

Use of *Caenorhabditis Elegans* and Zebrafish Embryo as an Alternative Model for Biocompatibility Assessment of Natural Latex Biocomposite

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Abstract

The number of animals killed used for research purposes has increased significantly in recent years. For this reason, several alternatives of animal models are widely used. In this study, two phases of calcium phosphates synthesized by the sol-gel method were added by precipitation to the surface of the natural rubber latex membranes for bone applications. Membranes cytotoxicity and toxicity was evaluated by *in vitro* and *in vivo* methods. Hemolysis levels were less than 3%, which indicates that there was no significant hemolytic activity. It was demonstrated that latex presents enzymes that can dissociate osteoblasts MC3T3, so it was necessary to eliminate these enzymes to obtain a good adhesion and cellular proliferation without morphological alterations. The *in vivo* toxicity test with *Caenorhabditis elegans* worms did not show evidence of toxicity with a sample of hydroxyapatite incubated for 5 days, exhibiting the lowest survival rate of the worms. In the Zebrafish embryotoxicity assay, 60% of embryos survived and there was no evidence of embryos with malformation or developmental delay. In summary, alternative animal models, which are not commonly used to assess biocompatibility, have provided reliable *in vivo* results that allow suggesting the use of these membranes in the bone biomedical.

Introduction

Bones and teeth are natural composites formed by collagen molecules linked in linear chains arranged in fiber shapes [1]. Among these molecules, there are small regularly spaced interstitial compartments where nanocrystals of a known inorganic solid, such as calcium phosphate, are present, accounting for 65% of the total bone mass [2]. Calcium phosphates (CaP's) have been widely used in bone regeneration applications because they show osteoconductive and, in some cases, osteoinductive features. The release of calcium and phosphorus ions regulates the activation of osteoblasts and osteoclasts, facilitating bone regeneration [3]. However, like any ceramic material, these compounds have high brittleness and low elasticity, which can impair their mechanical performance after implantation [4].

In this sense, incorporating CaP's into a polymeric matrix can be a possible solution. Combining CaP's with polymers has become a popular method for fabricating composite scaffolds, because CaP's play an active role in increasing cell adhesion and are able to induce differentiation of osteoprogenitor cells [5]. As the number of available polymers is almost unlimited, there is a wide potential for such combinations.

Natural rubber latex (NRL) is extracted from the *Hevea brasiliensis* tree, belonging to the *Euphorbiaceae* family, native to the Amazon basin in Brazil. This material consists of 40 to 45% by weight of polymeric molecules that form the rubber poly(*cis*-1,4-isoprene), 4 to 5% by weight of other organic constituents, such as proteins, lipids and carbohydrates, in addition to 50% of water [6]. The NRL membranes have been widely used in the clinical area, due to the excellent angiogenic properties presents in NRL [7].

Mrué [8] used the NRL membranes for the first time as prosthesis for a segment of the cervical esophagus of dogs, which demonstrated inducing property of tissue regeneration (neo-esophagus). Balabanian et al. [9] implanted latex granules in the alveolar bone cavities after dental extraction in rats

and observed that there was an acceleration of the new bone formation and a decrease in the presence of connective tissue around the implant. These results were further elucidated by Ereno et al. [10] where they used the NRL membranes for bone repair in rabbit calvaria. In this experiment, the NRL membrane was applied on the fracture, which prevented the migration of epithelial and connective tissue and facilitated the action of regenerative cells.

The increase in the number of researches in the biotechnology area has stimulated the search for alternative methodologies for assessing toxicity. Rat and mice are the conventional animal models used for toxicity and pharmacological tests. However, alternative methods seek to reduce the number of animals used in the experiment, as well as the cost of the experiments, since the animals used in the research need to be conditioned, fed and kept in the best possible health and hygiene conditions, otherwise they will not may be used for scientific purposes [11].

Caenorhabditis elegans is a small nematode that can be used as an animal model in biotechnological research. Unlike toxicity tests using cell cultures, *C. elegans* toxicity assays provide data for a complete organism, including systems such as digestive, reproductive, endocrine and neuromuscular, besides presenting 60–80% genome homology with humans [12]. These animals are small (1 mm long), proliferative, with a short lifecycle and have few maintenance requirements, which facilitates the study in a multicellular organism [13].

Another alternative model for the *in vivo* toxicity tests is the embryo test using the *Danio rerio* fish, better known as Zebrafish. Zebrafish genome shares approximately 70% homology with humans, so the bases of embryonic development are fundamentally similar between Zebrafish and mammalian embryos [14]. Due to the transparent nature of the eggs and embryos, Zebrafish allows the visualization of morphological and structural anomalies throughout the animal's body [15]. Despite their apparent simplicity, these models have aided biomedical research, particularly in the functional characterization of novel drug targets that has been identified using genomics technologies. However, their use in the field of biomaterials research has been limited.

In this work, two types of CaP's were synthesized via the sol-gel method (calcium pyrophosphate and hydroxyapatite) and incorporated separately into the NRL membranes. NRL used in this work, are composed of several proteins that can cause allergic reactions, and due to that clarification step was performed to reduce them. Membrane toxicity was assessed *in vitro* by hemolytic and cell culture (MC3T3 osteoblast) tests and *in vivo* using the nematode *C. elegans* and Zebrafish embryos.

Materials And Methods

Natural rubber latex

NRL was purchased from BDF Rubber Latex Co. *Ltd.* (producer and distributor of concentrated NRL, Guarantã, Brazil). This latex was obtained from the mixture of two clones: RRIM 600 and PB 235 (Lot:

Loading [MathJax]/jax/output/CommonHTML/fonts/TeX/fontdata.js liquid phase by the addition of ammonium

hydroxide NH_4OH (Sigma-Aldrich[®], USA), correcting the acidity and stabilizing the medium at pH 10.2 [16]. Subsequently, the polymer was centrifuged at 19.000 rpm for 2 h, aiming to decrease the amount of high molecular weight proteins responsible for hypersensitivity reactions.

