Published in final edited form as:

J Appl Toxicol. 2020 February ; 40(2): 257–269. doi:10.1002/jat.3901.

Nifedipine toxicity is exacerbated by acetyl L-carnitine but alleviated by low-dose ketamine in zebrafish in vivo

Bonnie L. Robinson¹, Qiang Gu¹, Volodymyr Tryndyak², Syed F. Ali¹, Melanie Dumas³, Jyotshna Kanungo¹

¹Division of Neurotoxicology, US Food and Drug Administration, Jefferson, Arkansas

²Division of Biochemical Toxicology, National Center for Toxicological Research, US Food and Drug Administration, Jefferson, Arkansas

³The Bionetics Corporation, Jefferson, Arkansas

Abstract

Calcium channel blocker (CCB) poisoning is a common and sometimes life-threatening emergency. Our previous studies have shown that acetyl L-carnitine (ALCAR) prevents cardiotoxicity and developmental toxicity induced by verapamil, a CCB used to treat patients with hypertension. Here, we tested whether toxicities of nifedipine, a dihydropyridine CCB used to treat hypertension, can also be mitigated by co-treatment with ALCAR. In the zebrafish embryos at three different developmental stages, nifedipine induced developmental toxicity with pericardial sac edema in a dose-dependent manner, which were surprisingly exacerbated with ALCAR co-treatment. Even with low-dose nifedipine (5 μ M), when the pericardial sac looked normal, ALCAR co-treatment showed pericardial sac edema. We hypothesized that toxicity by nifedipine, a vasodilator, may be prevented by ketamine, a known vasoconstrictor. Nifedipine toxicity in the embryos was effectively prevented by co-treatment with low (subanesthetic) doses (25–100 μ M added to the water) of ketamine, although a high dose of ketamine (2 mM added to the water) partially prevented the toxicity. As expected of a CCB, nifedipine either in the presence or absence of ketamine-reduced metabolic reactive oxygen species (ROS), a downstream product of calcium signaling, in the rapidly developing digestive system. However, nifedipine induced ROS in the trunk region that showed significantly stunted growth indicating that the tissues under stress potentially produced pathologic ROS. To the best of our knowledge, these studies for the first time show that nifedipine and the dietary supplement ALCAR together induce adverse effects while providing evidence on the therapeutic efficacy of subanesthetic doses of ketamine against nifedipine toxicity in vivo.

Keywords

acetyl L-carnitine; atropine; ketamine; nifedipine; ROS; developmental toxicity; zebrafish

Correspondence: Jyotshna Kanungo, Division of Neurotoxicology, National Center for Toxicological Research, US Food and Drug Administration, 3900 NCTR Road, Jefferson, AR 72079, USA, jyotshnabala.kanungo@fda.hhs.gov.

CONFLICT OF INTEREST

The authors have no conflict of interest to report.

1 | INTRODUCTION

Nifedipine, of the dihydropyridine subclass, is a calcium channel blocker (CCB) (Khan & Schaefer, 2019). CCBs have become very common therapeutics for hypertension and cardiovascular diseases (reviewed in Chakraborty & Hamilton, 2019). They are also popular in veterinary medicine (Hayes, 2018). CCBs inhibit the voltage-gated L-type calcium channels resulting in reduced intracellular calcium levels and accidental CCB overdose alone has been reported to induce severe toxicity and even mortality (reviewed in Chakraborty & Hamilton, 2019). Additionally, CCB toxicity can occur due to drug-drug interactions with other medications (Jamerson et al., 2008). Such toxicities often cause severe hemodynamic changes leading to fatalities (Dolphin, 2018). According to a report in 2011 by the American Poison Control Centers, cardiovascular drugs are the third highest among drug exposures that resulted in 11 764 CCB overdoses in adults, second only to beta-blocker overdoses (Dolphin, 2018). CCB toxicities include myocardial depression, dizziness due to hypotension, lethargy, seizures and pulmonary edema (Herrington, Insley, & Weinmann, 1986; Ramoska, Spiller, Winter, & Borys, 1993). An overdose of CCBs can also cause hyperglycemia, nausea, vomiting, atrioventricular block and cardiovascular collapse among other symptoms (Herrington et al., 1986; Ramoska et al., 1993).

Therapeutic CCBs include a diverse group of chemicals (reviewed in Chakraborty & Hamilton, 2019). Based on their structures, they are classified as diphenylalkylamines (e.g., verapamil), benzothiazepines (e.g., diltiazem) and dihydropyridines (e.g., nifedipine, amlodipine, nicardipine and nimodipine). Their binding sites on the L-type calcium channel are also distinct. Functionally, verapamil shows a strong effect on both cardiac myocytes and the vascular smooth muscle cells. Thus, verapamil suppresses cardiac contractility leading to reduced heart rate. It also depresses conduction of the sinoatrial and atrioventricular nodes resulting in vasodilation. Although dihydropyridines, such as nifedipine, cause potent vasodilation, they do not directly affect myocardial contractility (Kline, Raymond, Schroeder, & Watts, 1997; Ramoska et al., 1993; Ramoska, Spiller, & Myers, 1990).

