Development of Nutraceutical Emulsions as Risperidone Delivery Systems: Characterization and Toxicological Studies

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ABSTRACT: Emulsions are gaining increasing interest to be applied as drug delivery systems. The main goal of this work was the formulation of an oil/water nutraceutical emulsion (NE) for oral administration, enriched in omega 3 (ω3) and omega 6 (ω6), and able to encapsulate risperidone (RISP), an antipsychotic drug widely used in the treatment of autism spectrum disorders (ASD). RISP has low solubility in aqueous medium and poor bioavailability because of its metabolism and high protein binding. Coadministration of ω3, ω3, and vitamin E complexed with RISP might increase its bioavailability and induce a synergistic effect on the treatment of ASD. Here, we developed an easy and quick method to obtain NEs and then optimized them. The best formulation was chosen after characterization by particle size, defects of the oil-in-water interface, zeta potential (ZP), and *in vitro* drug release. The formulation selected was stable over time, with a particle size of around 3 μm, a ZP lower than −20 mV and controlled drug release. To better understand the biochemical properties of the formulation obtained, we studied *in vitro* toxicity in the Caco-2 cell line. After 4 h of treatment, an increase in cellular metabolism was observed for all RISP concentrations, but emulsions did not change their metabolic rate, except at the highest concentration without drug (25 μg/mL), which showed a significant reduction in metabolism respect to the control. Additionally, locomotor activity and heart rate in zebrafish were measured as parameters of *in vivo* toxicity. Only the highest concentration (0.625 μg/mL) showed a cardiotoxic effect, which corresponds to the decrease in spontaneous movement observed previously. As all the materials contained in the formulations were US FDA approved, the NE selected would be good candidate for clinical trials. © 2015 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci

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INTRODUCTION

The antipsychotic drug risperidone (RISP), 3-[2-[4-(6-fluoro-1,2-benzisoxazol-3-yl)-1-piperidinyl]ethyl]-6,7,8,9-tetrahydro-2-methyl-4hpyrido[1,2-a]pyrimidin-4-one, which belongs to the chemical class of benzisoxazoles, is one of the most widely used drugs in the treatment of autism spectrum disorders (ASD).[1–6] ASD include different neurodevelopmental disorders that manifest mainly in the earlier years of life, affecting the development of language, communication, and reciprocal social interaction,[7–9] and occur in one out of 110 individuals.[7] RISP has low solubility in aqueous and physiological media and, when orally administered, exhibits low bioavailability because of extensive first-pass metabolism and high protein binding (>90%).[10] Moreover, nontargeted delivery usually results in numerous side effects. As the target site of RISP is the brain, it is necessary not only to develop a strategy to improve drug bioavailability, by avoiding first-pass metabolism, but also to achieve the desired drug concentration at the site of action, and thus reduce undesirable side effects.[4,5]

In the last years, several strategies to overcome these issues, particularly the design of nano- and microstructured drug carrier systems, have been proposed.[1–6] However, these kinds of carriers (plain, ultradeformable, stealth or pH-sensitive liposomes, immunoliposomes, nanoparticles, dendrimers, and emulsions) must be carefully designed and/or chosen because their pharmacokinetics, biodistribution, and tissue selectivity will exclusively depend on the carrier's structure.[4,6,11,12]

Alanazi[9] reported that the use of nutraceuticals in ASD management can generate a successful integrative model with the current treatment that may allow achieving the desired results. Nutraceuticals offer several promising benefits that may include promoting a healthy gut, lowering body burdens of toxins, reducing cytotoxicity, improving antioxidant capacity, enhancing immunomodulatory systems, and minimizing stress and environmental contamination/hazards.[9] The nutraceuticals most used in ASD treatment are: multivitamins and mineral complexes, L-carnitine, and polyunsaturated fatty acids.[9]

Almost all autistic patients suffer from a deficiency of essential fatty acids, especially omega-3 (ω3) and omega-6 (ω6) fatty acids.[13] It has been reported that administration of ω3 fatty acids to autistic children for 6 weeks induces improvements in their behavior (i.e., a decrease in their hyperactivity and stereotyped movements), resulting in an efficient and well-tolerated treatment.[14] Furthermore, administration of a commercial ω3 complex to autistic children for 3 months has shown not only improvements in their behavior, but also enhancements at a biochemical level, with an increase in blood fatty acids.[15] Oils

Abbreviations used: ASD, autism spectrum disorders; bpm, beat per minute; dpf, day postfecundation; HF, hydrophobicity factor; HLB, hydrophilic–lipophilic balance; hpf, hours postfecundation; MC540, merocyanine 540; NE, nutraceutical emulsion; o/w, oil in water; PG, propylene glycol; RISP, risperidone; SL, soy lecithin; Smix, mix of surfactants; ZP, zeta potential; ω3, omega 3; ω6, omega 6

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with the highest ω3 and ω6 concentration (% of total ω) are cod liver (animal source, 21.5% ω3 and 8.7% ω6) and canola (vegetable source, 8.8% ω3 and 21.9% ω6).[16] Besides, it has been reported that children with ASD have increased oxidative stress and/or decreased antioxidant defenses, with decreased plasma levels of vitamin E.[17] In a group of 187 autistic children, a combinational supplementation of vitamin E and ω3 fatty acids produced dramatic improvements in speech, imitation, eye contact, coordination, behavior, and sensory function.[18] In addition, it has been demonstrated that ω3 can inhibit hepatic metabolism, increasing the bioavailability of drugs such as cyclosporin A, saquinavir, and midazolam in rats.[19] All this suggests that coadministration of ω3, ω6, and vitamin E complexed with RISP could inhibit the metabolic reactions catalyzed by hepatic enzymes, thus increasing its bioavailability and inducing a synergistic effect on the treatment of autism.

Although there are commercial formulations of microencapsulated RISP (RISPERDAL® CONSTA®), these are given by injection by a healthcare professional every 2 weeks and do not offer nutraceutical properties. The administration through the gastrointestinal tract is the most popular route for drug delivery[20,21] because of the convenience and patient's compliance, especially for treatment in children with multiple applications per day or long-term treatment.[22] Therefore, our objective was to obtain a microencapsulated RISP formulation suitable for oral administration and rich in nutraceuticals.

Oil-in-water (o/w) emulsions are promising as drug delivery systems, as lipids are known oral drug absorption enhancers.[6,23] Moreover, o/w emulsions retain the advantages of traditional colloidal systems[24–29]: enhanced physical stability, protection of drug molecules from degradation in the body, controlled drug release, specific targeting, biocompatibility, and laboratory-to-commercial scalability. Emulsions have been previously used as drug delivery systems for the following hydrophobic molecules: nalbuphine and its prodrugs,[30] paclitaxel,[25,29] resveratrol and doxorubicin,[29] carbamazepine,[31] cheliensisine A,[32] ibuprofene, ketoprofene, tamoxifen, testosterone, tolbutamide, and cyclosporin A.[33] As mentioned before, RISP has low solubility and exhibits extensive first-pass metabolism and high protein binding (>90%). In order to avoid these undesired facts, a drug delivery system is required and emulsions are ideal for this.

