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Original Research

Calcium Chloride Toxicology for Food Safety Assessment Using Zebrafish (*Danio rerio*) Embryos

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The salt calcium chloride ($CaCl_2$) is widely used in industry as a food additive; levels for human consumption are regulated by international or governmental agencies. Generally, the food industry relies on toxicity studies conducted in mammals such as mice, rats, and rabbits for determining food safety. However, testing in mammals is time-consuming and expensive. Zebrafish have been used in a range of toxicological analyses and offer advantages with regard to sensitivity, time, and cost. However, information in not available with regard to whether the sensitivity of zebrafish to $CaCl_2$ is comparable to the concentrations of $CaCl_2$ used as food additives. The aim of this study was to compare the $CaCl_2$ tolerance of zebrafish embryos and larvae with concentrations currently approved as food additives. Acute toxicity, embryotoxicity, cardiotoxicity, and neurotoxicity assays were used to determine the threshold toxic concentration of $CaCl_2$ in zebrafish embryos and larvae. The data showed that doses above 0.4% had toxic effects on development and on the activity of the cardiac and neuronal systems. Furthermore, all embryos exposed to 0.8 and 1.6% of $CaCl_2$ died after 24 hpf. These findings are consistent with the limits of $CaCl_2$ concentrations approved by *Codex Alimentarius*. Therefore, zebrafish embryos could be suitable for screening food additives.

Abbreviations: bw, body weight; $CaCl_2$, calcium chloride; ESFA, European Food Safety Authority; FAO, Food and Agriculture Organization of the United Nations; hpf, hours postfertilization; mpf, minutes postfertilization; WHO, World Health Organization

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Introduction

Calcium chloride ($CaCl_2$) is a white powder with a molecular weight of 111 g; it is odorless and highly water soluble. $CaCl_2$ is used widely in food manufacturing for products such as bread, potato snacks, dairy products, beverages, juices, coffee, tea, condiments, jams, meat products, and more. Fruit is often immersed in a water solution of $CaCl_2$ to aid in preservation. The use of $CaCl_2$ in food and nutritional supplement formulations is due to its stabilizing, thickening, and texturizing properties.[13,29,33]

Food additives and supplements for human and nonhuman consumption musts be manufactured with adherence to specific product specifications and quality standards in order to optimize quality and safety for users. Baseline product quality specifications are defined by the *Codex Alimentarius* Commission under the Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO). One of the main purposes of the Codex is to protect the health of consumers and to provide a benchmark standard for many nations, particularly those that do not yet have their own food standards. Other governmental or international organizations also base their legislation on the *Codex Alimentarius*, including the European Food Safety Authority (ESFA) in the European Union and the Food and Drug Administration (FDA) in the United States of America. For example, the FDA legislation establishes that the maximum level of $CaCl_2$ in food should be 0.1% for commercial jams and jellies, 0.2% for cheese and processed fruit and fruit juices, 0.2% for gravies and sauces, 0.22% for nonalcoholic beverages, 0.25% for meat products, 0.3% for baked and dairy products, 0.32% for coffee and tea, 0.4% for condiments and relishes, 0.4% for processed vegetables and vegetable juices; 2% for plant protein products, and 0.05% for all other food categories.[7]

Generally, the food industry relies on toxicity studies and levels of tolerance that are determined based on testing in animals such as mice, rats, and rabbits. The current study similarly tested zebrafish (*Danio rerio)* because it has high genetic similarity with humans, and the embryo's transparency in the development phase allows real-time analysis. However, a disadvantage of using zebrafish is the absence of current supporting legislation. Therefore, standards for use of this species should be developed by the scientific community, governmental and nongovernmental, public, and private entities.

The goal of the present study was to analyze the effects of permitted concentrations of $CaCl_2$ on zebrafish embryogenesis. We focused on cardiac and nervous system development in order to determine whether the published limits were safe for use in zebrafish. A zebrafish model could provide the basis for the development of a toxicological screening methodology

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that could be used before testing food preparations in other, costlier models.

Materials and Methods

The project was approved by the Animal Ethics Committee of the Federal University of São Carlos under Ref. 5085180319. $CaCl_2$ was purchased from a commercial supplier (Adicel Indústria e Comércio LTDA, Belo Horizonte, Brazil) and its toxicologic profile was tested using major endpoint requirements as required by the FDA, including assessment of acute embryotoxicity, cardiotoxicity, and neurotoxicity.

