A nanotoxicological approach to the effects of metformin and sodium metavanadate co-encapsulated in polycaprolactone nanoparticles under the biological parameters of *zebrafish (Danio rerio*)

ABSTRACT

The present study evaluated the sublethal and lethal effects, and heart rate of *Danio rerio* when exposed to antidiabetic drugs, metformin (M-10), and sodium metavanadate (V-10), their association (MV-10) and the polycaprolactone nanoparticles containing poloxamer 188 with (PPMV-1, PPMV-5, and PPMV-10) or without these antidiabetics (PP-10). Acute toxicity tests were carried out to evaluate these effects. Groups V-10, MV-10, PPMV-5, PPMV-10, and PP-10 had lethal and sublethal, such as effects as pericardial edema. Concerning the heart rate, the PPMV-1, PPMV-5, PPMV-10, and PP-10 groups had a reduction compared to the other groups, indicating toxicity of the constituents of the nanoparticles. PPMV-5, PPMV-10, and PP-10 groups had sublethal and lethal effects depending on the concentration. Antidiabetics were eliminated as a possible cause and the poloxamer 188 is non-toxic at the concentrations used. In the PP-10 group, there was a dynamic of sublethal and lethal effects like the PPMV-10 group. We conclude that the presence of PCL in the formulation of nanoparticles was harmful at the cardiovascular level in embryos and *eleuthero-embryos* of *D. rerio*, affecting their development and heart rate, regardless of their concentration.Because of the results obtained, we can conclude that the toxicological evaluation of a nanomaterial is important to anticipate problems in its veterinary application since such results show possible toxicity problems.

Keywords: PCL, toxicology, animal model, nanomaterial, antidiabetics.

1. INTRODUCTION

Nanotechnology currently offers significant contributions with applications to animal production and veterinary medicine, aiding in the prevention and treatment of diseases and applying new therapies that increase longevity and improve the quality of life of animals (Bai et al., 2018). About 25,000 patents and 2,000 articles on the application of nanotechnology in veterinary medicine have been filed and published to date (Lens, 2021), indicating a growing interest in the application of nanotechnology in this field. Among the areas of nanotechnology activity, there is research in the development of nanomaterials that have useful chemical and physical properties, which have attracted the attention of researchers. These nanomaterials have been widely explored for the controlled release of drugs and can be of different types such as metal-based, carbon-based, and polymeric. The latter are colloidal particles with a size between 10-500 nm. They are considered to have high

biological safetyand biodegradability, increasing the stability of antigens and drugs and improving bioavailability. However, due to this small size, it is necessary to consider the risk of accumulation of nanomaterials in different tissues (Han et al., 2018; Bueno, 2020). Therefore, it is important to assess the toxicity of polymeric nanomaterials such as those used in the production of nanoparticles (NPs), as well as the pharmaceutical excipients of these formulations (Hering et al., 2020). The toxicity of nanoparticles is defined by the field of nanotoxicology, which consists of understanding the interaction of nanoparticles with organisms in various ways, such as the interrelationship with fluids and tissues that can cause changes in cardiac functions or binding to mediators that can activate inflammatory responses (Tiple et al., 2020).

In the field of veterinary medicine, polycaprolactone (PCL) is a biocompatible and biodegradable polymer, approved by the *Food and Drug Administration*. It can be used in the formulation of nanoparticles. Its applications are diverse, such as in the encapsulation of antifungal, antiviral, and antiparasitic agents (Irache *et al*., 2011). Several surfactants are used in the production of nanoparticles, such as poloxamer 188. This surfactant is a hydrophilic triblock copolymer, approved by the *Food and Drug Administration* for oral and intravenous administration, being used as a pharmacological membrane stabilizer and rheological agent. It is efficient in repairing muscle injuries and reduces inflammation in rabbits (Cadichon *et al*., 2007).