On the basis of all the above-mentioned points, the aim of this work was to obtain a drug delivery system for oral administration based on an o/w nutraceutical emulsion (NE) enriched in ω3 and ω6 fatty acids and able to encapsulate RISP (NE-RISP), to improve its bioavailability and reduce secondary effects. The components selected were: canola oil, cod liver oil, vitamin E, Tween 80, soy lecithin (SL) and propylene glycol (PG). Several mixtures with different oil–surfactant ratios were analyzed. Also, several preparation methods were tested. The formulations selected were characterized by studying particle size, hydrophobicity factor (HF), morphology, zeta potential (ZP), and releasing profile. Finally, toxicity was analyzed *in vitro* in a cell line (Caco-2) and *in vivo* in an animal model (zebrafish) for the finally selected formulation.

The Caco-2 cell line was isolated by Fogh et al.[34] from human epithelial cells of colon adenocarcinoma. This cell line is most often used to test compounds to be orally administered.[20] However, it is known that *in vitro* assays in cells lack the complexity present in organisms, and that this complexity is required to test neurotoxicity, teratogenicity, cardiotoxicity, and the functions of the central nervous system. Thus, in several fields of biomedical research such as drug screening, drug safety, and toxicity tests, zebrafish embryos are a powerful alternative model to test toxicity and teratogenicity[35] as well as to study the cardiotoxicity[36] and hepatotoxicity[37] of drugs.

EXPERIMENTAL

Materials

Cod liver oil, canola oil, vitamin E, Tween 80, and SL were acquired from the local pharmacy and were suited for human intake. PG was from BioPack (Zarate, Buenos Aires, Argentina). Commercial solution of RISP (Risperdal®) was from Janssen-Cilag (Beerse, Belgium). Methanol, diethylamine, and dimethyl sulfoxide (DMSO) were from J. T. Baker (Buenos Aires, Argentina). The tetrazolium salt MTT [3-(4,5-dimethyl-thiazol-2-il)-2,5-diphenyl tetrazolium], minimum essential medium (MEM), fetal bovine serum (FBS), glutamine, and antibiotic antimycotic, all used in cell culture, were obtained from Gibco (Waltham, MA, USA).

Preparation of Emulsions

Optimization: Composition

Nutraceutical emulsions were developed by the spontaneous emulsification method.[5] The oily phase (66.55% cod liver oil, 33.28% canola oil, and 0.17% vitamin E) was flushed with nitrogen and stored. The surfactant (Tween 80) and cosurfactants (SL and PG) were mixed in fixed weight ratios as follows: 1:1:1 (A), 2:1:1 (B), 1:1:2 (C), and 4:1:1 (D). To simplify denominations, the mix composed of surfactant and cosurfactants was called Smix. In the optimization process, the drug was replaced by a PG solution in water (1:1), which was added to the oily phase. Briefly, each Smix was added to the oily phase and homogenized under vigorous stirring. The oil–Smix ratio varied as follows: 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, and 1:9 (w/w). A constant volume of water was added dropwise at room temperature to each oil–Smix mixture under vigorous stirring. After equilibrium, the samples were observed and those that presented phase separation were discarded. On days 1, 7, 14, and 21 post-preparation, the globule size was determined by light scattering with a Malvern Mastersizer 2000E (Malvern Instruments Ltd., Worcester, UK). Measurements were performed three times. After that, formulations with the best features were selected.

Optimization: Method of Preparation

The emulsions selected were prepared and the method was optimized based on obtaining globule size. During this step, formulations were prepared using an UltraTurrax homogenizer (UltraTurrax IKA T25 Digital with rotor S25-20 NK-186; Labortechnik, Wasserburg, Germany). Stirring speed and time were varied. Globule size was determined and samples that presented stable sizes were selected.

Preparation of NEs and NEs-RISP

Nutraceutical emulsions-RISP were obtained by mixing 1 ml of stock solution of RISP (1 mg/mL) with Smix and oily phase for 1 min at 10,000 rpm, using an UltraTurrax homogenizer. Water was added dropwise with continuous stirring at 20,000 rpm for 3 min. Samples were kept on ice during this process to avoid an increase in temperature. A NE (without RISP) was used as control.

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Characterization of NEs and NEs-RISP

Particle Size

The particle size distribution was determined by light scattering on days 1, 7, 14, and 21 postpreparation. Simultaneously, monitoring was performed by an optical microscope Leica DMI6000B (Leica Microsystems, GmbH, Wetzlar, Germany) with a digital camera (Leica DC100; Leica Microsystems, GmbH).

Determination of Defects of the Oil/Water Interface

To determine the spectral characteristics of the o/w interface, the visible spectra of the probe merocyanine 540 (MC540) were recorded between 400 and 600 nm. The MC540 probe is sensitive to polar environments. In water, the spectra show two maxima (a dimer at 500 nm and a monomer at 530 nm), whereas in hydrophobic environments, the maxima are shifted toward 530 nm (dimer) and 570 nm (monomer). The ratio of the absorbance at 570 nm with respect to 500 nm, called HF,[38,39] determines the degree of hydrophobic sites exposed to the interface and organization. MC540, freshly prepared in 1 mg/mL stock solution, was added in aliquots to give a final concentration of 2.19 μM. The sample was allowed to equilibrate for 2 min and then measured with a UV–Visible spectrophotometer (NanoDrop 2000; ThermoScientific, Waltham, MA, USA). To avoid scattering, NEs and NEs-RISP without MC540 were used as reference.

Zeta Potential

The surface electrical charge, expressed as the ZP, was measured on days 1 and 21 postpreparation with a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcester, UK).

In Vitro Drug Release Studies

The *in vitro* release of RISP from NEs-RISP was studied in water using a microdialysis Eppendorf tube diffusion technique.[1–3] This technique was developed *ad hoc* to quantify microquantities of the released drug. NEs-RISP or the free drug were sealed into the microdialysis Eppendorf tube (Sigma–Aldrich, Buenos Aires, Argentina; MW cut-off: 12,000 Da) and incubated in water under continuous stirring. The experimental design for RISP release consisted in collecting aliquots from the incubation medium at predetermined time intervals, and storing them at 4°C for quantitative analysis. The assay was repeated three times and the amount of RISP released was determined by RP-HPLC, according to the method previously validated.[3] Briefly, a RP-C18 column (LiChroCART® Lichrosper® 100 RP-18) and an isocratic mobile phase of methanol and solution of diethylamine in water 0.4% (v/v) (60:40, v/v, methanol:water) were used. The flow rate was 1.3 mL/min, the run time was 10 min, and RISP was measured by UV absorbance at 280 nm.