A total of 410 zebrafish embryos and larvae were supplied by the Central Vivarium of the Federal University of São Carlos, Brazil. Breeders were kept on automated racks at 28 °C (82.4 °F) at a pH 7.0 under a photoperiod of 14 h of light: 10 h of darkness, and were fed a commercial feed brand (Gemma for Zebrafish, USA) twice a day. Breeder were housed in a single fish tank overnight at a ratio of 2 males to one female. Embryos were collected the next morning at about 10 min post fertilization (mpf). At 60 mpf, the embryos were selected based on the observation of cell division under a microscope. $CaCl_2$ tests were conducted only when the fertilization rate was 90% or above. $CaCl_2$ embryotoxicity, cardiotoxicity, and neurotoxicity were tested using concentrations of 0.1%, 0.2%, 0.4%, 0.8%, and 1.6% (Table 1).

Table 1. Concentration of $CaCl_2$ used in this study

Concentrations ($CaCl_2$: mw = 110.984)		
%	**mM**	**ug/mL**
0.1%	9.01	1,000
0.2%	18.02	2,000
0.4%	36.04	4,000
0.8%	72.08	8,000
1.6%	144.16	16,000

Embryotoxicity. To assess acute toxicity, OECD Guideline 236 establishes that 200 individuals should be screened for each compound.[27] Embryos at 1.5 h post fertilization (hpf) were exposed to 5 logarithmic concentrations of $CaCl_2$ for 96 h in individualized 96-well plates. The viability of the embryos was assessed daily (6, 24, 28, 72 and 96hpf) based on the observation of lethality indicators such as embryo coagulation, absence of detachment of the yolk sac tail, changes in the formation of somites, and absence of heartbeat.[27] Developmental alterations were observed at 5.6 hpf; these changes included absence of

TIME-DOMAIN HRV PARAMETERS		
1	HR (bpm)	Average heart rate represented in beats per minute (bpm).[30]
2	SDHR (bpm)	Standard Deviation of Mean Heart Rate. Provides information on heart rate regularity. The bigger, the more irregular the heart rate.[30]
3	RR (ms)	Average time, in milliseconds (ms), of the cardiac cycle. The lower the time, the higher the average heart rate.
NONLINEAR METHODS		
4	SD1	The Poincaré chart is a nonlinear geometric method to assess heart rate variability. In clinical settings, Poincaré plot analysis of R-R intervals provides prognostic information in patients with heart failure and in patients vulnerable to life-threatening arrhythmias. HRV spectral and Poincaré plot analysis are widely applied to monitor sympathovagal change. The SD1 value represents the variations in heart rate measured between successive R-R intervals, represented in the Poincaré graph by the short axis length of the dispersion ellipse. In Poincaré charts, SD1 width reflects parasympathetic activity.[12]
5	SD2	The SD2 value represents the variations in heart rate measured between successive R-R intervals represented in the Poincaré plot by the long axis length of the dispersion ellipse. In Poincaré graphs, the SD2 width reflects the sympathetic modulation.
6	SD1/SD2	The Poincaré plot shape can be used to visually assess sympathovagal activity. An elongated torpedo-like shape with decreased SD1/SD2 ratio is associated with increased sympathetic tone, and a more oval fan-shaped configuration resulting from increased SD1/SD2 ratio indicates less sympathetic tone. Points are more dispersed when vagal activity increases or sympathetic activity decreases.
7	S	It corresponds to the area of an imaginary ellipse (S = × SD1 × SD2) with the length axes: SD2 (parallel to the identity line) and SD1 (perpendicular to the identity line). This descriptor is interpreted as a measure of the total variance of the RR intervals (HRV) because it increases with the growth of SD1 or SD2, or with both, and remains constant if SD1 increases at the rate that SD2 decreases.[11]
8	SampEn	Sample Entropy is a tool for investigating heart rate dynamics. SampEn is a similar measure, but less biased than the popular approximate entropy.[20,28]
9	BubbleEn	Bubble Entropy is a metric that aims to quantify the entropy of a series, having as its advantage the total elimination of the parameter and the low dependence on the length of the compared runs and the high tolerance to peaks.[23,24]
10	SVDEn	The SVD entropy is an indicator of the number of eigenvectors needed for an adequate explanation of the dataset. In other words, it measures the dimensionality of the data.[21]
11	ApEn	Approximate entropy (ApEn) measures the complexity or irregularity of the signal. Large values of ApEn indicate high irregularity and smaller values of regular signal ApEn.[28]
12	SpcEnt	The algorithm employs a general technique to quantify disorder in high-frequency event series: spectral entropy is a measure of disorder applied to the spectrum power of time series data periods. Spectral entropy has the ability to distinguish atrial fibrillation and flutter from other rhythms.[32]