In the veterinary field, nanoparticles are being used to increase the quality of treatment of patients.Several drugs, such as praziquantel, used in the treatment of fungi infection, and plant compounds, such as 4-nerolidylcatechol (Cheng *et al*., 2010; Greatti *et al*., 2020), have already been encapsulated in PCL nanoparticles as examples of veterinary products. Diabetes mellitus is a common disease in cats and dogs with an incidence that is increasing over the years (Hoening, 2002).Vanadium and metformin, drugs used to treat diabetes, were not yet co-encapsulated in the proposition of nanotechnological products for veterinary use. The first is a transitional element with several valence states (-3, -1, 0, and +1 to +5) and in recent decades this trace element has been reported to have insulin-mimetic/enhancer effects in orally administered diabetic patients. Due to this characteristic, vanadium was used in the stabilization of metabolic parameters of periparturient dairy cows (Heidari *et al*., 2016). The latter, metformin, on the other hand, is a drug of the biguanide class that acts to reduce hepatic glucose production and insulin resistance of peripheral tissues, thus being considered an insulin-sensitizing drug. It is used in the treatment of feline diabetes when these animals have a functional reserve of β-pancreatic cells, as well as in dogs affected by hyperadrenocorticism (Miceli *et al*., 2018).Evaluating the toxicity of metformin, the main drug in the treatment of diabetes, and vanadium, a new therapeutic alternative, is essential to ensure safety and efficacy in clinical use.

The zebrafish (*Danio rerio*) stands out as a widely used model organism for the rapid and economical evaluation of the safety and efficacy of new compounds for animal health, including nanopharmaceuticals. This model gains even more importance in a context in which more than a thousand new substances, such as vaccines, medicines, food additives, and agrochemicals, are introduced to the market annually (Böhme *et al*., 2017; Fukushima *et al*., 2020). Due to attributes such as genetic, anatomical (kidneys, brain, liver, intestine, heart, spine, eyes, mouth, ears, etc.), and physiological homology to mammals, external fertilization, high number of descendants through reproduction, embryo transparency, small size, and rapid development facilitate large-scale phenotypic approaches while maintaining the capacity to respond to the 3Rs (Macrae; Peterson, 2015). Therefore, in this work we propose the evaluation of the toxicity of PCL nanoparticles containing co-encapsulated metformin and vanadium and the constituents used in the production of these nanoparticles, evaluating their effects on the development of embryos and eleuthero-embryos of *D. rerio.*

2. MATERIAL AND METHODS

2.1Zebrafish husbandry and crossing

The experiments were carried out at the *Laboratório de Ecofisiologia e Comportamento Animal* – LECA of the *Universidade Federal Rural de Pernambuco* – UFRPE, a vivarium registered in the CIUCA-CONCEA Platform with a certificate of regularity issued by the regional council of veterinary medicine to perform tests with aquatic animals. All tests involving animals were previously approved by the Ethics Committee for Animal Use (License nº 071/2019). Adult wild-type fish (1 year) were bred and housed in 80 L aquariums, where they were quarantined to detect or confirm the absence of pathogens or diseases. They were housed under the following laboratory conditions: artificial aeration of 11 mg/L DO, temperature of 25 ± 1 °C, pH 7.5 ± 0.5, and 14/10 h cycle (light/dark). The water was partially renewed once a week. Abiotic parameters such as dissolved oxygen, ammonia, nitrite, and nitrate were also measured and maintained within ideal ranges (Gomes *et al*., 2024). The animals were fed three times a day, 2× with Fort Color® fish food (30% crude protein) and 1× with live brine shrimp nauplii (Artemia ssp). To obtain the embryos, the adult animals were separated in a 2:1 male-to-female ratio (OECD 236, 2013) and placed in spawning tanks (Alesco® Zebclean, Monte Mor, Brazil) for reproduction. Thirty minutes after the start of spawning, the eggs were collected, with the removal of unfertilized ones. Fertilized eggs (with normal blastula development) (OECD 236, 2013) were washed with distilled water and randomly transferred for exposure in sterile polystyrene pots (80 mL) and kept in an incubator at a controlled temperature (27 ± 1 °C) and photoperiod (14/10h light/dark).

