium (Ca^{2+}) levels (O'Shaughnessy and Lodge, 1988). Entry of cytosolic Ca^{2+} ions into the mitochondria is facilitated by a number of transporters which also have a direct role in stimulating the production of ATP (Boerries et al., 2007). It is possible that ketamine inhibits ATP production by reducing intracellular Ca^{2+} levels. We have previously shown a link between ALCAR and ATP synthase in the reversal of ketamine toxicity potentially involving Ca^{2+} (Guo et al., 2017). In rat heart, ALCAR facilitates fatty acid oxidation to support ATP production (Hagen et al., 2002). Our previous studies have shown that ALCAR can completely prevent several aspects (neuronal and cardiac) of ketamine-induced toxicities (Cuevas et al., 2013; Guo et al., 2017; Kanungo et al., 2012; Robinson et al., 2016).

Mitochondrial ATP synthase is composed of two multi-subunit complexes: the soluble catalytic core, F1, and the membrane-spanning component, Fo, comprising the proton channel. The catalytic core (F1) of mitochondrial ATP synthase consists of five subunits (α , β , γ , δ , and ϵ) assembled with a stoichiometry of three α , three β , and a single representative of each of the other three subunits, whereas the proton channel (Fo) consists of three main subunits (A, B, and C) (Cross and Muller, 2004). As a follow up investigation to explore how ATP synthase function may have been altered by ketamine, our current study focused on the transcriptional status of two ATP synthase subunits of the catalytic core, *atp5a1* and *atp5b*. Ketamine down regulated *atp5a1*, but upregulated *atp5b*, and ALCAR co-treatment prevented those alterations, indicating that ketamine and ALCAR differentially regulate the expression of these two transcripts.

A correlation between ATP synthase and cardiac health has been reported in various studies. An iron overload in the hypertrophied rat heart mitochondria resulted in an increase in both ATP synthase α and β subunits (Kim et al., 2008). In neonatal rat cardiomyocytes, a non-steroidal anti-inflammatory drug valdecoxib, increased the ATP synthase β subunit (Baek et al., 2010). Chronic exposure to ethanol caused a decrease in both ATP synthase α and β subunits in rat liver mitochondria (Venkatraman et al., 2004) and ethanol exposure for one week decreased ATP synthase α in the brains of fetal mice (Sari et al., 2010). Taken together, these reports suggest that the expression of both α and β subunit polypeptides of ATP synthase is altered in various organs upon exposure to drugs that cause mitochondrial dysfunction. On the other hand, estradiol that protects the brain cells from bio-energetic dysfunction caused by conditions such as hypoxia, stroke, and degenerative disease by stimulating the energy-producing pathway (Nilsen and Brinton, 2004), induces the expression of ATP synthase α protein (Tamrakar et al., 2015). In this context, our current study shows altered expression of the transcripts for both subunits (*atp5a1* and *atp5b*) of ATP synthase upon exposure to ketamine in the absence and presence of ALCAR. Ketamine-induced upregulation of *atp5b* expression, potentially contributed to an upregulation of the ATP synthase protein levels. Whether this altered expression influenced ATP synthase activity is not known since our attempts to measure ATP synthase activity in the embryo homogenates did not produce detectable results. Mitochondrial isolation and activity measurement using the mitochondrial fraction would be necessary for the ATP synthase activity assay. ATP synthase can both synthesize ATP and degrade ATP when

deregulated (Buchert et al., 2012). Whether ketamine can deregulate ATP synthase activity needs to be investigated.

A decrease in ATP synthase activity is linked to failing hearts (Casademont and Miro, 2002; Long et al., 2015) and reduced energy supply contributes to initiation and progressive transition to congestive heart failure (Neubauer, 2007). ATP5 α may act as a redox sensor and thereby modulate the functions of ATP synthase (Wang et al., 2011). The role of redox-mediated posttranslational modifications of the ATP synthase complex is implicated in heart failure and may serve as a target for possible clinical intervention (Wang et al., 2013). The current study shows ketamine-induced structural changes in the heart, which are not observed in the untreated control and ALCAR-co-treated embryos. This structural change in the heart could underlie the reduced heart rate in ketamine-treated embryos seen in our previous studies (Guo et al., 2017; Kanungo et al., 2012), although a reduced heart rate could also precede abnormal heart development.