By inhibiting L-type calcium channels in the pancreatic islet cells, CCBs reduce insulin secretion causing reduced glucose utilization by the heart (Graudins, Lee, & Druda, 2016). Along with bradycardia and hypotension, metabolic acidosis and vasodilatory shock are also caused by CCBs (DeWitt & Waksman, 2004). In vivo studies show that vasodilation induced by nifedipine is relatively greater than that produced by other CCBs (Beaughard, Michelin, Tisne-Versailles, & Lamar, 1986). Relative potency of the vasodilation produced by CCBs in isolated vessels has been shown to be highest in nifedipine followed by verapamil and diltiazem (Soward, Vanhaleweyk, & Serruys, 1986). The major difference between these three CCBs is that both verapamil and diltiazem induce bradycardia and hypotension, whereas nifedipine induces not only hypotension but also reflex sinus tachycardia (Proano, Chiang, & Wang, 1995).

In pharmacologic therapy for CCB toxicity, atropine is often considered the drug of choice initially to treat bradycardia by increasing heart rate; however, although effective in mild to moderate CCB toxicity, atropine is ineffective in severe CCB overdoses (reviewed in Chakraborty & Hamilton, 2019). Other therapeutic interventions in CCB toxicity include

treatment with methylene blue that inhibits guanylate cyclase and thus nitric oxide synthesis (Grunbaum, Gilfix, Gosselin, & Blank, 2012; Jang et al., 2015), lipid emulsion therapy to sustain a fatty acid energy source (Levine, Curry, Padilla-Jones, & Ruha, 2013), glucagon that improves heart rate and cardiac output (Jang, Spyres, Fox, & Manini, 2014), insulin (Holger, Engebretsen, Stellpflug, Cole, & Kerns, 2014), and catecholamines against vasodilatory shock (Jang et al., 2014).

Zebrafish (*Danio rerio*) are an ideal model system for studies on embryonic development as well as human disease modeling and health research (Ablain & Zon, 2013). Advantages of using the zebrafish model system are due to their physiological, genetic and functional similarity with higher-order vertebrates, including humans. Extensive studies on zebrafish genetics, anatomy and physiological processes have been useful in toxicological studies. Additionally, high fecundity of zebrafish and embryo transparency for in vivo visualization and monitoring of development, organogenesis and functions of certain organs have been instrumental in successfully using this model system. More importantly, rapid development of the zebrafish embryos, a unique advantage compared with higher-order vertebrates, makes it feasible to conduct acute toxicity studies and delineate potential mechanisms. For example, while the duration of the first trimester of the human embryonic development lasts ~10 weeks, the zebrafish embryonic period ends at 72 hours (Wilson, 2012).

CCBs are used to treat both adult and pediatric conditions, such as angina, hypertension, arrhythmias and Raynaud disease (Arroyo & Kao, 2009). Effective therapy against CCB toxicity is not well established (Salhanick & Shannon, 2003), particularly for children (Belson, Gorman, Sullivan, & Geller, 2000; Euwema & Swanson, 2019). We have previously shown acetyl L-carnitine (ALCAR) to be effective against calcium antagonists (verapamil, cyclosporine A and ketamine) in the zebrafish embryos, in which we were able to delineate a number of mechanisms based on the phenotypes produced upon drug treatments (Cuevas et al., 2013; Guo et al., 2017; Kanungo, Cuevas, Ali, & Paule, 2012; B. Robinson, Dumas, Gu, & Kanungo, 2018; B. Robinson, Gu, Ali, Dumas, & Kanungo, 2019; B. L. Robinson et al., 2017; B. L. Robinson et al., 2016). In continuation, we explored whether ALCAR can alleviate nifedipine toxicity as it did against toxicities induced by calcium antagonists, such as verapamil, cyclosporine A and ketamine.

2 | MATERIALS AND METHODS

2.1 | Animals (zebrafish)

Adult wild-type zebrafish (*Danio rerio*, AB strain) were purchased from the Zebrafish International Resource Center (www.zirc.org). The fish were housed in 3-L tanks (Aquatic Habitats) at the National Center for Toxicological Research/Food and Drug Administration zebrafish facility containing buffered water (pH 7.5) at 28.5°C. In total, eight adult females or males were kept in each tank. Handling and maintenance of zebrafish followed the NIH Guide for the Care and Use of Laboratory Animals and the zebrafish care protocol was approved by the National Center for Toxicological Research/Food and Drug Administration Institutional Animal Care and Use Committee. Under a day/night cycle at 14:10 hours, adult fish were fed twice daily with Zeigler dried flake food (Zeiglers) and once with brine shrimp. In the in-system breeding tanks, crosses of two to three males per one female were