At this point, the best formulation was selected for further analysis.

In Vitro Toxicity: MTT Assay

To study the influence of the delivery systems proposed on a cell culture and analyze possible cytotoxic effects, we used the Caco-2 cell line, a human cell line derived from a colon adenocarcinoma. This cell line is widely used in assays that involve drug absorption related to an oral route and was selected because its characteristics are similar to those of the absorptive intestinal epithelium.[20,21] The Caco-2 cell line was a gift from PhD Diego Mengual Gomez, from the Laboratorio de Oncología, Universidad Nacional de Quilmes, Buenos Aires, Argentina. Cell viability upon treatment with RISP, NE, or NE-RISP was measured as means of the activity of mitochondrial succinate dehydrogenase, using a tetrazolium salt MTT.[1,38,40] Cells were seeded at a cell density of 1×10^4 cells/well in a 96-well flat bottom microplate and grown at 37°C in MEM, supplemented with 10% FBS, 1% glutamine, and a 10% antibiotic-antimycotic solution in a 5% CO_2 atmosphere. After 24 h, the medium was replaced with 100 μL RISP, NE, or NE-RISP diluted in cell medium. The RISP concentration used ranged from 25 to 0.025 μg/mL. After 4 or 24 h of incubation,[38] solutions were removed and replaced by MTT at a final concentration of 0.5 mg/mL.[40] After 2 h of incubation, the MTT solution was removed, and the insoluble formazan crystals were dissolved in DMSO; absorbance at 570 nm was measured by using a Rayto RT-2100C microplate reader (Nashan, Shenzhen, China).

In Vivo Toxicity: Zebrafish

Animals

A breeding stock of heterogeneous wild-type zebrafish was inbred in our aquarium, as described previously by Kimmel et al.[41] Sexually mature females and males (8–12 months old) were kept in a glass aquarium filled with filtered tap water at 26 ± 1°C under a 14/10 h light/dark cycle. Fishes were fed with dry fakes (TetraMin PRO®) twice a day and nauplius larvae of Artemia once a day *ad libitum*. For embryo production, three females and two males were crossed the night before the spawning day in traps made of plastic mesh, to prevent the eggs from cannibalism. In this study, embryos refer to zebrafish prior to hatching [0–3 day postfecundation (dpf)], whereas larvae refer to posthatching animals (over 3 dpf). Embryos were obtained from natural mating, and all embryos and larvae used in these experiments were reared at 28.5°C under a 14/10 h light/dark cycle in conditioned E3 medium (NaCl 0.29 g/L, KCl 0.012 g, CaCl2 0.036 g/L and MgSO4 0.039 g/L in deionized water, and 50 ppb methylene blue to inhibit fungal growth). Only fertilized eggs in good condition were selected for further treatment; others were discarded. The characteristics of eggs were determined with a stereomicroscope (Leica Zoom 2000, Wetzlar, Germany).

Treatment

For the treatment, at 48 h postfecundation (hpf), three nonhatched zebrafish embryos were placed in each well of a 96-well plate containing E3 medium and incubated for additional 48 h at 28°C. At 4 dpf, the medium was replaced by 250 μL of solutions of the different NEs or E3 medium (control). For each assay, eight technical replicates and two biological replicates were used for each dilution ($n = 16$). The effect of serial dilutions of the NE-RISP medium in final concentrations from 0.625 to 0.00488 μg/mL was tested.

Automated Measurement of Larval Activity Events

Activity events were recorded for 15 min at 4, 5, 6, and 7 dpf after the addition of the NEs tested, at room temperature.[3] Changes in spontaneous locomotor activity events reflect the neurotoxicity of the different treatments.[42,43] The system is based on an infrared microbeam arrangement that detects light

refraction through the zebrafish body, essentially as described elsewhere.[3] Animals were placed in 96-well microplates and subjected to illumination with two infrared microbeams per well (100 μm wide and 880 nm wavelength each). A transient fluctuation in the signal is generated when larvae move across the light beam and is then received by a phototransistor array. The output of the light signals was digitalized by a multichannel ADC system (WMicrotracker; Designplus SRL, Buenos Aires, Argentina) at a sample rate of 10 samples/s and 10 bit resolution. Data were acquired with an IBM-PC connected via the RS232 protocol and processed by software programmed in MS-Visual Basic. Signal activity events (defined as the times that larvae crossed through infrared microbeams) were calculated in real time by detecting small fluctuations in the signal received. Variations greater than 3% in the signal received (empirically determined threshold) were considered as activity events. Swimming activity was calculated as the sum of the number of activity events for 15 min.

Heart Rate Measurements and Morphological Changes

The heart rate of zebrafish was assessed at 7 dpf. Control and experimental zebrafish larvae were individually transferred to a depression slide with methylcellulose and placed under a trinocular microscope Nikon SMZ800. The heart rate was determined by counting the number of beats every 15 s and expressed as beats per minute (bpm). Experiments were performed three times on five larvae per group for each time point. A difference between the heart rates of control and treated larvae was regarded as cardiotoxicity. Subsequently, to determine possible morphological changes, the fish was photographed with a Microsoft camera. Larval eye area, rostro caudal length, and spinal cord length were observed and analyzed with the Image J program (free software). Also, changes in the state of the liver were studied from these photographs. The liver of normal zebrafish is fairly globular in structure, has a clearly recognizable periphery against the neighboring tissues and is perfused with circulating blood cells, whereas after treatment with hepatotoxic drugs, it becomes darker with a brown or gray coloration and its texture becomes amorphous, indicating degeneration and/or necrosis.[37]

Ethics Statement

All animal procedures were performed in strict accordance with the guidelines for animal care and maintenance of the National Institutes of Health. The study protocols were approved by the Institutional Animal Care Committee of the Universidad Nacional de Quilmes (CE-UNQ 2/2014) (Buenos Aires, Argentina).

Statistical Analysis

Data are presented as mean ± standard deviation (SD) or as mean ± standard error of the mean (SEM) and analyzed by one-way ANOVA and Multiple Comparison post-test using GraphPad Prism v. 6.0. Only values with $p < 0.05$ were accepted as significant.