A link between mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling and ATP depletion in astrocytes has been shown to be mediated by a decrease in F1/F0 ATP synthase activity (Yung et al., 2004). Additionally, down regulated MAPK/ERK activity has been linked to ketamine-induced neurotoxicity in mice (Straiko et al., 2009). In human alveolar macrophages obtained from normal volunteers, inhibition of MAPK/ERK activity caused loss of mitochondrial membrane potential resulting in reduced ATP levels suggesting a role for MAPK/ERK in mitochondrial function (Monick et al., 2008). Mitochondrial functions have also been shown to be regulated by protein phosphorylation/dephosphorylation (Lim et al., 2016). Although MAPK/ERK is normally found in the cytosol and nucleus, reports have shown it to be localized to mitochondria in various organs such as mouse heart (Baines et al., 2002), renal epithelial cells (Nowak et al., 2006), hippocampus (Rumora et al., 2007) and human alveolar macrophages (Monick et al., 2008). Mitochondrial MAPK/ERK appears to be involved in mitochondrial function (Monick et al., 2008; Nowak et al., 2006) and dysfunction including mitophagy and apoptosis (Dagda et al., 2008; Hernandez-Resendiz and Zazueta, 2014; Zhuang et al., 2008). A potential mechanism for the differential effects of ketamine and ALCAR on MAPK/ERK in zebrafish was elucidated in our previous study (Kanungo et al., 2012). The current study indicates that ketamine-induced suppression of MAPK/ERK activity could be an upstream event of ATP depletion in zebrafish embryos.

Additional evidence linking MAPK/ERK to the expression of ATP synthase subunits was reported in studies using glioblastomas that highly expressed ATP5 α 1 and ATP5 β (Xu and Li, 2016) along with increased levels of activated MAPK/ERK (Popescu et al., 2016). Furthermore, ketamine reduced substance P-induced MAPK/ERK activity in human glioblastoma cells (Yamaguchi et al., 2017). These studies laid the ground work for a potential link between ketamine's ability to alter the expression of *atp5 α 1* and *atp5 β* and the modulation of MAPK/ERK signaling (Fig. 7). The current study shows that ketamine indeed down-regulated *atp5 α 1* expression, which was prevented by ALCAR co-treatment. However, ketamine up-regulated *ATP5 β* expression, which was also prevented by ALCAR co-treatment. It is possible that these two genes are differentially regulated by MAPK/ERK (Fig. 7). We speculate that the altered expression of these two ATP synthase subunits by

ketamine could negatively affect ATP synthase activity leading to reduced ATP synthesis, although the total ATP synthase protein levels were higher in ketamine-treated embryos, whereas ALCAR prevented these adverse effects (Fig. 7). The other possibility is, since ATP synthesis and hydrolysis are both carried out by ATP synthase with specific subunit configurations (Adachi et al., 2007), disproportionate subunit expressions could adversely affect ATP synthesis by enhancing ATP hydrolysis. Further work is needed to analyze these distinct functions of ATP synthase in ketamine-treated embryos. In conclusion, the current findings are consistent with reported adverse effects of ketamine on the mitochondria and ATP production in human cells (Baker et al., 2016; Chang et al., 2009; Ito et al., 2015), dysfunctional cardiac function in infant non-human primates (Hotchkiss et al., 2007) and newborn human infants (Saarenmaa et al., 2001), additionally providing mechanistic data that show ALCAR as a protectant against these adverse effects.

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Fig. 1.

Experimental design using zebrafish embryos to study the effects of ketamine and ALCAR on the mitochondrial function. Manually dechorionated embryos at 28 hpf were exposed for 20 h to ketamine and in the presence or absence of ALCAR. Post-exposure, the embryos (actual age 48 hpf) were analyzed for various experiments including assessment of ATP content, mitochondrial potential, ATP synthase gene expression and heart morphology.

Fig. 2.