Figure 1. Parameters used to characterize the dynamics of cardiac movement.

epiboly, failure to hatch, swim bladder insufflation, and changes in brain, eye, heart, ear and spine.[18] Changes in the development of the embryos were recorded daily for evaluation of teratogeny. All tests were evaluated against a negative control group (Embryonic medium E3, $n = 25$), a $CaCl_2$ plate control (Embryonic medium E3, $n = 4$), a positive control group (3.4 dichloroaniline - 4 mg L^{-1}, $n = 25$) and a solvent control group (1% DMSO, $n = 25$).

Cardiotoxicity. One of the most used parameters to evaluate the cardiotoxicity in Zebrafish larvae is heart rate and its dispersion interval (standard deviation), which can be measured by recording the dynamics of cardiac movement under microscopy. However, given the complexity of changes that cardiotoxic compounds can produce in the normal activity of the heart, we suspected that the quantification of these parameters might not be sufficiently predictive to identify cardiotoxic effects of some compounds, especially at low concentrations. Therefore, we used several parameters to measure the variation in cardiac cycles that were obtained from sequential images of an electrocardiogram that was captured on video and used to characterize cardiac Kymograph movement (Figure 1). To do this, we developed a computational algorithm in Python that captured the temporal sequence of the ventricular wall movement and quantified a series of linear and nonlinear parameters associated with the intensity and variability of the heart rate.

To assess cardiotoxicity, 60 zebrafish larvae were exposed at 96 hpf to one of 3 different concentrations of $CACl_2$ for 4 h (0.1, 0.2 and 0.4%). After that, using recorded videos of 50 frames per second and CardioCount V3 (in-house Python software, Laboratory of Applied Immunology, UFSCar, São Carlos Brazil), we measured variables associated with cardiac frequency and rhythm, including heart rate, Poincaré (SD1, SD2, SD1/SD2, S) and entropy (sample, bubble, SVD, approximate, and spectral)[30] and characterized the dynamics of cardiac movement (Figure 1). All cardiac parameters were normalized against the DMSO means and standard deviation values (Z-Score).

Neurotoxicity. We assessed spontaneous movement at 24 hpf to evaluate neurotoxic effects. We followed the design used in another study[25] that screened 150 individuals. At 10 mpf, embryos were exposed to 3 base 2 logarithmic dilutions of $CaCl_2$ ($n = 30$) and one negative group control (DMSO 1%) for 24 h. The spontaneous movement of the embryos was captured over a period of 6 min by using a digital camera attached to an optical microscope. For each embryo, the number of spontaneous movements per minute were counted using the EspMovCont, an in-house Python software (Laboratory of Applied Immunology, UFSCar, São Carlos Brazil). Movements were counted beginning at 60 s after filming began, which provided 5 min of movement counting.

Statistical analysis. Group comparisons of cardiac (Z-Scores) and neurotoxicity variables were performed using the Kruskal–Wallis statistical test and the Dunn analysis for the posthoc assessment,[31] with all groups compared with medium group (G1). Results of the univariate analyzes of the cardiac variables could be visualized in boxplot graphs. The data were also analyzed using multivariate tests to identify differences that could not be found by univariate statistical analyses. Linear Discriminant Analysis (LDA) was used to reduce all cardiac parameters to 2 components, LDA1 and LDA2, which were used to visualize clustering patterns. The cluster map shows the patterns, the correlations of the variables, and the proximity associations between the groups. Multiclass Logistic Regression