Synthesis and characterization of calcium phosphate phases

The CaP's phases synthesis was performed according to the methodology of Dos Santos et al. [17] The reagents used were: calcium nitrate (P.A.) and phosphoric acid (85%) acquired from Sigma-Aldrich[®] (USA), which were dissolved separately in methanol (P.A.) also obtained from Sigma-Aldrich[®] (USA) under stirring for 70 min. The phosphoric acid solution was added to the calcium nitrate solution in an ice bath with stirring for 1 h. The obtained solution was dried at 100°C until solvent evaporation and the obtained powder was fractionated and treated at 400°C to obtain calcium pyrophosphate (CPP) and, at 600°C, for hydroxyapatite (HA), both with a heating rate of 5°C/min for 4 h. The Fourier Transform Infrared spectroscopy (FTIR) spectra were obtained using a spectrometer (VERTEX 70, Bruker[®], USA) in Attenuated Total Reflectance (ATR) mode from 400 to 4000 cm^{-1} at 4 cm^{-1} resolution and 32 scans. Structural analysis was realized by X-ray diffraction (XRD) technique using an X-ray diffractometer (DIFFRACplus XRD Commander, Bruker[®], USA) with Cu ($\text{K}\alpha$) radiation, range at 4°–70° with step size of 0.02 s (2θ) and acquisition time of 3 s. The crystalline phases identification was accomplished by comparing the data obtained with the datasheets of the Crystallographica Search-Match[®] (CSM) database, indexed in the International Center for Diffraction Data. The particle morphology of the CaP's phases was investigated by Field Emission Gun Scanning Electron Microscopy (FEG-SEM, model 7500F, JEOL[®], Japan) at an accelerating voltage of 20 kV.

NRL/calcium phosphate membranes preparations

The membranes were obtained depositing 5 mL of NRL on the circular plate, maintaining them at room temperature (25 °C) for 24 h (NRL-m). After the latex polymerization, 10 mL of CPP, dissolved in water (10 mg/mL), were added to the NRL membrane and kept at 50°C for 72 h (CPP-m). The same procedure was performed with 10 mL of HA; (HA-m) with final concentrations of 10 mg/mL. The membranes surface was visualized by FEG-SEM (model 7500F, JEOL[®], Japan) and Energy Dispersive X-ray Spectroscopy (EDX) at an accelerating voltage of 20 kV.

In vitro toxicity assays

Samples preparations for cytotoxicity test

The NRL-m, CPP-m and HA-m membranes were incubated in phosphate buffered saline solution (PBS) obtained from Sigma-Aldrich[®] (USA) at pH = 7.4 in the proportion 0.2 mg of samples per mL of PBS, during 24 h and 120 h. For the *in vitro* toxicity assay with osteoblasts, the α -Modified Eagle's Medium (α -MEM, LGCBio, Brazil) was used instead of the PBS solution. After the incubation period, the PBS

solutions (or α -MEM) were collected for later use in *in vitro* and *in vivo* toxicity tests. These solutions were called eluates and identified as: NRL-e24, NRL-e120, CPP-e24, CPP-e120, HA-e24 and HA-e120.

Hemolysis assay

The protocol was carried out according to Onuma et al. [18] where sheep blood cells (Newprov®, Brazil) were used in this assay. 50 μ L of the NRL-e24, NRL-e120, CPP-e24, CPP-e120, HA-e24 and HA-e120 were incubated together with 50 μ L of a 5% (w/v) erythrocyte solution (0.9% (w/v) saline solution) at 37°C for 1 h. After this period, the eluates were centrifuged at 3000 rpm for 3 min and 50 μ L of the supernatant were pipetted into 96-well microplates. The absorbance measurements at 540 nm were determined using a microplate reader (PowerWave Epoch2, BioTek Instruments®, USA). The 100% hemolysis value was defined using the positive control (50 μ L of PBS solution with 100 μ L of 1% (v/v) Triton X-100 (Sigma-Aldrich®, USA), while the 0% hemolysis value was obtained using the negative control (10 μ L of PBS solution) [19]. The hemolysis rate was calculated using following Eq. (1):

$$\text{Hemolysisrate}(\%) = \frac{ABS_{\text{sample}} - ABS_{\text{negativecontrol}}}{ABS_{\text{positivecontrol}} - ABS_{\text{negativecontrol}}}$$

1

ABS_{sample} is absorbance of the samples, $ABS_{\text{negative control}}$ is absorbance of negative control, and $ABS_{\text{positive control}}$ is absorbance of positive control.

Cell viability assay

Cytotoxicity assays were performed with MC3T3 osteoblasts obtained from LGCBio (Cotia, São Paulo, Brazil) and according to International Standard Organization (ISO) 10993-5 recommendations.^[19] Cells were cultured in α -MEM (Cell Resource Center) supplemented with 10% fetal bovine serum (Thermo Fisher®, USA) and 1% penicillin/streptomycin (M&C Gene Technology®, China) at 37°C in a fully humidified atmosphere with 5% CO₂. A total of 2x10⁴ cells/cm were seeded onto 96-well plates placed under cell culture conditions and incubated with 100 μ L of samples eluates (NRL-e24, NRL-e120, CPP-e24, CPP-e120, HA-e24 and HA-e120) for 48 h in an incubator at 37°C. After these times, the culture medium was removed and 10 μ L of MTT reagent [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] (Sigma-Aldrich®, USA) was added (5 μ g/mL in PBS; pH = 7.2). After 4 h, the supernatants were removed and 100 μ L of dimethylsulfoxide (DMSO) (Synth®, Brazil) was added in order to dissolve the formation of crystals and the absorbance at 570 nm was read using a microplate reader (PowerWave Epoch2, BioTek Instruments®, USA). DMSO was used as positive control and as a negative control, polystyrene culture plate. The experiments were fulfilled in triplicate.

Adhesion assay on the surface of membranes

The NRL-m was incubated with culture medium for 48 h to extract and neutralize the enzymes that make the adhesion assay impossible and after that time, the medium was discarded [20]. A new complete

medium enriched with the CPP and HA (1 mg/mL) was added to the pure NRL-m. After 48 h, the medium was carefully removed from the corner of the wells so as not to extract the phosphates that precipitated on the membrane surface. The membranes were sterilized by ultraviolet light for 20 min. The adhesion assay was realized in 24-well plates, seeding 2×10^4 cells/cm of MC3T3 osteoblast directly on the membranes surface and cultivating with the α -MEM medium for 48 h. The membranes were washed with PBS solution (pH = 7.2), fixed in 2.5% (v/v in PBS) glutaraldehyde (Sigma-Aldrich[®], USA) for 1 h at room temperature. Subsequently, the samples were dehydrated in increasing series of ethanol (50%, 60%, 70%, 80%, 90% and 100%) and dried at room temperature. The analyzes were evaluated by FEG-SEM (model 7500F, JEOL[®], Japan) at 20 kV.

In vivo toxicity assays

Toxicity assay in *Caenorhabditis elegans*

C. elegans worms of wild-type strain N2 were synchronized by treatment with sodium hypochlorite (Sigma-Aldrich[®], USA) and the 4th larval stage (L4) was used for the experiment. The worms were washed with NaCl 50 mM (Sigma-Aldrich[®], USA) and about 20–25 worms were added to the wells of 96-well plates containing 54 μ L NaCl 50 mM, 36 μ L Brain Heart Infusion medium, 10 μ g/mL cholesterol, 90 μ g/mL kanamycin and 200 μ g/mL ampicillin. 100 μ L of samples of NRL-e24, NRL-e120, CPP-e24, CPP-e120, HA-e24 and HA-e120 were added to the wells and the plates were kept at 25 °C for 24 and 48 h. Toxicological results were analyzed by worm shape and mobility (rod-shaped worms were considered dead and sinusoidal worms were considered alive) and by nematode reproduction. The visualization was carried out using a Zeiss[®] Discovery V12 (Germany) microscope attached to a camera. The statistical analysis of the results was performed using the GraphPad Prism 5[®] software (San Diego, CA, USA).