set up the previous day with partitions keeping the sexes separate. The partitions were taken off the following morning at the time of light onset to stimulate spawning and fertilization. Fertilized eggs were collected from the bottom of the tank in the system water (temperature 28.5°C, pH 7.5, dissolved oxygen 7.0, conductivity 800 μ S) containing methylene blue, placed in Petri dishes after being washed thoroughly in the system water and transferred to the incubator. For experimental set-up, egg water (reverse osmosis water containing 60 mg sea salt [Crystal Sea®; Aquatic Eco-systems, Inc.] per liter of water [pH 7.5]) was used and the eggs/embryos were kept in an incubator at 28.5°C to develop. The embryos were killed after the completion of experiments was performed according to the procedure in our approved Institutional Animal Care and Use Committee protocol. Briefly, embryos will be anesthetized first by prolonged treatment (>10 minutes) of tricaine by adding buffered tricaine (4% stock solution) to the water containing the embryos to a final concentration of 0.04% (<http://oacu.od.nih.gov/ARAC/documents/Zebrafish.pdf>).

2.2 | Reagents

Ketamine hydrochloride (10 mg/mL vials) (product no. VINV-KETA-0VED) was purchased from Vedco, Inc. ALCAR (cat. no. A6706), 2',7'-dichlorodihydrofluorescein diacetate (H2DCF-DA) (cat. no. D6883), nifedipine (N7634), atropine (A0132) and other reagents were purchased from Sigma unless stated otherwise.

2.3 | Treatment of zebrafish embryos with various compounds

In the first set of experiments, exposure of zebrafish embryos ($n = 10$ /dose group) to nifedipine at different doses (0, 2.5, 5, 10, 25 or 50 μ M) began at 6 hours postfertilization (hpf) and continued until 72 hpf. In the second set of experiments, 28 hpf embryos ($n = 10$ /dose group) were exposed to 5–50 μ M nifedipine for 20 hours. From these experiments, a highly toxic dose (25 μ M) was chosen to induce similar toxicity in both 28 hours and 52 hpf embryos, so that efforts could be made to reverse the adverse effects. We chose 28 and 52 hpf embryos as our previous studies showed that these embryos as whole organisms respond to ketamine anesthesia (Cuevas et al., 2013; Kanungo et al., 2012) and are therefore more appropriate to exhibit a specific response to other drugs. Embryos at 28 and 52 hpf have also been successfully used for drug efficacy studies by others (Morash et al., 2011). For each experiment, eggs laid at the same time from several females were pooled. For each treatment, 10 embryos were placed in one of the wells of the six-well plates containing 5 mL buffered egg water. At the specific experimental time points, body length and hatching rates were recorded. The experiments were reproduced at least three times. When using 28 and 52 hpf embryos, nifedipine treatment continued for 20 hours (static exposures) in the presence or absence of ALCAR (1 mM), ketamine (25, 50, 100 μ M or 2 mM) or atropine (0.5, 5, 10 or 50 μ M). Untreated control groups were examined in parallel.

2.4 | Scoring hatching rate and survival, and body length measurement

Live images of the embryos in the six-well plates, used to treat the embryos with the drugs, were acquired using an Olympus SZX 16 binocular microscope and DP72 camera. The number of embryos/larvae still alive and the number of larvae that had hatched out of the chorion were scored at 72 hpf. The data are presented as the percentage of the control (100%). Data for each group were used to calculate the mean and standard deviation. Body

lengths were measured following procedures described in our previous studies (Guo et al., 2017; Robinson, Dumas, Gu, et al., 2018). The statistical significance of the effects of the various treatments on hatching rate, survival and body length was determined by one-way ANOVA (Sigma Stat) using Holm-Sidak pairwise multiple comparison post-hoc analysis. Statistical significance was set at $P < .05$.

2.5 | Measurement of the pericardial sac area

Live images of the embryos were acquired using an Olympus SZX 16 binocular microscope and DP72 camera. The extent of pericardial sac edema in the embryos was determined by quantitation of the pericardial sac area (Carney, Peterson, & Heideman, 2004; B. L. Robinson et al., 2017). First, the pericardial sac area was outlined in lateral view images of the embryos and the area was quantitated using the DP2 BSW microscope digital camera software (Olympus). The statistical significance of the effects of the various treatments of the pericardial edema was determined by one-way ANOVA (Sigma Stat) using Holm-Sidak pairwise multiple comparison post-hoc analysis. Statistical significance was set at $P < .05$.