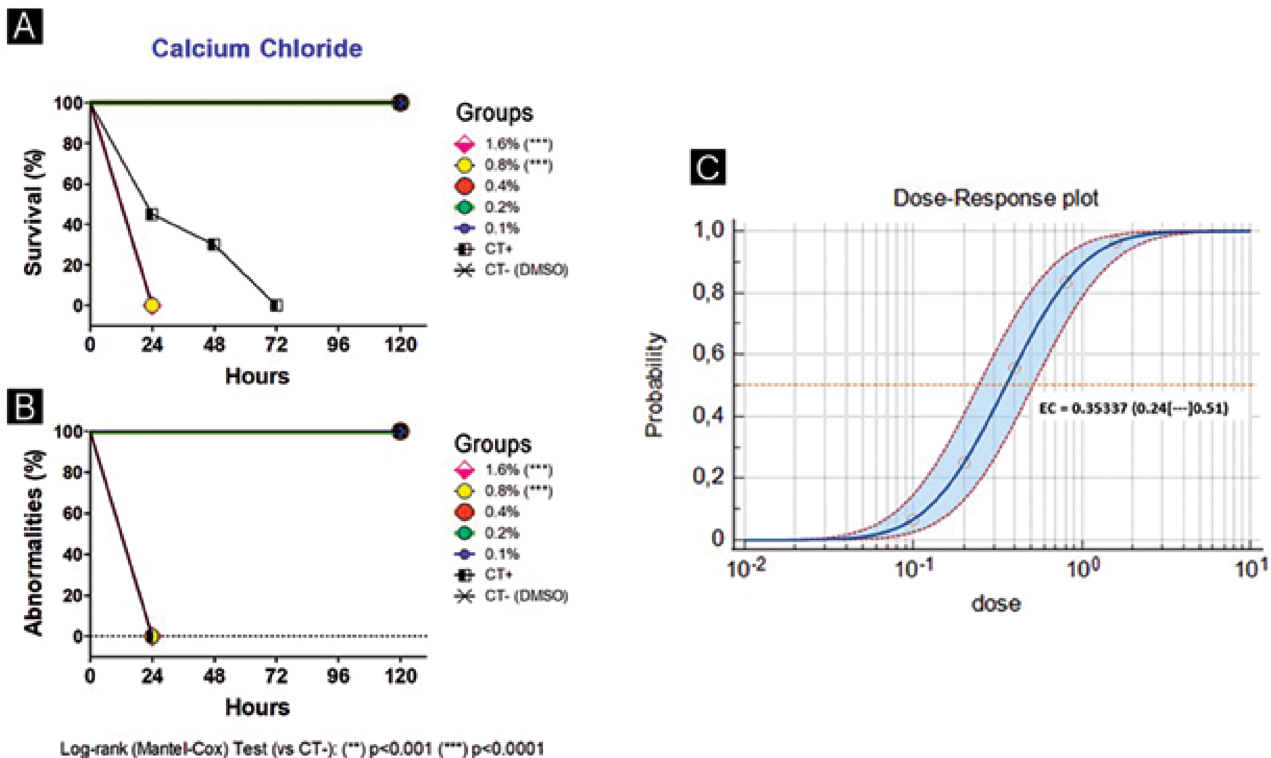

Figure 2. (A) Percentage survival and death of embryos exposed to $CaCl_2$ at 96 hpf, compared with the DMSO control; (B) Dose-response curves showing the percentages of abnormalities or death at 96 h after treatment with each concentration (0.1, 0.2, 0.4, 0.8, 1.6%) of $CaCl_2$. The Log-rank Statistical Test (Mantel-Cox) was performed to compare all groups with the DMSO control. The P values were corrected by the Bonferroni methodology and considered significant for values $P < 0.001$. The blue line represents the fit model of dead embryos calculated by Probit Regression ($R2 = 0.865$, $P < 0.0001$) and its 95% confidence interval (IC95) (dashed blue line) calculated by Medcalc.