RESULTS AND DISCUSSION

Preparation of Emulsions

The type of NEs formed depends on the properties of the oil, surfactant, and cosurfactant. The cosurfactant also ensures that the interfacial film is flexible enough to deform readily around each droplet as its intercalation between the primary surfactant molecules decreases the polar head group interactions.[5] In this study, Tween 80 and SL were selected as the surfactant–cosurfactant system and PG as a solvent. An important criterion for the selection of the surfactants is that the hydrophilic–lipophilic balance (HLB) value required to form the oil–water NE needs to be greater than 10. The right mixture of low and high HLB surfactants leads to the formation of a stable NE formulation.[5] Tween 80 had an HLB value of 15[5] and the cosurfactant SL had an HLB value of 8.[44] Both are FDA approved and widely used in emulsion systems for oral administration.[45] Formulations were developed by varying the Tween 80–SL–PG ratios as 1:1:1 (Smix A), 2:1:1 (Smix B), 1:1:2 (Smix C), and 4:1:1 (Smix D). The globule size was determined on days 1, 7, 14, and 21 postpreparation (Table 1).

Formulations A2, A4, and A5 presented stable sizes along time, whereas formulations A6 and A7 were variable. Formulations C were also variable in size and this could be because PG acts as a cosolvent instead of as a surfactant. By reducing the amount of active surfactant (Tween 80 and SL), oil drops could not become stabilized correctly. The smaller sizes of formulations B and D respect to formulations A could be because of the increase in the amount of Tween 80. This reagent acts as the main surfactant in the mixture. An increase in the amount of this component leads to quick stabilization processes and smaller particles during stirring.[27,45,46]

The above-mentioned results are in agreement with those by Kumar et al.[5] Particle size decreased with the increase in the concentration of Tween 80 as well as with the increased concentration of Smix. It has been previously reported that the increasing concentration of surfactant helps to stabilize the emulsion and reduce particle size.[27,45,46] These observations can be explained by considering the mechanism proposed by Pichot et al.[27] to stabilize o/w emulsions with the surfactant mixtures. Initially, low molecular weight surfactants may stabilize the oil–water interface formed during the emulsification process, lowering the interfacial tension and promoting the separation of the droplets in others of smaller sizes. Then, the surfactant adsorbed to the surface reduces coalescence processes and avoids the increase in particle size over time.[26,27]

Formulations A were selected based on their temporal stability and large amounts of oil that lead to the possibility of incorporating more hydrophobic drug. The remaining samples were discarded because they showed widely scattered results or contained high amount of Tween 80, which could induce toxic effects.

The NEs were optimized on the basis of globule size and stability in time, and the results are shown in Table 2. During this step, formulations were prepared using an UltraTurrax homogenizer, varying the stirring speed and time. Züge et al.[45] reported that when the energy input to the system is increased, particle size decreases because a larger interfacial area can be formed, leading to more uniform droplets that tend to be more stable. This agrees with the results obtained using the UltraTurrax homogenizer, as compared with magnetic stirring.

Method E was selected because it led to formulations with smaller sizes. In this method, the PG solution, the Smix, and the oil were mixed for 1 min at 10,000 rpm, and water was added dropwise while mixing at 20,000 rpm for 3 min.[45] Three formulations with the following composition: A2 1:8:2:9; A4 1:6:4:9,

Table 1. Globule Size of Initial Formulations Prepared by Magnetic Stirring over Time

			Globule Size (μm) ± SD			
Formulation	Oil–Smix Ratio	NE Formation	Day 1	Day 7	Day 14	Day 21
A1	9:1	Yes	96.916 ± 14.769	76.599 ± 3.549	145.649 ± 8.495	134.226 ± 5.564
A2	8:2	Yes	32.905 ± 0.168	32.680 ± 0.041	31.544 ± 0.020	34.546 ± 0.029
A3	7:3	Yes	84.397 ± 9.310	61.434 ± 1.413	59.747 ± 1.971	89.882 ± 3.504
A4	6:4	Yes	8.301 ± 0.008	7.971 ± 0.021	7.884 ± 0.022	7.727 ± 0.013
A5	5:5	Yes	2.578 ± 0.012	2.585 ± 0.002	2.560 ± 0.006	2.470 ± 0.003
A6	4:6	Yes	2.442 ± 0.067	2.309 ± 0.009	2.411 ± 0.019	2.095 ± 0.003
A7	3:7	Yes	6.891 ± 0.540	1.464 ± 0.004	1.129 ± 0.072	1.198 ± 0.010
A8	8:2	Yes	4.667 ± 1.999	2.199 ± 0.061	2.136 ± 0.005	2.160 ± 0.001
A9	1:9	No				
B1	9:1	No				
B2	8:2	No				
B3	7:3	No	72.004 ± 6.098	77.876 ± 2.494		
B4	6:4	Yes	10.256 ± 0.018	13.717 ± 6.535	9.945 ± 0.034	10.087 ± 0.012
B5	5:5	Yes	17.837 ± 0.095	12.634 ± 0.069	13.668 ± 0.100	13.519 ± 0.015
B6	4:6	Yes	9.200 ± 0.018	9.007 ± 0.021	9.349 ± 0.297	9.009 ± 0.023
B7	3:7	Yes	1.766 ± 0.000	1.796 ± 0.001	1.756 ± 0.001	1.781 ± 0.018
B8	8:2	Yes	1.859 ± 0.001	1.863 ± 0.001	1.883 ± 0.002	1.830 ± 0.000
B9	1:9	No				
C1	9:1	No				
C2	8:2	No				
C3	7:3	No	106.136 ± 7.326			
C4	6:4	Yes	61.901 ± 0.653	53.822 ± 0.576	50.686 ± 0.313	40.239 ± 0.080
C5	5:5	Yes	4.436 ± 0.006	4.468 ± 0.008	4.315 ± 0.001	4.315 ± 0.016
C6	4:6	Yes	5.371 ± 0.017	5.513 ± 0.001	5.480 ± 0.009	5.363 ± 0.012
C7	3:7	Yes	4.197 ± 0.009	4.190 ± 0.017	4.112 ± 0.029	4.044 ± 0.021
C8	8:2	Yes	15.949 ± 0.036	15.709 ± 0.135	14.946 ± 0.092	14.747 ± 0.039
C9	1:9	No				
D1	9:1	Yes	90.801 ± 4.111	98.666 ± 3.163	95.070 ± 2.406	92.033 ± 2.888
D2	8:2	Yes	89.693 ± 4.061	93.143 ± 2.721	102.802 ± 3.647	124.831 ± 6.939
D3	7:3	Yes	22.468 ± 0.019	21.386 ± 0.027	21.846 ± 0.036	21.541 ± 0.024
D4	6:4	Yes	10.426 ± 0.003	10.213 ± 0.287	10.607 ± 0.295	9.208 ± 0.004
D5	5:5	Yes	3.429 ± 0.001	3.479 ± 0.002	3.479 ± 0.001	3.394 ± 0.001
D6	4:6	Yes	1.489 ± 0.001	1.484 ± 0.000	1.480 ± 0.000	1.492 ± 0.000
D7	3:7	Yes	3.194 ± 0.099	2.594 ± 0.008	3.407 ± 0.107	2.963 ± 0.006
D8	8:2	Yes	1.443 ± 0.001	14.871 ± 3.867	1.498 ± 0.001	1.480 ± 0.001
D9	1:9	No				

and A5 1:5:5:9 (H2O/PG or RISP:Oils:Smix:H2O) were selected. A5 was selected because of its small size, whereas A2 and A4 were selected because of their high content of oils.