2.6 | Detection of reactive oxygen species in the larvae in vivo

The detection of reactive oxygen species (ROS) in the zebrafish larvae was performed by fluorescent microscopy as described previously (B. Robinson, Gu, Ali, Dumas, Kanungo, 2019). A cell membrane-permeable probe, H2DCF-DA fluorescent dye, was used to detect ROS in vivo. H2DCF-DA is a permanent ROS marker (Chen, Zhong, Xu, Chen, & Wang, 2010). When deacetylated by cellular esterases, H2DCF-DA is converted to non-fluorescent 2',7'-dichlorofluorescein. 2',7'-Dichlorofluorescein, in the presence of cellular ROS, is converted to the green fluorescent dichlorofluorescein (DCF). Fluorescence of DCF is captured by fluorescence microscopy. In brief, larvae were treated with H2DCF-DA for 30 minutes in the dark at 28.5°C. The fluorescence of the whole larva was captured using a DP2 BSW fluorescence microscope (Olympus).

3 | RESULTS

3.1 | Nifedipine-induced developmental toxicity in early stage embryos is dose dependent

To assess nifedipine toxicity, zebrafish early embryos (6 hpf) were treated with 2.5, 5, 12.5, 25 or 50 μM nifedipine. Static exposure continued until the larval stage (72 hpf) (Figure 1). Compared with the control, there were no clear morphological changes in the larvae exposed to 2.5 or 5 μM nifedipine (Figure 1A). However, nifedipine at 10, 25 and 50 μM doses showed gradual increase in developmental arrest as evidenced by changes in body length and hatching rate (Figure 1B and 1C). Compared with the control, larvae treated with 25 or 50 μM nifedipine had significantly reduced body lengths ($P < .001$) and hatching rate began to decline gradually from 10 to 50 μM nifedipine-treated larvae ($P < .001$). These results indicated that nifedipine doses at 10 μM and higher are developmentally toxic in zebrafish embryos.

3.2 | Nifedipine-induced developmental toxicity in late-stage embryos

To determine whether nifedipine also induces similar toxicities in the 1-day-old embryos, 28 hpf were treated with various doses (5–50 μM) of nifedipine for 20 hours. Measurement of

body lengths was the endpoint to assess overall growth. The data showed that nifedipine reduced growth of the 48 hpf embryos (Figure 2). Statistically significant reduction in body length was apparent in embryos treated with 25 and 50 μM nifedipine. The pericardial sac edema (marked by an asterisk) and stronger adverse effect of nifedipine on growth were seen in the 25 and 50 μM groups (Figure 2A and 2B). These data suggest that the effects of nifedipine are consistent through different stages of embryos and the dose-dependent effects at all age groups tested are specific.

3.3 | Acetyl L-carnitine exacerbates nifedipine toxicity

Our previous studies have shown that ALCAR prevents developmental toxicity, cardiotoxicity and neurotoxicity induced by the calcium antagonists, such as ketamine, verapamil, cyclosporine A and TMB-8 (Cuevas et al., 2013; Guo et al., 2017; Kanungo et al., 2012; B. L. Robinson, Dumas, Paule, Ali, & Kanungo, 2015). Here, we used ALCAR to prevent nifedipine (an L-type CCB) toxicity believing that the same phenomenon (ability of ALCAR to activate L-type CCBs) would work against nifedipine toxicity. We used the 52 hpf embryos as these embryos not only express one of the major drug metabolizing enzymes (CYP3A65, an ortholog of the mammalian CYP3A4), but also modulate the expression of this enzyme upon drug exposure (B. L. Robinson et al., 2017). Surprisingly, 1 mM ALCAR co-treatment exacerbated nifedipine toxicity at its highest dose (50 μM) (Figure 3A). The most adverse effect was detected at the pericardial sac area and quantification of the edematous pericardial sac area showed that the adverse effect of nifedipine was exacerbated by ALCAR (** $P < .001$) (Figure 3B). As body length was severely affected by nifedipine, a ratio of pericardial sac area to body length is also shown (Figure 3C).

After establishing that nifedipine and ALCAR co-treatment is more toxic than nifedipine treatment alone, we tested whether lowering nifedipine doses could prevent the occurrence of ALCAR and nifedipine combination toxicity. For this purpose, we used 52 hpf embryos for a 20-hour exposure as these embryos express the drug metabolizing enzyme CYP3A65, an ortholog of the mammalian CYP3A4. We reduced nifedipine doses to 5 and 10 μM to evaluate whether the mild toxicities by both doses could be prevented by 1 mM ALCAR. Surprisingly, ALCAR still accentuated the effects that were not observed in the nifedipine treatments alone (Figure 3D). Compared with the control, the most obvious deformity at the pericardial sac area was significantly exacerbated by ALCAR (** $P < .001$) (Figure 3E).