Embryotoxicity assay in *Danio rerio* (Zebrafish)

Zebrafish were raised according to standard protocols ($28 \pm 0.5^\circ\text{C}$ with 14:10 day/night photoperiod). Zebrafish embryos used for chemical exposure were obtained from spawning adults in appropriate tanks overnight with a male/female ratio of 2:1. Embryos were collected within 1 h post fertilization (hpf) and were transferred to 96-well plates. 100 μ L of NRL-e120, CPP-e120 and HA-e120 samples were tested in the static mode and embryonic medium (NaCl; KCl; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.03% methylene blue) was used as control. Plates were maintained at 25°C. The development status of Zebrafish embryos was observed with a Zeiss[®] Discovery V12 (Germany) microscope attached to a camera at 24, 48, 72 and 96 hpf, respectively.

Results And Discussion

Characterization of calcium phosphate phases

In this study, the choice of the sol-gel method for the CaP's synthesis was based on the simplicity of the Loading [MathJax]/jax/output/CommonHTML/fonts/TeX/fontdata.js structure, aiming at the *in vivo*

biomineralization process. Using the same chemical precursors and different temperatures, it is possible to obtain two different CaP's phases, namely: CPP and HA, with properties needed as biomaterial, such as osteoinductivity, bioactivity and nontoxic.

Figure 1A shows the FTIR analysis for CPP and HA. The CPP spectrum presents characteristic bands of OH stretching and H-OH deformation of H₂O at 3434 cm⁻¹ and 1646 cm⁻¹, respectively. Several bands observed at 1131, 1018 and 926 cm⁻¹ were attributed to the symmetric P-O stretching of PO₄³⁻ groups. The peak at 555 cm⁻¹ corresponds of the O-P-O deformation (PO₄³⁻). The existence of all these bands indicates the CPP formation [17]. In the HA spectrum, the water absorption appears around 3638 cm⁻¹ as stretching vibrations of OH groups. We observed the characteristic absorbance of the PO₄³⁻ asymmetric deformation between 1029 cm⁻¹ and 1100 cm⁻¹ and around 874 cm⁻¹, associated with the P-OH stretch in HPO₄²⁻. The band near 600 cm⁻¹ corresponds to the asymmetric deformation of P-O bond in the phosphate network. Whereas the peak at 562 cm⁻¹ is related to the asymmetric deformation of P-O (H) bond [21]. The XRD patterns (Fig. 1B) confirmed the existence of the CaP's phases obtained in each heat treatment performed in the sol-gel precipitate [17]. Both samples revealed broad peaks at 2θ = 20–40°, characteristic of nanocrystalline phases. The intense peaks at 2θ = 25°, 32° and 35° indicated that product obtained at 400°C was Ca₂P₂O₇ base on CSM card #96-100-1557. The thermal treatment at 600°C confirmed the formation the Ca₅(PO₄)₃OH, identified by the peaks 26°, 31° and 33° (card #96-900-1234). In addition, morphological characterization was carried out (Fig. 1C). The CPP micrographs showed filiform particles grouped in the form of agglomerates with a size range of 170–650 nm, while the particles of HA presented sizes between 110–620 nm and hexagonal shape.

Calcium phosphate incorporation into the NRL membranes

The NRL membranes were prepared according to the Sect. 2.3. The proteins responsible for the allergic reactions of NRL have a molecular weight of about 14 kDa, and due to that, they can be easily separated by clarification unit (centrifugation), [22] where reduction of 27% of the proteins were achieved.

The incorporation process of CaP's phases was fulfilled by precipitation, unlike the casting method commonly used in the literature [23–25]. Figure 2 (A-C) shows SEM micrographs of the surfaces of the NRL membranes without and with the inclusion of the phases containing CPP and HA. The pure NRL membrane (NRL-m) exhibited a smooth surface, with evidence of some irregularities (Fig. 2A), which can be explained by the fact that, in solution, some of the NRL proteins are released and, after drying, they are precipitated on the surface [26]. With a magnification of 2000x, it was possible to observe that the CPP particles are homogeneously dispersed in the NRL matrix (CPP-m) (Fig. 2B). In the micrograph of the membrane incorporated with HA (HA-m), a heterogeneous layer of HA was denoted on the material surface (Fig. 2C). The lower uniformity of the HA particles in the HA-m membrane in relation to the CPP-m membranes may be related to the different solubility of each CaP's phase, because the higher the Ca/P ratio, the lower the solubility in water [27]. HA has the highest Ca/P ratio with a value of 1.67, while CPP

Loading [MathJax]/jax/output/CommonHTML/fonts/TeX/fontdata.js makes CPP easier to be adsorbed by the

polymeric membrane. To confirm the presence of CPP and HA particles in the membranes, we used the EDX technique coupled to the SEM. In Fig. 2 (D-E), the EDX spectra are demonstrated and the presence of both chemical elements Ca and P, constituents of CPP and HA, can be observed on the membranes surface.

The obtaining of CaP's-NRL composites with different CaP's has been the object of study by different researchers [28, 29]. However, there are few studies that have precisely explained the molecular interactions between the non-rubber constituents of NRL and inorganic particles. However, it is known that, on the CaP's particles surface, there are Ca^{2+} ions that are attracted by the negative charges found in the NRL proteins and lipids.

In vitro toxicity assays

Evaluation of hemolytic activity

Hemolytic activity assay was accomplished to assess whether the components released by the samples NRL-e24, NRL-e120, CPP-e24, CPP-e120, HA-e24 and HA-e120 are able to rupture the red blood cell membrane, causing hemoglobin release [16, 30]. Figure 3 shows the positive control with red color, indicating that hemolysis occurred due to the release of hemoglobin, and the transparent samples (negative control and other samples), where there was no hemolysis. According to Fischer et al. [31] rates below 10% of hemolysis suggest that the samples are considered non-toxic. The released hemoglobin percentages were calculated and it was proved that the eluates at 24 and 120 h exhibited hemolysis levels less than 3%, confirming that there was no damage to the red blood cell membrane and the type of CaP's did not influence hemolytic activity. Our results are complemented by those obtained by De Barros et al. [32] who tested the hemolytic activity of the NRL membranes with oxytocin, and values below 5% were achieved. Floriano et al. [33] also verified that the NRL membranes coated with CaP's and with ketoprofen respectively, did not present hemolytic effect.