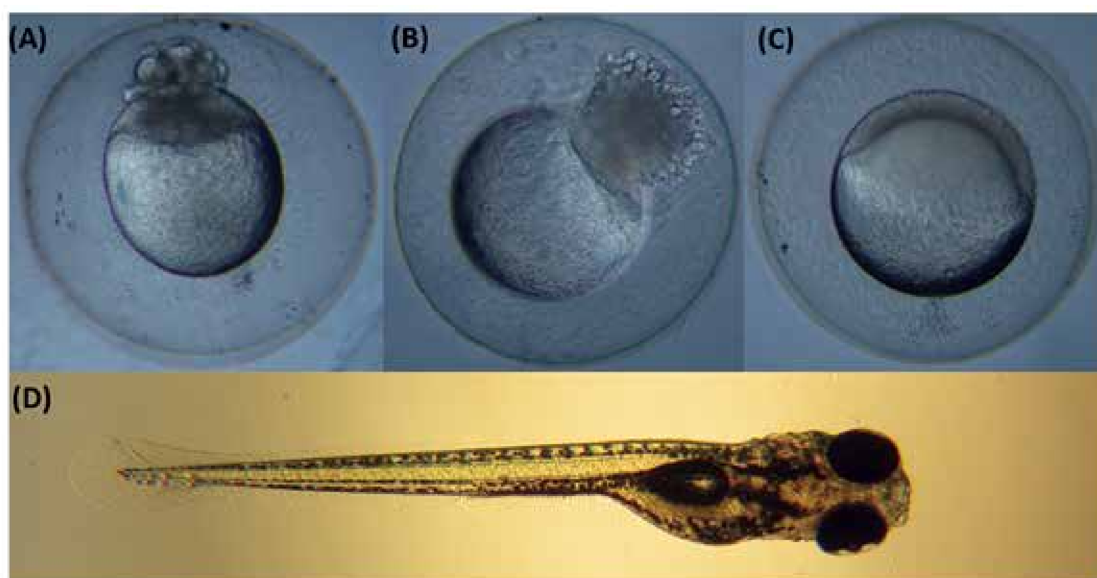

Figure 3. (A) Embryos exposed at 1.6% of $CaCl_2$ showing the absence of epiboly (embryonic maldevelopment) at 5.3 hpf ; (B) Embryos exposed at 5.3 hpf to 0.8% of $CaCl_2$ showing absence of epiboly; (C) Embryos exposed at 5.3 hpf to up to 0.4% of $CaCl_2$, showing standard development, with the gastrula period at 50% epiboly; (D) Larvae exposed at 120 hpf showing standard development at concentrations up to 0.4% $CaCl_2$.

analysis was used to build the mathematical model that quantified the probability of larval cardiotoxicity. Compounds were considered cardiotoxic when the mean value of the probability of normality of the larvae of the respective group, analyzed by the t-student test (1 sample), was statistically lower than 0.5. The difference between survival curves were analyzed by the Log-rank (Mantel–Cox) Test with *p*-values corrected by the Bonferroni method. The software used was the Prism 5 (GraphPad Software, San Diego, CA, USA) Dose–response curves were plotted and analyzed by Probit Regression using the MedCal 19.8 software (MedCalc Software, Ostend, Belgium). *P* values of < 0.05 were considered significant.

Results

The acute toxicity assessment showed that embryos exposed to $CaCl_2$ concentrations above 0.8% had significantly more deaths than did the DMSO control group. The cumulative mortality for each concentration was 100% of survival for 0.1, 0.2 nd 0.3% at 96 hpf, and 100% of mortality for 0.8 and 1.6% at 24 hpf (Figure 2). No abnormalities were observed with 0.1 to 0.4%, although 0.8 and 1.6% were associated with abnormalities that included absence of epiboly, failure to hatch, swim bladder insufflation, and changes in brain, eye, heart, ear and spine in 100% of animals at 24 hpf. The LC50% at 96 h was 0.35%. No deaths occurred in the control groups, and no incidents that may have influenced the results occurred during the tests. Survival and teratogenic effects (abnormalities) and photos of groups in different concentrations are shown in Figures 2 and 3, respectively.

The highest tested concentration of $CaCl_2$ used to assess cardiotoxicity and neurotoxicity was the LC50% (0.35%). Neither the heart rate (HR, RR) nor the variability of heart rate (SDHR) showed differences between the control groups DMSO and $CaCl_2$ groups. Variables associated with arrhythmia (Poincaré: SD1, SD2, SD1/SD2, S; Entropy: sample, bubble, SVD, approximate, and spectral) of the $CaCl_2$ groups also failed to show elevation of the heart rate as compared with the control DMSO group, indicating absence of arrhythmia. The Heatmap (right side, Figure 4) shows the results of using the Dunn test to compare the groups analyzed in pairs. The values on the Heatmap represent the ×100 significance level. Values lower than 5.0% were considered significant. Cardiac parameters including heart rate, SD1, SD2, Sd1/SD2, bubble, SVD, and approximate and spectral entropies of the $CaCl_2$ groups were normalized to those of the DMSO group. The effects in the 0.4% $CaCl_2$ group were highly variable, and differences were not statistically significant. None of the zebrafish larvae groups had significant differences in measures of cardiac activity at 96 hpf when exposed for 4 h to $CaCl_2$ as compared with larvae exposed to DMSO.