As our previous studies showed counteracting effects of ALCAR and ketamine (Cuevas et al., 2013; Guo et al., 2017; Kanungo et al., 2012; Robinson et al., 2018; B. L. Robinson et al., 2016; B. L. Robinson et al., 2015), we hypothesized that if ALCAR exacerbates the effect of nifedipine, ketamine would alleviate it. In the presence of 2 mM ketamine in water, which we have used in our previous studies (Guo et al., 2017; Kanungo et al., 2012; Kanungo, Cuevas, Ali, & Paule, 2013; Robinson, Dumas, Gu, et al., 2018; B. L. Robinson et al., 2017; Robinson, Dumas, Ali, et al., 2018; B. L. Robinson et al., 2016; Trickler et al., 2013) that gives an internal dose of $\sim 8.4 \mu\text{M}$ in the zebrafish embryos (Trickler et al., 2013), nifedipine toxicity appeared to be milder (Figure 4). Nifedipine-induced axial curvature was no more apparent in ketamine and nifedipine co-treated embryos (Figure 4). However, these embryos, in the presence of ALCAR, exhibited a stunted body with axial curvature

suggesting that ALCAR exacerbated nifedipine toxicity even in the presence of ketamine (Figure 4).

3.4 | Atropine was ineffective against nifedipine toxicity

To determine whether atropine, a drug of choice to treat moderate but not severe nifedipine toxicity, can alleviate the adverse effects, 52 hpf were co-treated with various doses (0.5–50 μM) of atropine for 20 hours. Nifedipine at 25 μM significantly reduced growth of the 72 hpf larvae (** $P < .001$) (Figure 5A and 5B). A statistically significant reduction in body length (** $P < .001$) was apparent in larvae co-treated with 25 μM nifedipine and atropine with no improvements over embryos treated only with nifedipine. Atropine at 50 μM had no effect on the embryos. These data suggest that nifedipine toxicity in these embryos were too severe to respond to atropine treatment.

3.5 | Ketamine alleviates nifedipine toxicity

Of all the therapeutic approaches for nifedipine toxicity hitherto described in the literature (e.g., atropine), ketamine is not one of them. Nifedipine, which is a vasodilator, blocks the voltage-gated L-type calcium channels, whereas ketamine blocks the calcium-permeable *N*-methyl-D-aspartate receptors and is a vasoconstrictor (Park et al., 2016). We hypothesized that the vasodilatory shock of nifedipine (Smith, Ayon, Tang, Makino, & Yuan, 2016) would be counteracted by subanesthetic doses of ketamine resulting in the reversal of the nifedipine-induced developmental toxicity. We used 52 hpf embryos with a 20-hour co-treatment with 25 μM nifedipine. We chose 25 μM nifedipine, a dose that induced significant adverse effects and these effects were not mitigated by various doses (0.5–50 μM) of atropine (Figure 5). However, ketamine at 25–50 μM doses showed significant improvement ($P < .001$) from nifedipine-induced developmental toxicity (Figure 6A and 6B). Specifically, the nifedipine-induced reduction in body length was restored with 25–100 μM ketamine co-treatment (Figure 6A and 6B); however, there was ~30% mortality in the nifedipine and 100 μM ketamine co-treatment group (Figure 6C). These results indicate that in subanesthetic concentrations, the vasoconstrictive effects of ketamine may be counteracting the vasodilatory shock of nifedipine.

3.6 | Ketamine prevents nifedipine-induced abnormal reactive oxygen species in the trunk muscles

To determine whether ROS that are generated downstream of calcium signaling did play any role in the drug effects in the zebrafish embryos, we stained the embryos with dichlorofluorescein diacetate. The data showed that in the 72 hpf larvae (treated for 20 hours at 52 hpf), ROS in the digestive tract (particularly the intestine) was present in the control and embryos treated with 25 and 50 μM ketamine, but not in the nifedipine-treated larvae in the presence or absence of ketamine (Figure 7). Most importantly, in the absence of ketamine, ROS was present in the trunk myotomes in the nifedipine-treated larvae (Figure 7 and Table 1). These data indicated that although nifedipine in the presence or absence of ketamine reduced metabolic ROS predominantly visible in the rapidly developing digestive system (indicated by an asterisk), the stunted trunk myotomes under duress potentially produced pathologic ROS (arrowheads) (Figure 7 and Table 1).

4 | DISCUSSION

The current study showed that the CCB nifedipine stunted the development of the zebrafish embryo body axis that is made up of musculoskeletal components (reviewed in Holley & Nusslein-Volhard, 2000). Reduced intracellular calcium by nifedipine and other CCBs has been shown to inhibit skeletal myoblast differentiation into mature myotubes in vitro (Porter, Makuck, & Rivkees, 2002) and skeletal growth in rabbits in vivo (Danielsson, Danielson, Rundqvist, & Reiland, 1992; Duriez, Flautre, Blary, & Hardouin, 1993). Calcium signaling is essential for body axis development in vertebrates including zebrafish (Langenbacher & Chen, 2008). Additionally, calcium signaling plays a critical role in the differentiation and proper development of embryonic muscle (Berchtold, Brinkmeier, & Muntener, 2000; Ferrari, Podugu, & Eskew, 2006; Fujita, Nedachi, & Kanzaki, 2007; Webb, Cheung, Chan, Love, & Miller, 2012; Webb & Miller, 2011). The calcium antagonism of nifedipine may have played a role in bringing about the disrupted muscle and bone development resulting in a stunted body axis in the zebrafish embryos.