Cytotoxicity assay in osteoblasts

The *in vitro* test was performed with MC3T3 osteoblasts. The samples NRL-e24, NRL-e120, CPP-e24, CPP-e120, HA-e24 and HA-e120 were placed in indirect contact with the cells for 24 and 48 h. After 24 h of plating, it was found that the cells were adhered and the culture medium was replaced. The MTT assay was carried out at 48 h and it was observed that all samples, except the positive control (100%), demonstrated percentage values of cell viability less than 50%. This result indicates that the eluates tested showed cytotoxic behavior, which is in contradiction with the countless studies in the literature that demonstrate the excellent biocompatibility and bioactivity *in vivo* of NRL [9, 10, 34, 35].

However, in this study, biocompatibility assays were evaluated in the serum (eluates) not in the NRL membranes. It is known that NRL contains proteolytic enzymes, such as serine and cysteine proteases, [16, 36–38] which can influence the cell viability assay. To prove this hypothesis, a cell culture test was accomplished using only NRL serum previously centrifuged (at 19000 rpm), and after 5 min of

incubation, it was microscopically verified that there was no evidence of cell adhesion and the MC3T3 osteoblast cell body was dissociated. This result indicated that the proteolytic enzymes present in NRL may be able to dissociate the MC3T3 cells, causing the low values of cell viability obtained in the MTT assays. Borges et al. [20] obtained similar results when studying the eluate of the incubation of the NRL membrane without dilution, exhibiting a cell viability of approximately 48%.

From these results, a new cell adhesion test was realized. At this time, to eliminate the enzymes that prevent adhesion, the membranes were prepared according to Sect. 2.7. The micrographs of the membranes surface after 48 h of culture (Fig. 4(A-C)) revealed that the cell line established points of adhesion and the cells did not undergo morphological changes. The existence of lamellipodia and filopodia of osteoblasts (red arrows), as well as intercellular connections (blue arrows), indicated that there was a good adhesion of osteoblasts to the membranes surface [20]. In tissue engineering, in addition to cell adhesion, cell proliferation and differentiation are necessary to guarantee greater bioactivity of the material [39]. In this study, it was possible to observe the cell division in the NRL-m, CPP-m and HA-m membranes (Fig. 4 (D-F)) through the FEG-SEM analysis, suggesting the cell proliferation qualitatively.

According to Deligianni et al. [40] osteoblasts present better proliferation, adhesion and mineralization on rough and sandy surfaces. Thus, the CaP's phases are excellent candidates for polymer coating, because, in addition to these characteristics, CaP's are formed by the main bone constituents, bonding chemically and directly to the bone, resulting in a better bone repair. Figure 4 (D-F) exhibits a close connection between clusters of CPP and HA and osteoblasts on the NRL membranes surface (green arrows). These results suggest good cell viability on the membranes. Finally, the *in vitro* biocompatibility of the prepared membranes did not show appreciable differences between them.

In vivo toxicity assays

Toxicity assay in *Caenorhabditis elegans*

The toxicity assay using *C. elegans* followed the methodology described in Sect. 2.4.1 where all membranes were incubated separately in PBS solution for 24 and 120 h. The eluates resulting from these incubations were placed in contact with the worms and analyzed after 24 and 48 h. Figure 5 (A-C) reveals the percentages of survival, where in the first 24 h of the experiment, all samples did not significantly reduce the survival of *C. elegans* worms. After 48 h of assay, the survival rates of the animals exposed to the samples were analyzed again and the eluates NRL-e24, NRL-e120, CPP-e24, CPP-e120 and HA-e24 did not show mortality. However, the worms exposed to the HA-e120 sample presented toxic effects, with only 42% survival.

In this study, the toxicity also was verified by the worm development and by monitoring the body shape. Live worms (sinusoidal shape) were observed for all samples and a predominant amount of dead worms were identified by their rod-shape in the HA-e120 sample (Fig. 5 (D-F)). The toxicity of this sample may be

Loading [MathJax]/jax/output/CommonHTML/fonts/TeX/fontdata.js eluate (5 days). As previously mentioned, the

difference in the Ca/P ratio between the two CaP's resulted in a lower solubility for HA, which caused the HA particles to not be fully adsorbed on the NRL membranes surface (Fig. 2C), being easily released in the HA-e-120 eluate. Ca^{2+} plays an essential role in physiological processes and in cell proliferation or differentiation. Alvarez et al. [41] summarized evidence from various experiments on *C. elegans* worms that helped to understand the role of Ca^{2+} in living systems. However, their results showed that the system is quite complex and there is only a fine line that separates the levels of calcium flows that can be considered normal from those that can lead to pathology. Therefore, an excess of Ca^{2+} ions in the HA-e120 eluates (after 5 days of incubation) may have induced the death of a greater number of worms. Nematode reproduction was also evaluated (Fig. 5E) and in all samples, except HA-e120, the presence of the 1st larval stage (L1) from the 4th larval stage (L4) reproduction indicates the non-toxic character of these samples.

Embryotoxicity assay in *Danio rerio* (Zebrafish)

A second *in vivo* model was used to investigate the biocompatibility of the eluates incubated for 5 days (NRL-e120, CPP-e120 and HA-e120). Recently, Zebrafish proved to be an ideal model for studying biocompatibility and bone disease [42]. Zebrafish embryos were analyzed by determining different physiological parameters, such as percentage of viability and phenotypic morphological alteration. Figure 6A shows the survival rate of the three samples and the control in relation to the contact time with the embryos. The samples were obtained employing the same methodology used in the *C. elegans* assay and the eggs were exposed for 24, 48, 72 and 96 hpf. Quantitatively, all samples, including the control, exhibited dead embryos from 24 hpf. The survival rate in the solvent control was approximately 76%, which was reduced to nearly 60% when the embryos were incubated in the studied eluates. It was noted that the reduction was not dependent on the exposure time or the type of eluate. These results suggest low embryotoxicity, as the values obtained by the eluates were near to those of the control sample (containing only embryonic medium, without embryos), which is widely used as control in this type of assay [43].

In view of these results and knowing that the embryonic stages are extremely sensitive for the determination of toxicity, [15] the number of live, dead, delay in development and deformed embryos was counted. This study showed only the existence of live or dead animals (more than 50% of Zebrafish embryos were found alive) and none of the tested samples induced deformation or delay in embryonic development (Fig. 6B). This result was supported by the analysis of the images obtained from the development of live embryos. Through the transparency of the embryos, it was possible to observe different stages of normal development, according to the phases of gastrula, pharynx, early larvae and larva (Fig. 7(A-D)), which were found in all samples [44]. The certification that the embryos were alive was carried out through microscopic observation of the sequence of development and movement of the embryos. The existence of worms with curvature of the tail and pericardial edema were considered dead.