2.2Preparation and characterization of nanoparticles

The concentrations of the compounds PCL, poloxamer 188, metformin, and sodium metavanadate used in the production of nanoparticles whose toxicity was evaluated, are presented in Table 1. To obtain the NPs, the technique of deposition of performed polymers was used, where the PCL was dissolved in acetone and dichloromethane and heated at 30º C for 5 minutes for total dissolution, forming the organic phase. This solution was then poured into an aqueous phase containing phosphate buffer (0.1 M, pH 7.4), metformin, sodium methavanadate, and poloxamer 188. The mixture was kept under magnetic stirring with a relative centrifugal force of 21 *g* for total evaporation of organic solvents for 24 hours (patent BR 102020020499). To produce the white nanoparticle, there was no addition of metformin and/or sodium metavanadate. The waste generated during the experiment underwent treatment in an advanced oxidative process in a reactor using UV photo-oxidation and H_{2O2} before disposal (Wols*et al*., 2013). The average size (nm), polydispersity index (PDI), and zeta potential (mV) of the nanoparticles were evaluated using the standard photon correlation spectroscopy (PCS) technique fixed at 90º to 25 ºC using a Zetasizer Nano ZS (Malvern, UK) (Cadena et al., 2013; dos Santos Magnabosco et al., 2022). The analysis was performed at the*Laboratórios Associados em Rede de Nanotecnologia* (LARnano) of the *Universidade Federal de Pernambuco* (UFPE). The data were measured in triplicate.

Table 1. Concentrations of the compounds used in the experiments. Acronyms: C – Control; V – Sodium metavanadate; M – Metformin; MV – Sodium metavanadate associated with Metformin; PP – Polycaprolactone Nanoparticles containing Poloxamer 188; PPMV - Polycaprolactone nanoparticles containing Poloxamer 188 with association of co-encapsulated sodium methavanadate and metformin. Legends: * - Xu*et al*., 2003; # - Kumar *et al*., 2017; ## - Mazzarino*et al*., 2012.

Group / Compound	**C**	**V-10**	**M-10**	**MV-10**	**PP-10**	**PPMV-1**	**PPVM-5**	**PPMV-10**
Sodium Metavanadate*	0	0.025 mg/mL	0	0.025 mg/mL	0	0.0025 mg/mL	0.0125 mg/mL	0.025 mg/mL
Metformin[#]	0	0	1,875 mg/mL	1,875 mg/mL	0	0.1875 mg/mL	0.9375 mg/mL	1,875 mg/mL
Polycaprolactone[##]	0	0	0	0	0.313 mg/mL	0.031 mg/mL	0.156 mg/mL	0.313 mg/mL
Poloxamer 188[##]	0	0	0	0	0.0125 mg/mL	0.000125 mg/mL	0.00625 mg/mL	0.0125 mg/mL

2.3 Toxicity test

For the evaluation of the toxicity of the nanoparticles, the development of embryos and *eleuthero-embryos* of *D. rerio* was evaluated at 24, 48, 72, and 96hpf. With the aid of an optical microscope (400x, 1000x), pictures were taken of the embryos and *eleuthero-embryos* for the identification of possible lethal and sublethal effects (Lammer *et al*., 2009; Bittencourt *et al*., 2018; Cadena *et al*., 2020). Also, for lethal effects, mortality was observed daily. Mortality was determined as coagulation, tail not detached, no somite formation, absence of heartbeat, and/or lack of hatching (OECD 236, 2013). In addition to the assessment of sublethal effects, heart rate was measured by manually counting heartbeats (Bittencourt *et al*., 2018). Pigmentation Reduction (ReP), Pericardial Edema (PE), Yolk Sac Edema (YSE), and Spine Deformation (SDef) were also evaluated (Lammer *et al*., 2009; Bittencourt *et al*., 2018). The lethal and sublethal effects observed were first analyzed by dichotomous response (presence and absence), considering the affected animal when it had at least one lethal or sublethal effect (Cadena *et al*., 2020). This response was analyzed by its relative frequency, and its data was presented in the frequency of affected animals (%).

2.4Statistical analysis

These results were analyzed by *one-way* ANOVA followed by Tukey's test ($p < 0.05$). Also, as validation criteria, the animals in the control group did not present any of the sublethal effects analyzed, and the mortality of the group did not exceed 10% (OECD 236, 2013). For heart rate, the mean and standard deviation of each group were determined, and the results were analyzed by *two-way* ANOVA followed by Tukey's test ($p < 0.05$). Statistical analyses were performed using the *Origin Pro Academic* 2015 software (Origin Lab. Northampton, MA, USA).

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Physiochemical characterization of nanoparticles

The results of the physicochemical characterization show nanoparticles with an average size between 165.3 and 341.07 nm, polydispersity index (PDI) between 0.025 and 0.198, and zeta potential (ζ) between -7.83 to -3.03 mV.