In case of CCB overdose, intravenous calcium infusion is used (Salhanick & Shannon, 2003). Although cardiovascular toxicity by some CCBs is manifested by bradycardia and tachycardia, nifedipine, a vasodilator, does not have a direct effect on the heart but can induce reflex tachycardia (Botta et al., 2018). Guidelines of the Society of Critical Care Medicine recommends the use of catecholamines, such as norepinephrine or epinephrine, particularly norepinephrine to treat vasodilatory shock (Graudins et al., 2016). Treatment of drug-induced cardiovascular toxicity needs intervention in restoring the hemodynamic status. In this study, we provide evidence that vasoconstrictors may relieve nifedipine toxicity (Figure 8). Ketamine (10–100 μ M) has been shown to be a vasoconstrictor in isolated rat arteries (Park et al., 2016). Ketamine can also cause vasoconstriction in patients (Spotof, Korshin, Sorensen, & Skovsted, 1979). Zebrafish embryos need 0.5 mM ketamine at a minimum in water to be anesthetized (our unpublished data). In the current study, we used low subanesthetic doses (25–100 μ M added to water) that do not have any effect on the zebrafish embryo heart rate (data not shown), as our earlier studies showed that 0.5 mM ketamine does not have any effect on the 52 hpf embryo heart rates after 2 hours of treatment (Kanungo et al., 2012). Interestingly, these low doses of ketamine, which possibly had mild vasoconstrictive properties, alleviated nifedipine-induced severe developmental toxicity.

First, using 2 mM ketamine and 1 mM ALCAR doses, which we have used in many of our previous studies (Guo et al., 2017; Kanungo et al., 2012, 2013; Robinson et al., 2016; Robinson et al., 2017; Robinson, Dumas, Ali, et al., 2018; Robinson, Dumas, Gu, et al., 2018; Robinson, Gu, Ali, Dumas, & Kanungo, 2019; Trickler et al., 2013), we discovered that, in the 28 hpf embryos, 2 mM ketamine with 50 μ M nifedipine was less toxic than 50 μ M nifedipine alone. Surprisingly, embryos treated with 50 μ M nifedipine and 2 mM ketamine showed a better body axis than the embryos treated only with 50 μ M nifedipine. However, ALCAR and nifedipine were more toxic together than either of the drugs alone in these embryos. The exacerbating effect of ALCAR towards nifedipine toxicity was further confirmed when in the 52 hpf embryos treated for 20 hours, even non-toxic (5 μ M) or mildly toxic (10 μ M) doses of nifedipine became very toxic in the presence of ALCAR as evidenced

by pericardial sac edema. In the zebrafish embryos, we have previously shown that ALCAR prevents toxicities induced by verapamil, another CCB (Guo et al., 2017). As nifedipine and verapamil are structurally-different CCBs, the inability of ALCAR to prevent nifedipine toxicity indicates that in the presence of nifedipine, the ability of ALCAR to activate the L-type calcium channel (Tewari, Simard, Peng, Werrbach-Perez, & Perez-Polo, 1995) was possibly lost. Another reason could be the potential vasodilatory property of ALCAR (McMackin et al., 2007), which would exacerbate the effect of nifedipine.

One striking observation was that nifedipine, 2 mM ketamine and ALCAR in combination were more toxic than either of the drugs alone indicating that ALCAR does not prevent toxicity induced by nifedipine alone or nifedipine and ketamine together. Therefore, we concluded that if the ketamine dose could be appropriately adjusted, it might be possible to counteract nifedipine toxicity to a greater extent. In the 52 hpf embryos, first, we used 1–10 μM ketamine for 20 hours to reverse nifedipine toxicity. However, the results showed no significant improvement on the nifedipine toxicity exhibited by the embryos (data not shown). We further increased ketamine doses to 25–100 μM . At these doses, the embryo development that was stunted with only nifedipine treatment showed improvement. From the overall developmental aspects, 25 μM ketamine could improve nifedipine toxicity effectively with no pericardial sac edema and axial curvature. At a 100- μM dose, ketamine induced mortality in a few (20%) of the nifedipine co-treated embryos. These results indicated that careful titration of doses of drugs having counteracting effects on the cardiovascular system can lead to alleviation of toxic effects by one of the drugs alone. Here, at a lower dose (25 μM), ketamine did not have any negative effect on the heart. It could therefore stabilize the heart rate when nifedipine was about to induce reflex tachycardia. We tried to monitor the heart rate from time to time to detect nifedipine-induced reflex tachycardia but to no avail, which suggests that the tachycardic episodes may be very subtle and not continuous; however, they were enough to induce cardiotoxicity as reflected by pericardial sac edema.