These results are in line with those reported by Makkar et al. [45] who used the Zebrafish model to study [the biocompatibility of two dental bioceramics, one of which is the mineral trioxide aggregate \(MTA\)](#)

composed mainly of inorganic elements containing calcium. The eluates of MTA contain silicates, aluminates and calcium oxides, which facilitated the increase in abnormal metabolic activity in embryonic cells, causing a percentage of embryonic deaths from oxidative stress and cellular apoptosis.

Conclusion

In this work, the sol-gel method was used to obtain two different CaP's that were incorporated into the NRL membranes. The cytotoxicity of the samples was tested by conventional *in vitro* methods, in addition to two alternative *in vivo* models that have so far been little explored to evaluate the biocompatibility of biomaterials. The membranes were obtained by the precipitation method of the CaP's phases in the NRL matrix and, by means of FEG-SEM micrographs, it was observed that the CaP's phases were incorporated into the membrane surface. The hemolytic activity assay did not confirm cell damage, with hemolysis level less than 3%. The cytotoxicity assay by MTT in MC3T3 osteoblasts was not efficient, because enzymes contained in NRL dissociated the cells, making the test impossible. Therefore, it was necessary to wash the pure NRL membranes with complete α -MEM medium and then incorporate the CaP's phases. After these procedures, it was observed that the cells adhered to the surface of all membranes with intimate contact through lamellipodia and filopodia. The *in vivo* biocompatibility study using the *C. elegans* nematode did not demonstrate significant toxicity in the samples incubated for 24 and 48 h. During the larvae counting, the worm reproduction capacity was observed, due to the presence of L1 larvae from the reproduction of L4 larvae. The embryotoxicity of all samples was tested using embryos of the *D. rerio* fish (Zebrafish) and it was found that all samples contained live and dead embryos, without malformation or developmental delay, suggesting low embryotoxicity. The alternative animal models used in this study allowed assessing the *in vivo* biocompatibility of the studied membranes, indicating their potential to be used in biomedical applications.

Declarations

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Conflict Interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Figures

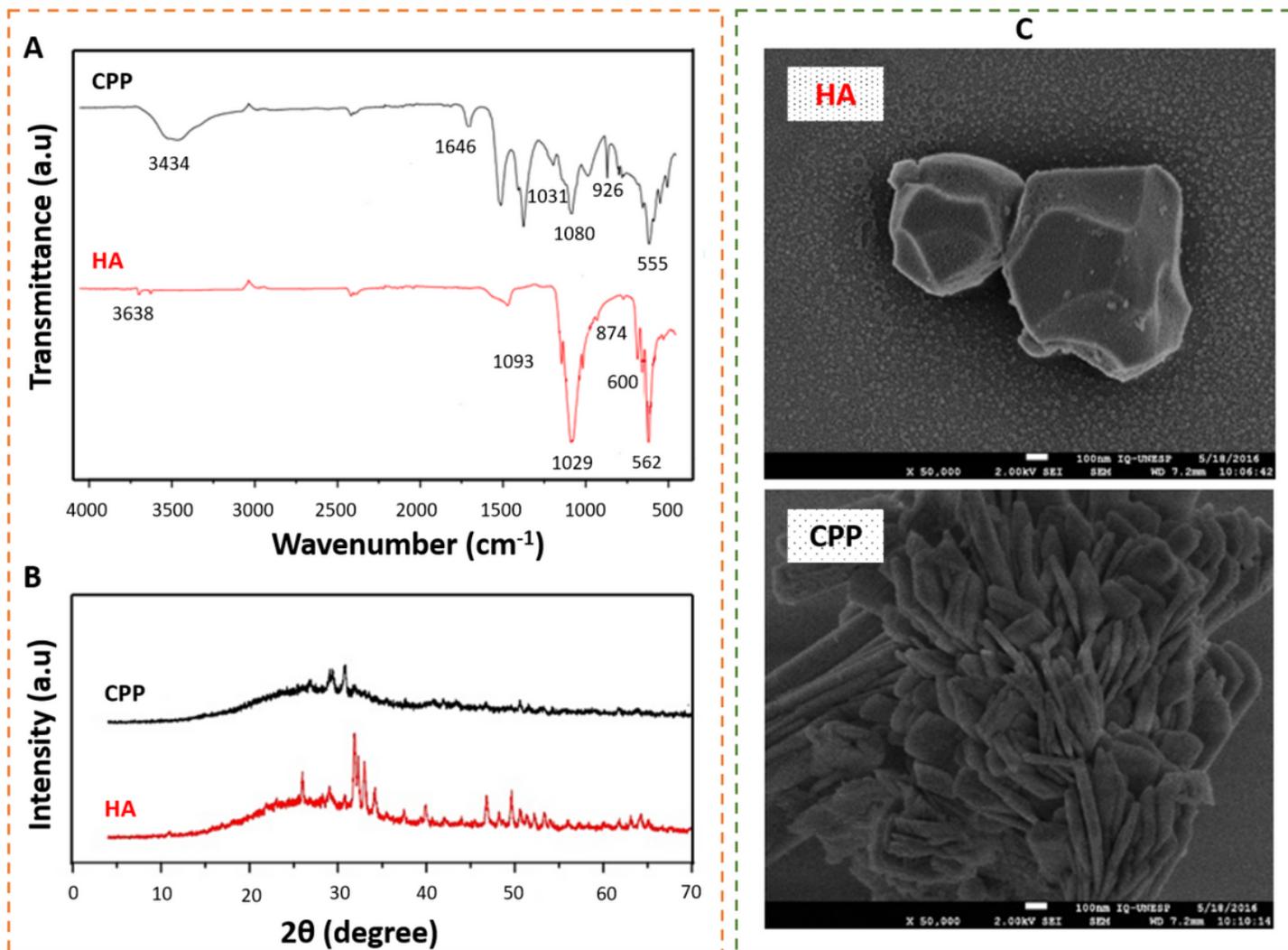


Figure 1

(A) FTIR spectra of CPP and HA, (B) XRD patterns of CPP and HA and (C) SEM micrographs of the CPP and HA particles.