Spontaneous movement was analyzed in embryos exposed at 24 hpf to DMSO or treated $CaCl_2$ (Figure 5). The analysis showed that spontaneous movement during exposure to 0.1 and 0.2% were not significantly different from those of the negative control group, with an average number of movements of 7.20 and 7.15, respectively. Exposure to 0.4% was associated with an average movement score of 5.17.

Discussion

$CaCl_2$ has a low molecular weight and high water solubility and is thus rapidly dissociated in water into calcium and chloride ions, which are efficiently absorbed by the intestine. Once absorbed, calcium and chloride ions are regulated separately. These 2 ions are essential constituents of all animal species.[13] Calcium is the most abundant metallic element in all animal species, located mostly in the skeleton, and is essential for the formation and maintenance of bones and teeth. It also regulates several important physiologic functions, such as blood coagulation, bone mineralization, neuromuscular activity, enzymatic activity, and acid-base balance. In humans, hormonal systems regulate plasma calcium concentrations at approximately 100 µg/mL by controlling the intestinal absorption of dietary calcium, release of calcium from bone, and renal absorption and excretion. Chloride is the most abundant anion in animals and is important for maintaining osmotic and acid–base balance; most of the chloride is located in extracellular fluids. Plasma concentrations in humans are 3.6 to 3.9 mg/mL.[13]