Our current study showed that metabolic ROS, a downstream product of calcium signaling (B. Robinson, Gu, Ali, Dumas, Kanungo, 2019), was reduced in the rapidly developing intestine in the nifedipine-treated embryos in the presence or absence of ketamine. Ketamine at lower doses (25–50 μM) did not have any effect on the intestinal metabolic ROS as opposed to 0.5–2 mM ketamine, which can significantly reduce intestinal ROS (B. Robinson, Gu, Ali, Dumas, Kanungo, 2019). In contrast to metabolic ROS, there was some pathologic (abnormal) ROS production induced by nifedipine in the trunk region. These findings suggest that while nifedipine inhibits metabolic ROS production, it may induce disruption of tissues that may be deprived of circulating blood due to severe vasodilation and thus under stress could produce pathologic ROS. Besides, muscle development requires calcium signaling (Webb et al., 2012; Webb & Miller, 2011) that could have been inhibited by nifedipine at the dose we used in this study. We used low doses of ketamine that may not have interfered with calcium signaling; therefore, resulting in no change in metabolic ROS production.

Dihydropyridine CCBs (e.g., nifedipine, amlodipine and nicardipine) are strong vasodilators with very little negative effect on cardiac conduction and contractility, whereas verapamil and diltiazem, both non-dihydropyridine CCBs, are weak vasodilators but adversely affect

cardiac function (Katz, 1993). Owing to the difference of their affinity for specific target organs, while verapamil (affinity for heart) toxicity was reversed (Guo et al., 2017), nifedipine (affinity for arterial vascular smooth muscle) toxicity, however, was exacerbated by ALCAR, a cardioprotective dietary supplement (Hagen, Moreau, Suh, & Visioli, 2002). Under physiological conditions, L-carnitine is involved in the transport of long-chain fatty acids from the cell cytoplasm to the mitochondria for ATP synthesis, which is the major energy source for the heart (Lango, Smolenski, Narkiewicz, Suchorzewska, & Lysiak-Szydlowska, 2001). It is possible that ALCAR, a derivative of L-carnitine, could exacerbate the tachycardic effect of nifedipine, thereby manifesting the effect in pericardial sac edema. Low doses of ketamine did not have any effect on development and heart rate but could potentially act as vasoconstrictors (Park et al., 2016) thus counteracting the vasodilation-mediated adverse effects of nifedipine (Figure 8).

Although the literature search did not reveal any report on drug-drug interaction between ALCAR and nifedipine, our findings raise concern on potential adverse effects when these compounds are consumed together. In an isolated case study, although L-carnitine was beneficial in a patient with amlodipine (another CCB) toxicity (St-Onge et al., 2014), L-carnitine is not indicated in treating CCB or beta blocker toxicity (Palatnick & Jelic, 2014). The likely reason could be that ALCAR is a vasodilator (McMackin et al., 2007) and related compounds, L-carnitine and propionyl-L-carnitine, are also known vasodilators (Cipolla, Nicoloff, Rebello, Amato, & Porter, 1999; Guclu et al., 2013) that may have the potential to exacerbate nifedipine toxicity. All these carnitines could possibly cause vasodilation in a manner that is vascular smooth muscle-independent as propionyl-L-carnitine-induced vasodilation is endothelium-dependent (Cipolla et al., 1999). On the other hand, the vasodilatory effect of nifedipine is through calcium blockage in the vascular smooth muscle cells (reviewed in Chakraborty & Hamilton, 2019). These independently activated pathways could synergize to exacerbate vasodilation (Figure 8).

While many other countermeasure options to treat nifedipine toxicity are available, ketamine may not be the drug of choice to treat the condition. Atropine is often considered the drug of choice initially to treat mild to moderate CCB toxicity; however, atropine is ineffective in severe CCB overdoses (Chakraborty & Hamilton, 2019). Atropine was ineffective in mitigating nifedipine toxicity in our study indicating that at a dose of 25 μM , nifedipine-induced vasodilation in the zebrafish embryos was severe. In conclusion, our *in vivo* studies show, to the best of our knowledge, for the first time, that vasoconstrictors, such as low subanesthetic doses of ketamine, can alleviate severe nifedipine toxicity when the first-line therapy with atropine fails.

Funding information

NCTR/FDA, Grant/Award Number: E0767501

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FIGURE 1.

Dose-dependent effect of NIF on zebrafish development when exposure begins at an early stage. Zebrafish early stage embryos at 6 hpf were exposed to various doses (2.5–50 μ M) of NIF until the larvae were 72 hpf. A, Morphology of the larvae: arrows indicating pericardial area and arrowhead shows the yolk sac. B, Body length. C, Hatching rate. Untreated embryos were used as control. Values are presented as mean $\pm$ SD. ** $P < .001$, a statistically significant difference with control. Scale bar = 105 μ m. hpf, hours postfertilization; NIF, nifedipine

FIGURE 2.

Effect of NIF on zebrafish late-stage embryos. Zebrafish embryos at 28 hpf were exposed to various doses (5–50 μ M) of NIF until the embryos were 48 hpf after a 20 h static exposure. Untreated embryos were used as controls. A, Morphology: arrows indicate pericardial sac and asterisks show pericardial sac edema. B, Body lengths of the embryos. Values are presented as mean $\pm$ SD. ** $P < .001$, statistically significant difference with control. Scale bar = 150 μ m. hpf, hours postfertilization; NIF, nifedipine

FIGURE 3.