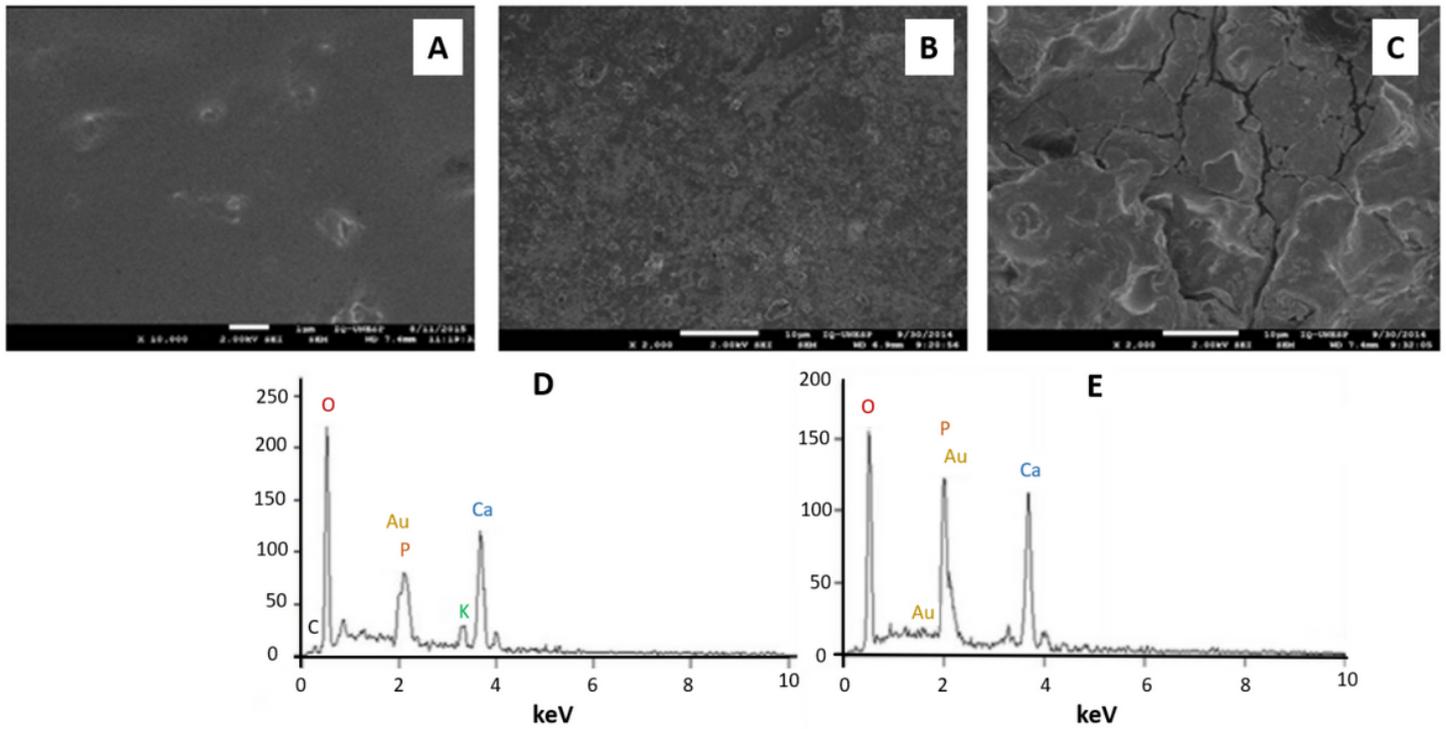


Figure 2

SEM micrographs of the surfaces membranes: (A) NRL-m, (B) CPP-m and (C) HA-m membranes. EDX spectrum: (D) CPP-m and (E) HA-m.

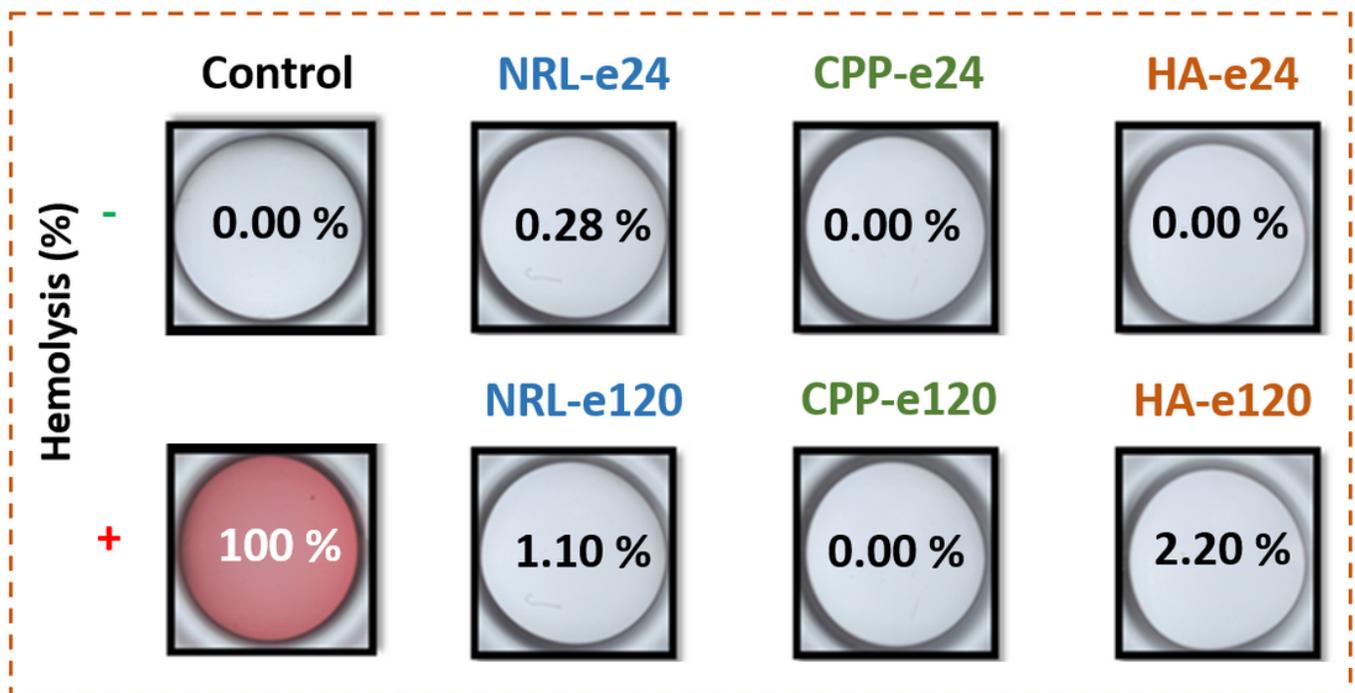


Figure 3

Hemolytic activity of the different eluates evaluated in this study.