Characterization of NEs and NEs-RISP

Particle Size

The selected formulations A2, A4, and A5 were obtained as previously described and size was determined on days 1, 7, 14, and 21 postpreparation. Results are shown in Figure 1. In agreement with that reported by Hung et al.,[29] the particle size increased significantly after the addition of RISP, indicating the inclusion of RISP in the oily phase. In the case of formulation A2, size did not significantly increase over time, showing that it is more stable than others.

After 1 week of storage, three phases were observed in all formulations, evidencing a creaming process.[45] Nevertheless, in all cases, the appearance of the original emulsion was restored by shaking.

Table 2. Globule Size of Formulations Prepared by an UltraTurrax Homogenizer over Time

		Globule Size (μm) ± SD			
Formulation	Method	Day 1	Day 7	Day 14	Day 21
A5	A	4.480 ± 0.545	7.373 ± 0.555	9.207 ± 0.201	9.260 ± 0.278
A5	B	1.898 ± 0.029	1.582 ± 0.002	1.790 ± 0.001	1.889 ± 0.003
A5	C	3.750 ± 1.958	0.515 ± 0.001	1.515 ± 0.001	1.583 ± 0.001
A5	D	9.782 ± 1.079	0.618 ± 0.005	1.480 ± 0.010	1.528 ± 0.006
A5	E	0.701 ± 0.001	0.475 ± 0.000	0.710 ± 0.018	0.670 ± 0.001
A5	F	0.832 ± 0.004	0.488 ± 0.000	0.783 ± 0.001	0.791 ± 0.001

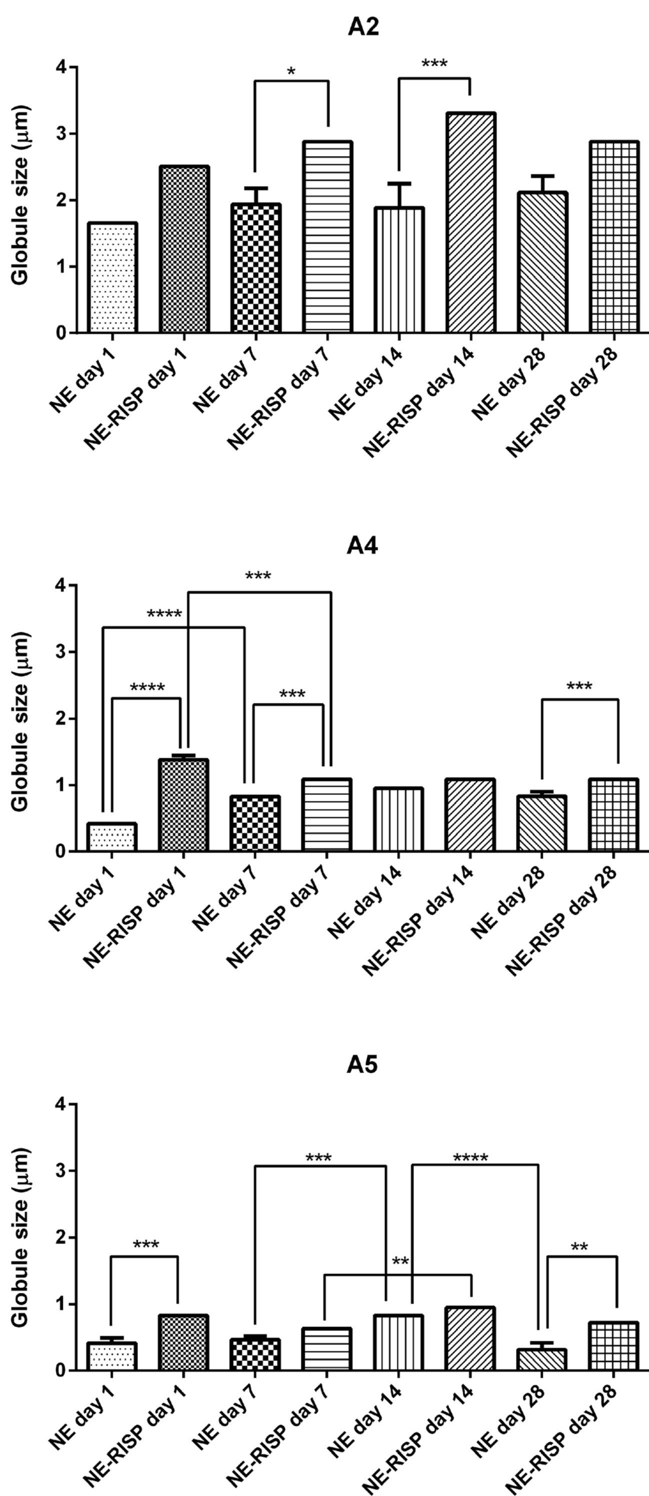

Figure 1. Size of selected formulations over time. Results are shown as mean ± SEM of three independent measurements. Statistical analysis was performed by ANOVA and Tukey's test (* $p < 0.05$, ** $p < 0.001$, *** $p < 0.001$, **** $p < 0.0001$).

Optical microscopy was performed simultaneously with the sizing; results for days 1 and 21 postpreparation are shown in Figure 2. No aggregation was observed in any day of analysis (data not shown).