A, ALCAR exacerbates NIF toxicity. Zebrafish embryos at 28 hpf were exposed to 50 μ M NIF until the larvae were 48 hpf after a 20-h static exposure. Untreated embryos were used as control. A, Morphology: asterisks show pericardial sac edema. Scale bar = 120 μ m. B, Relative pericardial sac areas are shown. C, Ratios of the pericardial sac to body length. D, Toxicities of lower doses of NIF in the presence of ALCAR are shown in the zebrafish embryos at 52 hpf that were exposed to 5 and 10 μ M NIF in the presence or absence of 1 mM ALCAR until the larvae were 72 hpf after a 20-h static exposure. Untreated embryos were used as control. D, Morphology: asterisks show pericardial sac edema. Scalebar = 155 μ m. E, Only relative pericardial sac areas are shown, as body lengths were not severely affected. Data are presented as mean $\pm$ SD. ** $P < .001$, a statistically significant difference with control. ALCAR, acetyl L-carnitine; hpf, hours postfertilization; NIF, nifedipine

FIGURE 4.

Effect of ALCAR and 2 mM Ket alone or in combination with NIF on the development of zebrafish embryos. Embryos at 28 hpf were exposed for 20 h to 50 μ M NIF alone and in the presence of either 2 mM Ket, or 2 mM Ket and 1 mM ALCAR. Images show morphology of the 48 hpf embryos. Dotted line indicates body axis, straight vs. curved. Scale bar = 175 μ m. ALCAR, acetyl L-carnitine; hpf, hours postfertilization; Ket, ketamine; NIF, nifedipine

FIGURE 5.

Atropine (0.5–50 μ M) does not prevent NIF-induced developmental toxicity in 72 hpf zebrafish larvae. Embryos at 52 hpf were exposed to 25 μ M NIF in the presence or absence of various doses of atropine for 20 h. A, Larvae morphology (72 hpf). B, Body length measurements as percentage change over control are presented as mean $\pm$ SD. $**P < .001$, a statistically significant difference with control. Scale bar = 270 μ m. hpf, hours postfertilization; NIF, nifedipine

FIGURE 6.

Subanesthetic doses of Ket alleviates NIF toxicity. Zebrafish embryos at 52 hpf were treated for 20 h with 25 μ M NIF in the presence of Ket (25, 50 and 100 μ M). Untreated embryos were used as control. A, Representative images show morphology. Scale bar = 260 μ m. B, Body length. C, Survival percentage. Data are presented as mean $\pm$ SD. * P < .05, ** P < .001, a statistically significant difference with control. hpf, hours postfertilization; Ket, ketamine; NIF, nifedipine

FIGURE 7.

Effect of NIF on ROS in the presence or absence of Ket. Zebrafish embryos at 52 hpf were treated for 20 h with 25 μM NIF alone or in the presence of Ket (25 and 50 μM). Untreated embryos were used as control. Embryos were also treated with Ket (25 and 50 μM) alone. Representative images of post-treatment larvae show ROS levels in vivo. Whole larvae are shown on the left and magnified views of specific trunk regions are shown to highlight the intestinal area. Arrowheads indicate ROS in the axial region. Asterisk indicates the location of the developing digestive system (intestine). Scale bar = 180 μm . hpf, hours postfertilization; Ket, ketamine; NIF, nifedipine; ROS, reactive oxygen species

FIGURE 8.

Schematic presentation of a potential mechanism to explain the effects of nifedipine, ketamine and ALCAR on the zebrafish embryos. Based on their known physiological effects, ketamine and nifedipine have counteracting effects on the vascular system, ketamine being a VC at low doses while nifedipine is a VD. Effect of ketamine as a VC must have been milder to induce any visible adverse effects on the zebrafish embryos, which had normal morphological features and no change in physiologic ROS levels. Nifedipine at higher doses (25–50 μM) could have caused severe vasodilation potentially leading to abnormal development with stunted body axis and abnormal ROS levels in the trunk. Ketamine and nifedipine combined would have annulled the VD effect of nifedipine manifesting in normal embryo development and normal ROS levels. ALCAR, acetyl L-carnitine; ROS, reactive oxygen species; VC, vasoconstrictor; VD, vasodilator

TABLE 1

Effect of NIF and Ket on ROS generation

	Control	25 μ M NIF	25 μ M NIF + 25 μ M Ket	25 μ M Ket	25 μ M NIF + 50 μ M Ket	50 μ M Ket
Normal ROS (intestine)	+	-	-	+	-	+
Abnormal ROS (axial muscle)	-	+	-	-	-	-

Ket, ketamine; NIF, nifedipine; ROS, reactive oxygen species.