Effect of ketamine and ALCAR on the ATP levels in zebrafish embryos. Manually dechorionated embryos at 28 hpf were treated with 2 mM ketamine or 2 mM ketamine plus 1.0 mM ALCAR alongside the control untreated group. Exposure was for 20 h (static exposure). Total ATP contents of the embryos were measured for each group and the values were normalized against total protein content. Data from three separate experiments were averaged and shown as mean $\pm$ SD. Significance was set at $P < 0.05$ (*).

Fig. 3.

Effect of ketamine and ALCAR on the mitochondria. Manually dechorionated embryos at 28 hpf were treated with 2 mM ketamine or 2 mM ketamine plus 1.0 mM ALCAR alongside the control untreated group. Exposure was for 20 h (static exposure). (A) Total mitochondrial proteins and (B) mitochondrial membrane potential were measured for each group. The experiment was repeated three times. Data are shown as mean $\pm$ SD. Significance was set at $P < 0.05$ (*).

Fig. 4.

Effect of ketamine and ALCAR on ATP synthase gene expression. Manually dechorionated embryos at 28 hpf were treated with 2 mM ketamine or 2 mM ketamine plus 1.0 mM ALCAR alongside the control untreated group. Exposure was for 20 h (static exposure). Relative expression of the two subunits of the catalytic core of ATP synthase, *atp5 α 1* (A) and *atp5 β* (B) is presented following qRT-PCR assessment of the cDNA synthesized from total RNA isolated from these larvae. Data are presented as mean $\pm$ SD. Statistical significance ($P < 0.05$) is indicated (*).

Fig. 5.

Effect of ketamine and ALCAR on ATP synthase protein levels. Manually dechorionated embryos at 28 hpf were treated with 2 mM ketamine or 2 mM ketamine plus 1.0 mM ALCAR alongside the control untreated group. Exposure was for 20 h (static exposure). Relative levels of ATP synthase protein were measured for each group and the values are expressed as OD (optical density). The experiment was repeated three times. Data are shown as mean $\pm$ SD. Significance was set at $P < 0.05$ (*).

Fig. 6.

Effect of ketamine and ALCAR on the developing heart in the zebrafish embryos. Manually dechorionated embryos at 28 hpf were treated with 2 mM ketamine or 2 mM ketamine plus 1.0 mM ALCAR alongside the control untreated group. Exposure was for 20 h (static exposure). Hearts of the live embryos were imaged. The ventricle (arrows) and auricle (arrowhead) are shown in the images of Control (A, D), ketamine-treated (B, E) and ketamine/ALCAR co-treated (C, F) embryos. Yolk sac is indicated. Magnified images of the heart are shown in D–F. The solid red outline shows the heart with the upper ventricle (v) and lower auricle (a) and the dotted red line shows the looping point of the heart, which is absent in the ketamine-treated embryos (E). Scale bar (A–C) = 2 mm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 7.

Schematic presentation of the effects of ketamine and ALCAR on mitochondrial function. Ketamine by reducing cellular calcium via its antagonism of the Ca^{2+} -permeable NMDA receptors can suppress MAPK/ERK activity, which may be responsible for altered expression of the two catalytic subunits of ATP synthase, *atp5α1* and *atp5β*. ATP synthase is known to modulate mitochondrial membrane potential. ALCAR, on the other hand known to normalize ketamine-induced reduction in MAPK/ERK activity could therefore counteract the altered expression of *atp5α1* and *atp5β*, thus restoring normal mitochondrial function and ATP generation.

Table 1

List of primers used in real-time quantitative polymerase chain reaction (RT-qPCR) assays.

Gene	Forward primer	Reverse primer
<i>GAPDH</i>	5'-GATACACGGAGCACCAGGT-3'	5'-GCCATCAGGTCACATACACG-3'
<i>atp5a1</i>	5'-GCTTTCGCTCAGTTTGGTTC-3'	5'-AAGGCCTTCTCGAATTGTT-3'
<i>atp5β</i>	5'-GACATGAGTGTCTGAGCAGGA-3'	5'-ACGGAGTAACCACCATGAGC-3'