Determination of Defects of the Oil–Water Interface and ZP

By using the probe MC540, which interacts only with the surfactant layer in the oil–water interface, the HF was calculated. Results are shown in Figure 3. It has been previously reported that when HF is less than 2, the interface is organized, whereas when HF is greater than 2, the interface is disorganized.[39]

In all cases, HF was less than 2, which reveals that the surfactants were organized. Formulation A5 showed an HF of around 1.25, formulation A4 showed an HF of around 1, and formulation A2 showed an HF of around 0.5. So it can be thought that an increase in the surfactant concentration leads to a decrease in the organization of the oil–water interface. On the contrary, RISP incorporation induced variations in the surfactant organization. In the case of formulation A2, the addition of RISP promoted an initial disorganization (day 1), followed by a stabilization process (days 7–21) where no significant changes were found with respect to the NE. In the case of formulation A4, the variations observed in time indicate that RISP induced the organization of the surfactant. Finally, in the case of formulation A5, RISP disorganized the surfactant on day 21. This demonstrates that RISP, which is a lipophilic drug, can be located not only in the oily phase, but also in the interface, giving rise to different patterns of hydrophobicity. Tests conducted with other antipsychotic drugs such as haloperidol, olanzapine, and sulpiride have shown that antipsychotic drugs can show high affinity for biomembranes because of their amphipathic nature.[47]

Zeta potential is defined as the difference in the potential between the particle surface and the electroneutral region of the solution. When ZP is high (25 mV or more, absolute value), the repulsive forces exceed the attractive forces, preventing the coalescence.[24,28,48] Results are shown in Table 3. All formulations showed ZP values lower than −20 mV. The negative charge is provided by phosphatidylserine, phosphatidic acid, phosphatidylglycerol, and phosphatidylinositol (all present in SL) and by PG.[29,32]

Formulations A2 showed stable ZP values, without significant changes when comparing the NE and NE-RISP, indicating stability over time. Also, this formulation showed no increase in size and presented no aggregation processes, indicating that the characteristics of the interface are not affected over time. Formulations A4 showed significant changes in ZP values between the NE and NE-RISP on both days of analysis and, as observed by light scattering and MC540, the particles changed slightly in size and their interface remained fairly organized. Formulation A5 showed significant changes in ZP values between the NE and NE-RISP over time. These changes correlate with a dynamic system in which the interface is constantly changing and were probably because of some instability process that affected the system, as observed by light scattering.

In Vitro Drug Release Studies

The RISP releasing profile was studied by microdialysis. A2 and A5 emulsions were analyzed to compare between formulations with high and low content of fatty acids, respectively. Results are shown in Figure 4.

Formulation A2 with RISP retained a higher amount of drug than the control, whereas formulation A5 released more RISP.

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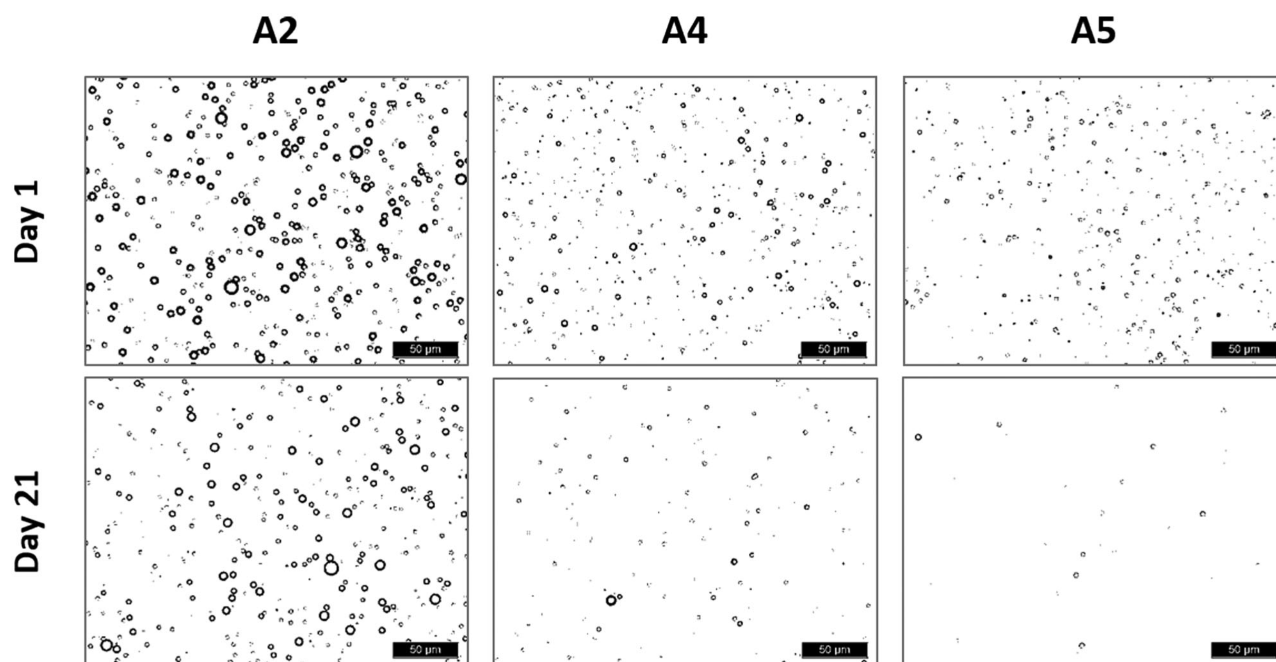

Figure 2. Optical microscopies of formulations A2, A4, and A5 on days 1 and 21 postpreparation.

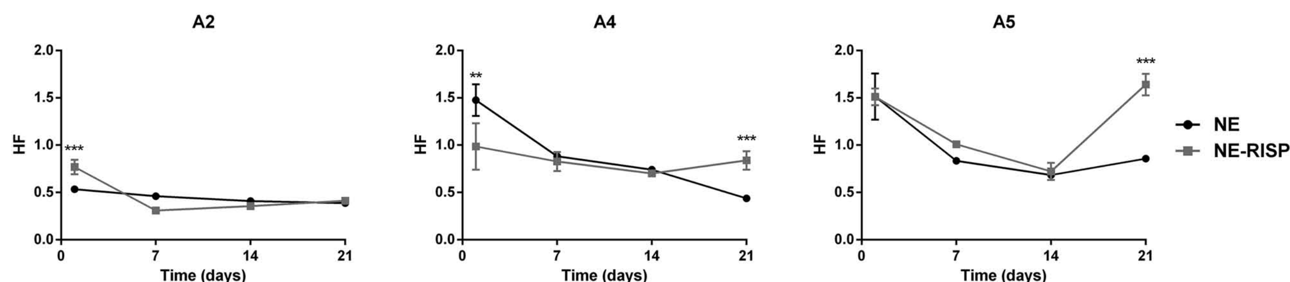

Figure 3. Hydrophobicity factor of samples over time. Results are shown as mean ± SEM of four independent measurements. Statistical analysis was performed by ANOVA and Tukey's test (* $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$).

Subsequently, after 28 h, the release of RISP from A2 was similar to that from the control.

Formulation A2, despite having larger sizes, did not suffer a destabilization process and had high fatty acid content. It presented organized and stable interfaces with negative ZP, without aggregation of the droplets. Finally, it retained more RISP than the control, with subsequent release of it. Therefore, formulation A2 was selected for further testing (*in vitro* and *in vivo* toxicity).