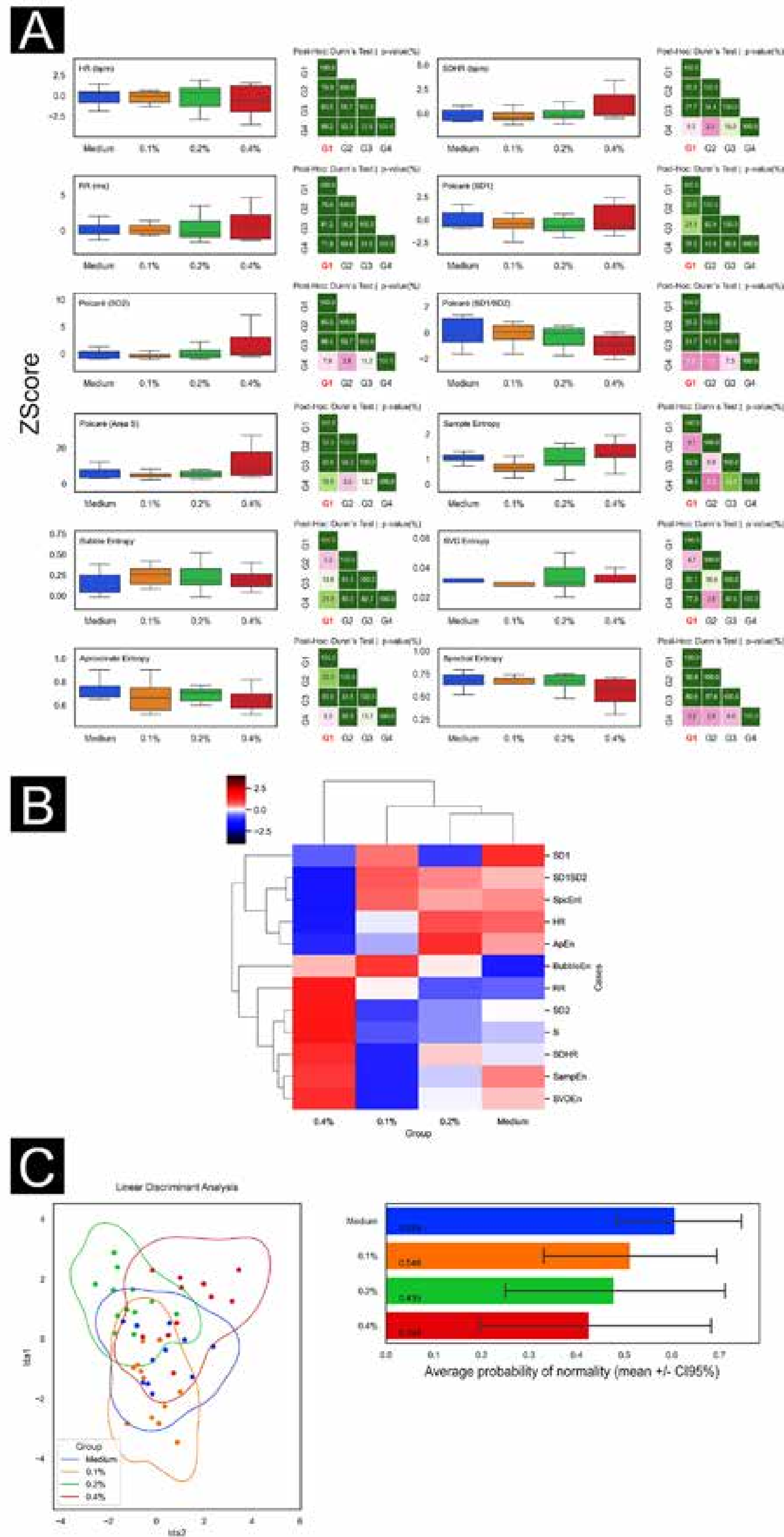

Figure 4. (A) Boxplot graphs of the distribution of relative levels of variables normalized in relation to medium; (B) The multivariable methodology of LDA Analysis accompanied by the Student *T* Test; (C) Clustermap showing the results of the cluster analysis.

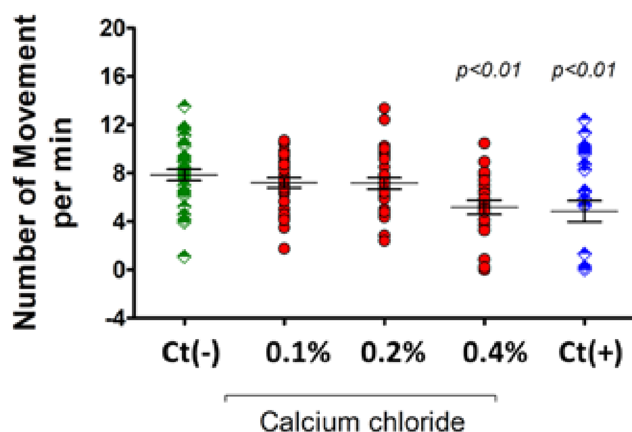

Figure 5. Means values ± SEM of the number of spontaneous movements per minute of Danio rerio embryos exposed at 24 hpf to different CaCl2 concentrations.

Zebrafish embryos are excellent organisms for testing pharmacological and food compounds because they are permeable to small molecules and drugs during organogenesis. This feature simplifies drug administration and vital dye staining because embryos have already formed most of their organs by 96 hpf.[16] However, little information is available on $CaCl_2$ toxicity in zebrafish.

In mice, the Lethal Dose 50% (LD50) values were 2,045 mg/kg body weight (bw) (0.20%) in male mice and 1,940 mg/kg bw (0.19%) in female mice.[1] In rats, oral LD50 values were 3,798 mg/kg bw (0.38%) jn males and 4,179 mg/kg bw (4.42%) in females.[1] Thus, rats required almost twice the concentration than did mice. Furthermore, the oral LD50 for rats was approximately 5,000 mg/kg bw (0.5%),[5] whereas in dogs it was over 2,000 mg/kg bw (0.2%).[22] In acute oral toxicity studies in rabbits, oral LD50 values for $CaCl_2$ ranged from 500 to 1,000 mg/kg/bw; gross postmortem examination revealed perforation and severe ulceration in the stomach of dead rabbits.[19]

A series of developmental toxicity studies examined the effects of $CaCl_2$ on embryo lethality and teratogenicity in mice, rats, and rabbits.[7] The highest doses of $CaCl_2$ used were 189 mg/kg body weight /day (or 0.02%) for mice, 176 mg/kg/day (0.02%) for rats, and 169 mg/kg/day (0.02%) for rabbits.[8] These doses were much lower than those used in our current study. The mouse, rat and rabbit studies found no effects on maternal or fetal development or survival, and no differences were found in the number of soft tissue or skeletal abnormalities between treated and control animals, leading to the conclusion that $CaCl_2$ at these doses did not produce toxic effects on development.[8]