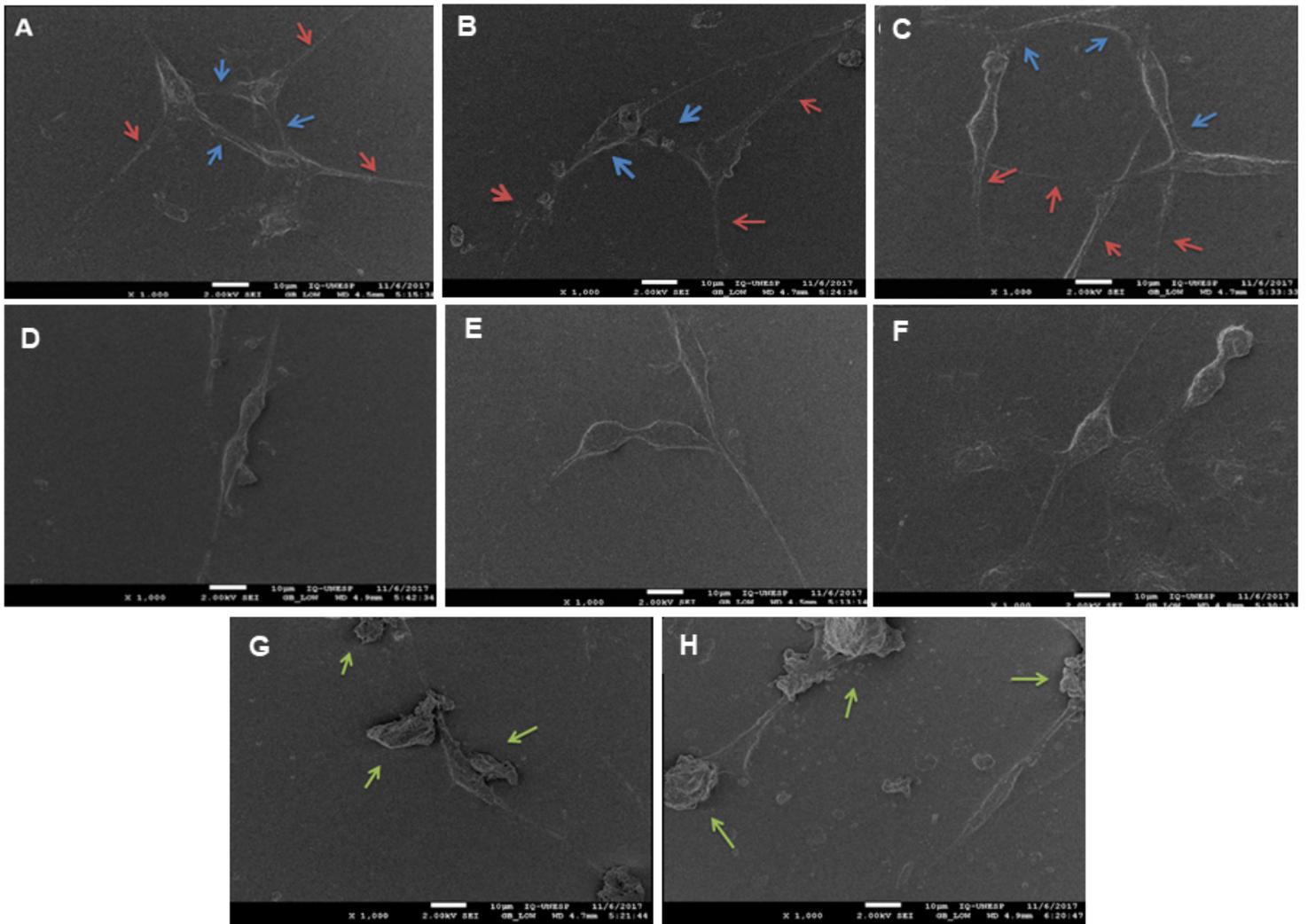


Figure 4

SEM micrographs of the MC3T3 cells seeded on (A) NRL-m, (B) CPP-m and (C) HA-m. Osteoblasts cells divisions on (D) NRL-m, (E) CPP-m and (F) HA-m. Intimate bonds between (G) CPP-m and (H) HA-m agglomerates and osteoblasts.

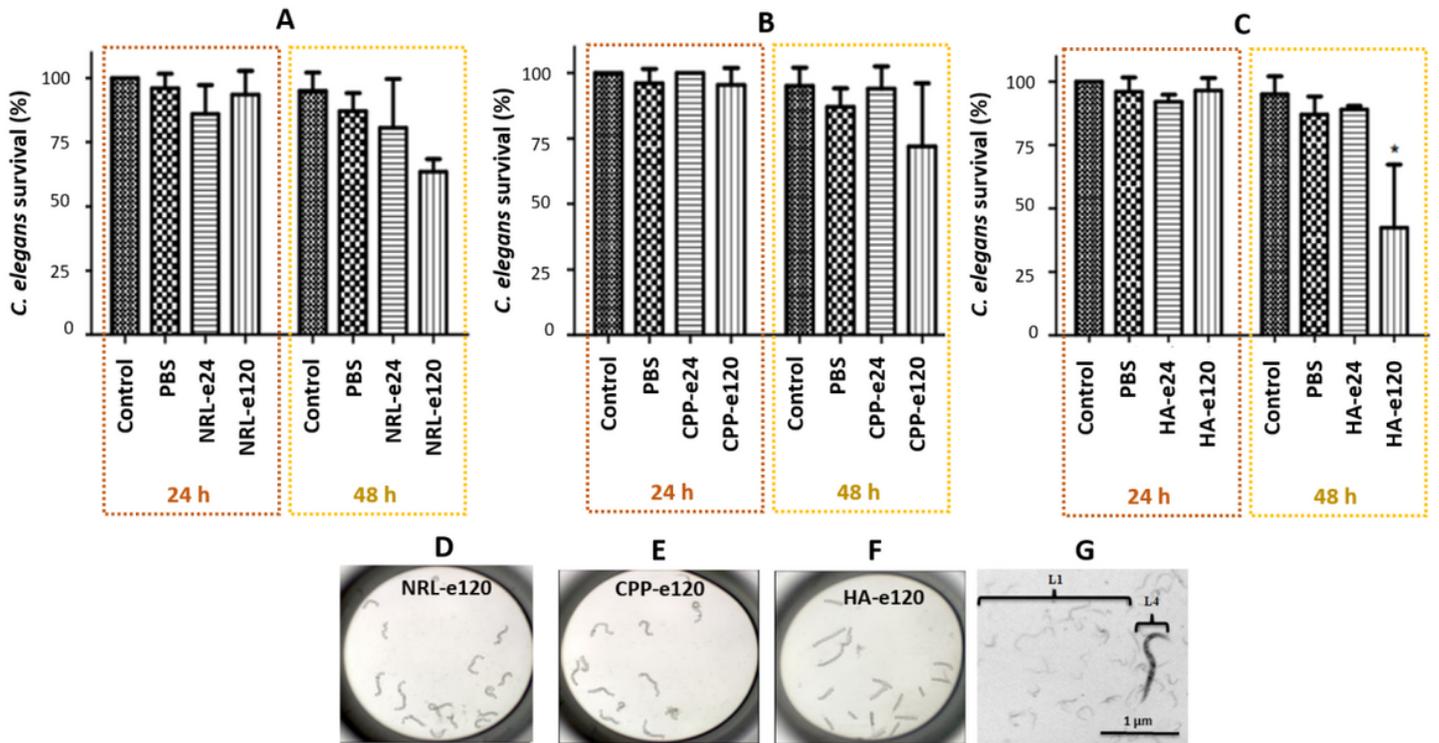


Figure 5

Survival rate of *C. elegans* worms exposed for (A) NRL-e, (B) CPP-e and (C) HA-e at 24 and 48 h. Optical images of *C. elegans* worms: (D) NRL-e120, (e) CPP-e120, (F) HA-e120 and (E) 1st larval stage (L1) from 4th larval stage (L4) reproduction.