Table 3. Zeta Potential on Days 1 and 21 Postpreparation

	ZP (mV) ± SD	
Formulation	Day 1	Day 21
NE A2	−28.27 ± 5.37	−26.78 ± 5.20
NE-RISP A2	−26.74 ± 5.97	−30.92 ± 4.14
NE A4	−29.15 ± 2.09	−53.95 ± 6.23
NE-RISP A4	−21.41 ± 2.69	−20.36 ± 3.91
NE A5	−35.71 ± 1.13	−47.28 ± 4.22
NE-RISP A5	−15.98 ± 1.23	−29.10 ± 1.14

Results are shown as mean ± SD of five independent measurements.

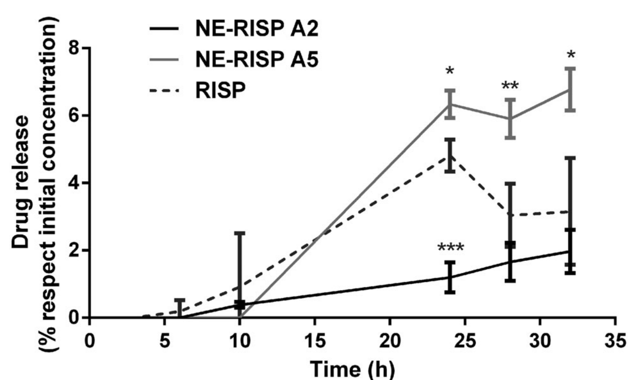

Figure 4. Releasing profile of RISP. The formulations studied were A2 and A5; they were compared against the control (RISP without NE). Results are shown as mean ± SEM of three independent measurements. Statistical analysis was performed by ANOVA and Tukey's test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

In Vitro Toxicity: MTT Assay

The metabolic activity on monolayers of Caco-2 cells was studied by the MTT method. Results are shown in Figure 5. After

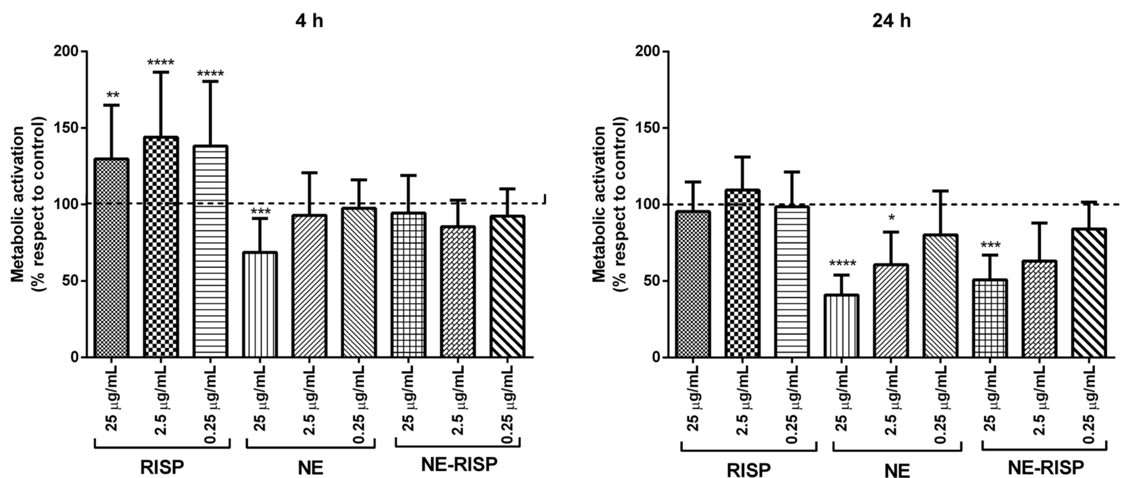

Figure 5. Percentage of metabolic rate respect to the control (cells without treatment) of cells treated with different concentrations of RISP, NE, and NE-RISP (formulation A2). Results are shown as mean ± SD of three independent measurements. Statistical analysis was performed by Tukey's test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

Figure 6. Percentage of spontaneous movement respect to the control (untreated larvae) of larvae incubated with different concentrations of NE-RISP (formulation A2). Results are shown as mean ± SEM ($n = 16$). Statistical analysis was performed by ANOVA and Dunnett's post-test comparing all samples to the control (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

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Table 4. Heart Rates Measured at 7 dpf

Treatment	Concentration (µg/mL)	Heart Rate (bpm)
Control	–	197.2 ± 23.8
NE-RISP A2	0.6250	175.3 ± 39.7
	0.3125	185.3 ± 29.1
	0.1562	200.0 ± 23.1
	0.0780	206.7 ± 30.9
	0.0390	195.1 ± 26.0
	0.0190	204.7 ± 24.5
	0.0098	190.1 ± 15.8
	0.0049	188.4 ± 28.6

Results are shown as mean ± SD of five independent measurements.

4 h of treatment, an increase in cellular metabolism was observed for all RISP concentrations. On the contrary, formulations A2 did not change their metabolic rate, except at the highest concentration without drug, which showed a significant reduction in metabolism respect to the control. This reduction was recovered after 24 h. There was a concentration-dependent effect in the case of NE and NE-RISP. This could be because of their viscosity, which would interfere with a proper metabolite exchange with the medium.[49]

In Vivo Toxicity: Zebrafish

Automated Measurement of Larval Activity Events

Larvae were incubated with various concentrations of NE-RISP at 4 dpf and the spontaneous movement was measured at 4, 5, 6, and 7 dpf (Fig. 6). A concentration-dependent response was observed: at high concentrations, larval movement decreased respect to that of the control; at intermediate concentrations, movement increased showing an excitatory effect; and at lower concentrations, no significant changes were observed with respect to the control. The safe incubation concentration for formulation A2 was less than 0.3125 µg/mL. In zebrafish, 4 dpf coincides with the initial appearance of raphe neurons distributed throughout the entire length of the spinal cord.[43] Therefore, at

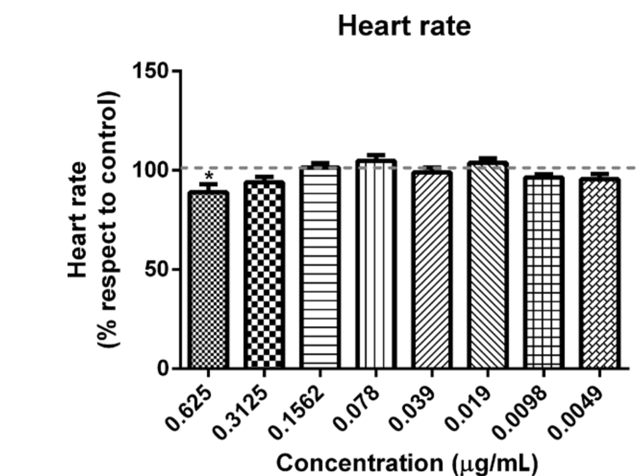

Figure 7. Heart rate of larvae incubated with different concentrations of NE-RISP (formulation A2). Results are presented as percentage of heart rhythms respect to the control (untreated larvae). Data are shown as mean ± SEM ($n = 16$). Statistical analysis was performed by ANOVA and Dunnett's test, comparing all samples against the control ($^{*}p < 0.05$).

this time, the embryos are very sensitive to the action of antipsychotic drugs.