In our study of zebrafish, the lethal concentration (LC)50 of $CaCl_2$ was 0.35% or 3,533 mg/kg, and at doses above 0.8%, or the equivalent of 8,000 mg/kg, all embryos died. Similar results were presented previously for mice, rats, and rabbits [1,5,19,22] (Table 2). However, a few articles in the literature discuss zebrafish and $CaCl_2$ toxicology, as shown in the table.[17]

In a previous study, incubation of zebrafish embryos in 1 mM $CaCl_2$ (0.11 mg/kg, or less than 0.0001%) did not cause developmental morbidity or mortality at 120 hpf.[17] The same study determined, by titration approach, that 62.5 µM $CaCl_2$ was low sufficient to permit a normal development up to 120 hpf,[17] but these concentrations were lower when compared with those used in this and other studies[1,5,19,22] (Table 1 and 2).

At the present study, concentrations of 0.1, 0.2, and 0.4% $CaCl_2$ produced no significant differences in cardiotoxicity as compared with the control group. However, all embryos exposed to concentrations of 0.8 and 1.6% died after 96hpf, so cardiotoxicity could not be assessed. Neurotoxicity testing was performed at 24 hpf using concentrations of 0.1, 0.2 and 0.4%. The effects of 0.1 and 0.2% were the same as those of the negative control group. However, the effects of 0.4% were significantly different from control. At concentrations of 0.8% and 1.6%, all embryos had died before the scheduled test and therefore could not be evaluated.

The results of our study support the use of zebrafish embryos for testing exposure to the $CaCL_2$ concentrations present in foods as established in the FDA *Codex Alimentarius*, with maximum concentrations of 0.4%. Nonetheless, chemical interactions that might occur during processing could further reduce the $CaCl_2$ concentration in the final product before the time of consumption. We suggest that zebrafish embryos can be used to prioritize chemical testing in mammalian species with the ultimate goal of protecting human health. In this way, the time and costs of the safety assessment of new food compounds could be substantially reduced. The potential of the zebrafish embryo for food toxicity testing is already known,[2] but regulatory agencies do not yet generally encourage or accept its use. Such acceptance would require additional studies to standardize zebrafish protocols and strains.[3,10]

In conclusion, numerous studies have documented similarities in the toxicity profiles of zebrafish and mammals. Because test compounds can be dissolved in water and zebrafish embryos and larvae absorb through their skin, gut, and gills, using zebrafish can simplify food safety assessments can simplify the process. Also, because fish embryos are very sensitive to acute, chronic, and other harmful toxins, biologic testing technologies based on fish embryos can quickly and comprehensively determine the overall safety of food ingredients and products.[6] The current study verified the acute toxicity of $CaCl_2$ in zebrafish during embryogenesis with regard to mortality, teratogenic effects, and cardiac and neural system development using a real-time analysis. Our study found that the limits allowed by international legislation were safe for ze-

Table 2. Benchmarks of LD50 in zebrafish, mice, rats, rabbits, and dogs.

Animal	Sex	Lethal Dose (50%)	(%)	References
Mice	Male	2045 mg/kg/bw	0.2	Akatsuka et al., 1977, as cited in OECD 2002[1]
	Female	1940 mg/kg bw	0.19	Akatsuka et al., 1977, as cited in OECD 2002[1]
Rats	Male	3798 mg/kg/bw	0.38	Akatsuka et al., 1977, as cited in OECD 2002[1]
	Female	4179 mg/kg/bw	0.42	Akatsuka et al., 1977, as cited in OECD 2002[1]
Rats	---------	5000mg/kg/bw	0.5	Barnes and Eltherington, 1964, as cited in SCOGS-45, 1975[5]
Rabbits	---------	500–1000mg/kg/bw	0.05-0.1	Koopman & Pot, 1986[19]
Dogs	---------	2000 mg/kg/bw	0.2	Mahorner, 1937, as cited in SCOGS-45, 1975[22]
Zebrafish	---------	3533mg/kg	0.35	Present study

brafish embryos. We demonstrated the value of this species in toxicologic screening and its likely applicability to toxic effects in humans. Although more studies are necessary to develop food safety indices based on zebrafish, we expect that our study can provide a basis for the development of a toxicologic screening methodology for use prior to the testing in other animals.

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