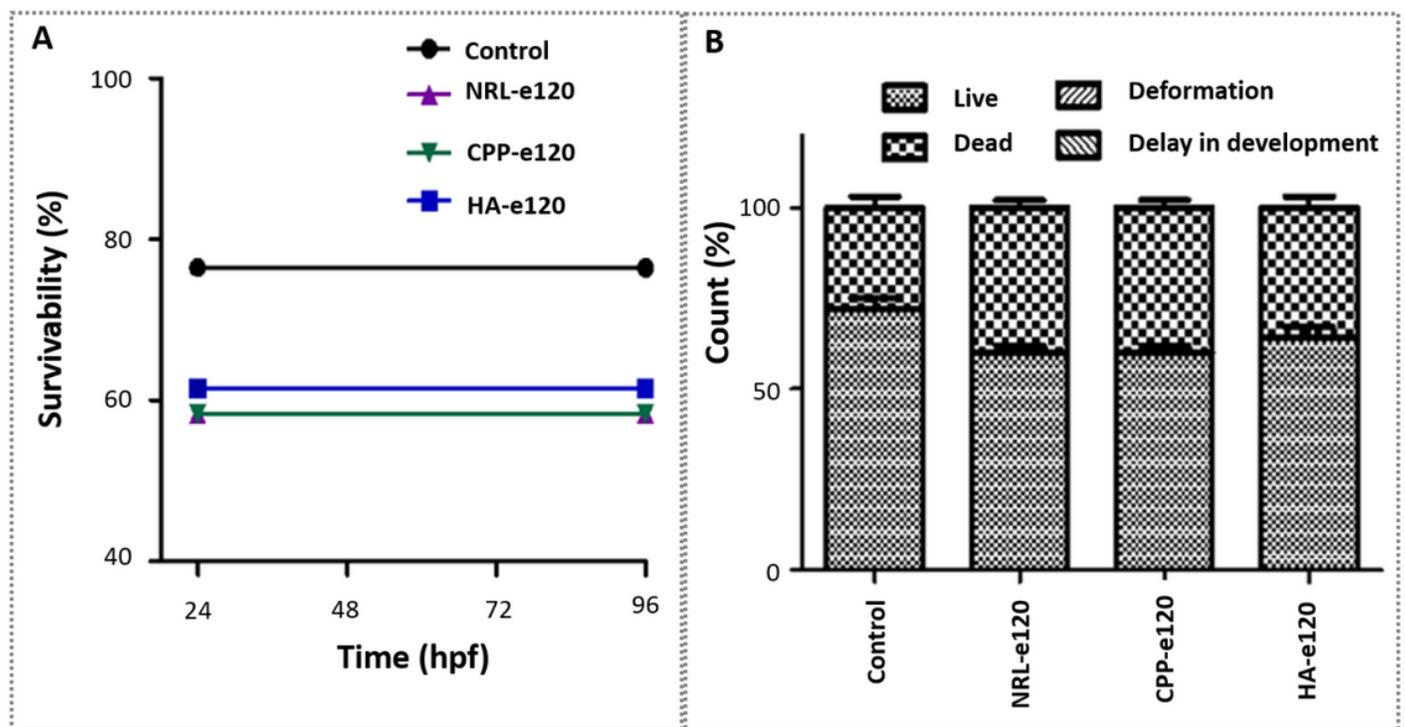


Figure 6

(A) Survivability of embryonic Zebrafish exposed to control sample, NRL-e120, CPP-e120 and HA-e120 and (B) average the embryos number of live, dead, delayed in development and deformed counted in each sample after 96 hpf.

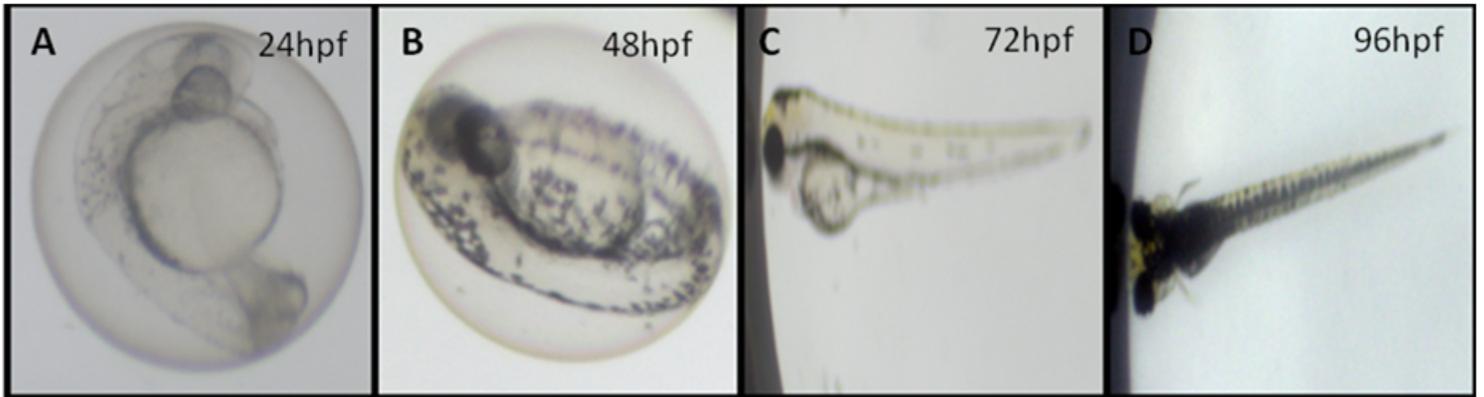


Figure 7

Morphological and anatomical changes in Zebrafish embryos incubated with NRL-e120, CPP-e120 and HA-e120 for different time: (A) Gastrula, (B) Pharynx, (C) Early larvae and (D) Larvae.