Heart Rate Measurements

Larvae were incubated with various concentrations of NE-RISP at 4 dpf and the heart rates measured at 7 dpf (Table 4; Fig. 7). Only the highest concentration, 0.625 g/mL, showed a cardiotoxic effect, which agrees with the decrease in spontaneous movement previously observed.

Morphological Changes and Hepatotoxicity

At 7 dpf, the treated larvae were photographed to analyze the morphological changes. Results are presented in Figures 8 and 9. Only treatment with 0.625 µg/mL showed a significant

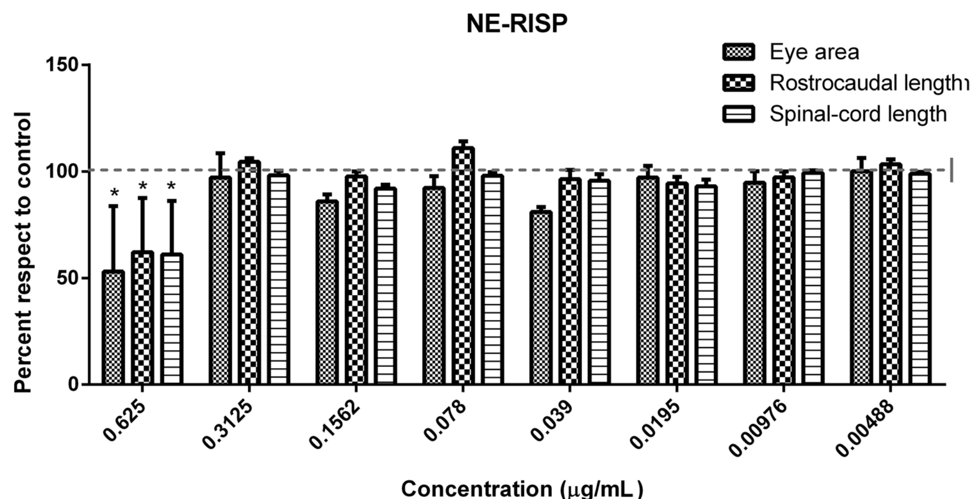

Figure 8. Percentage of larval eye area, rostrocaudal length and spinal cord length compared with the control (untreated larvae) of larvae incubated with different concentrations of NE-RISP (formulation A2). Results are shown as mean ± SEM of five independent measurements. Statistical analysis was performed by ANOVA and Dunnett's test comparing all samples against the control ($^{*}p < 0.05$).

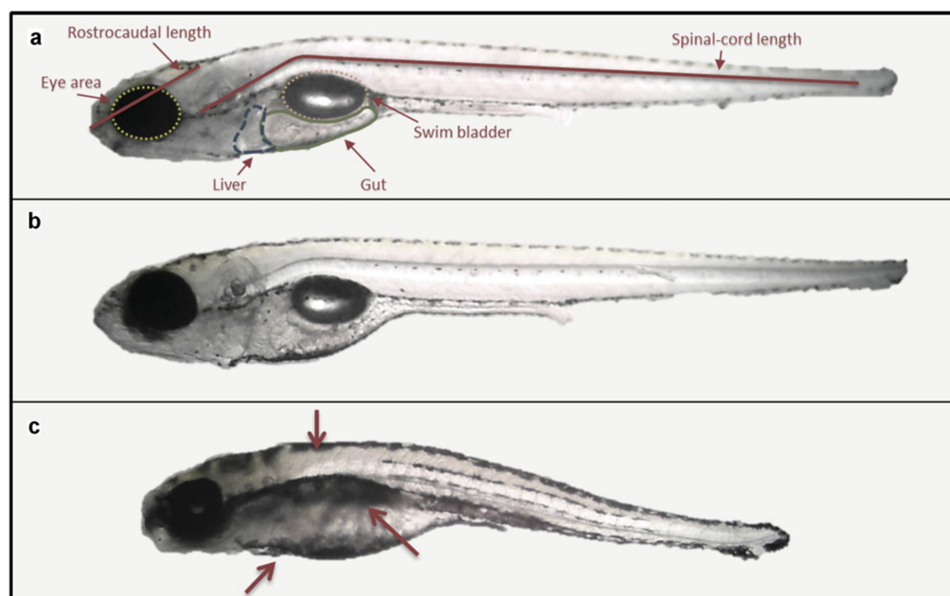

Figure 9. Pictures of larvae of 7 dpf incubated at 4 dpf with (a) 0 μg/mL (control), (b) 0.078 μg/mL (intermediate concentration), and (c) 0.6258 μg/mL (high concentration) of RISP in NE-RISP.

reduction in larval eye area, as well as in the rostrocaudal and spinal cord length. Furthermore, 20% of the larvae incubated at this concentration showed an arched body, whereas 40% showed absence of the swim bladder and the presence of ulcerated tissue. Larvae incubated at intermediate concentrations showed only an arched body. Particularly, 20% of the larvae treated with 0.078 μg/mL showed an arched body (data not shown). On the contrary, none of the treatments used introduced changes in the color of the liver tissue.

These results demonstrate that incubation with 0.625 μg/mL of NE-RISP produces neurotoxic effects (a decrease in the spontaneous movement), cardiotoxic (a decrease in the heart rate), and morphological defects (arched body, ulcerated tissue, and the absence of the swim bladder). Concentrations less than 0.3125 μg/mL showed no significant toxic effects in zebrafish larvae.

CONCLUSIONS

In the present study, we designed a quick and easy method to produce NEs with controlled release. The NE selected was stable in size over time and presented high levels of RISP incorporation. It also had high content of essential fatty acids and was able to properly maintain organization of their interface. In cell culture, the formulation developed was safe at concentrations under 2.5 μg/mL, whereas in the zebrafish animal model it was safe at concentrations under 0.3125 μg/mL. This decrease in the safe level relative to that observed in cell culture may be because the larvae are developing whole organisms, which are more sensitive.

Also, as all the materials used in the preparation were approved for consumption by the FDA, the NE-RISP selected would be easy to incorporate into clinical trials, to test its efficiency in comparison with the current oral treatment, which uses free RISP.

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