3.1.2Effects of metformin, sodium metavanadate, white PCL nanoparticles, and different concentrations of antidiabetic drugs co-encapsulated in PCL on zebrafish embryonic development

Examples of typical sublethal effects presented in all experimental groups can be seen in Fig. 1. V-10 and MV-10 induced pericardial and yolk sac edema and spinal deformation. PP induced a reduction of pigmentation and pericardial edema. PPMV-5 induced spine deformation. PPMV-10 induced yolk sac edema.

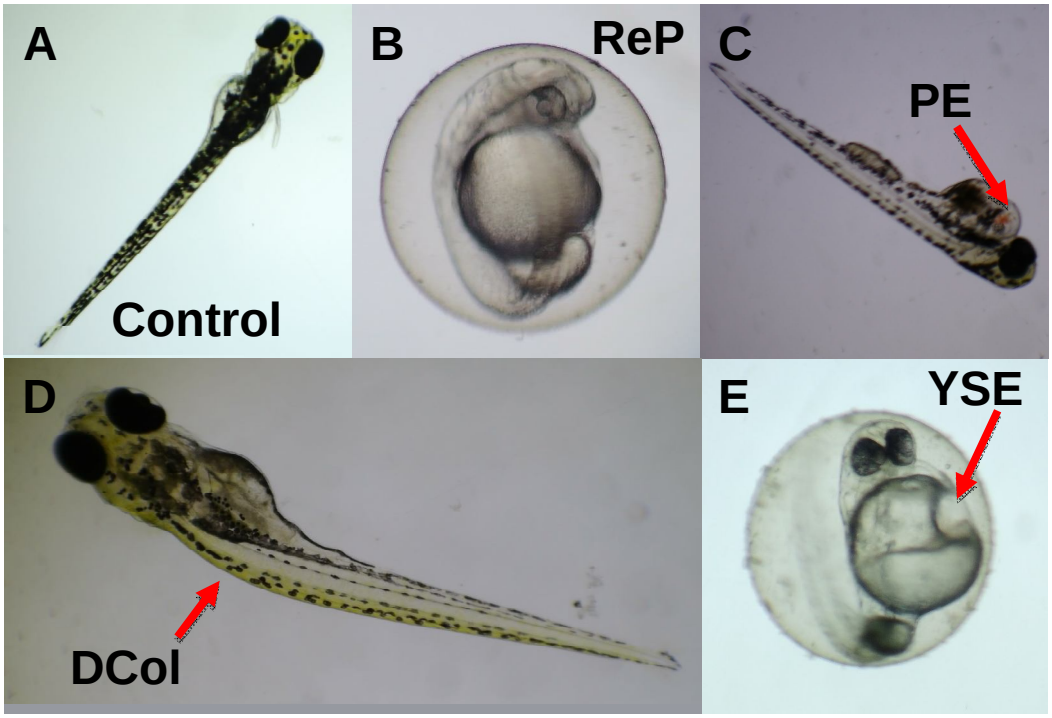

Fig. 1.Typical sublethal effects were observed by exposure to white polycaprolactone (PCL) nanoparticles and the different concentrations of metformin and sodium metavanadate co-encapsulated in PCL nanoparticles in the period between 24-96 hpf. Legend: A – Group C in dorsal view; B and C – Sublethal effects in the PP group; D – Sublethal effect in PPMV-5; E – Sublethal effect in PPMV-10. Acronyms: ReP – reduction of pigmentation; PE – pericardial edema; SDef – spine deformation; YSE – yolk sac edema.

Embryos and *eleuthero-embryos* were exposed to free antidiabetics, white PCL nanoparticles, and different concentrations of antidiabetic drugs co-encapsulated in PCL nanoparticles to evaluate the lethal and sublethal effects. The results are presented in Fig. 2. V-10 and MV-10 caused sublethal and lethal effects at 96hpf. PP-10 caused sublethal effects at 24 and 72hpf and lethal effects at 96hpf. PPMV-5 caused sublethal effects at 48hpf and lethal effects at 96hpf. PPMV-10 caused sublethal and lethal effects at 24, 72, and 96hpf, with mostly lethal effects at 96hpf.

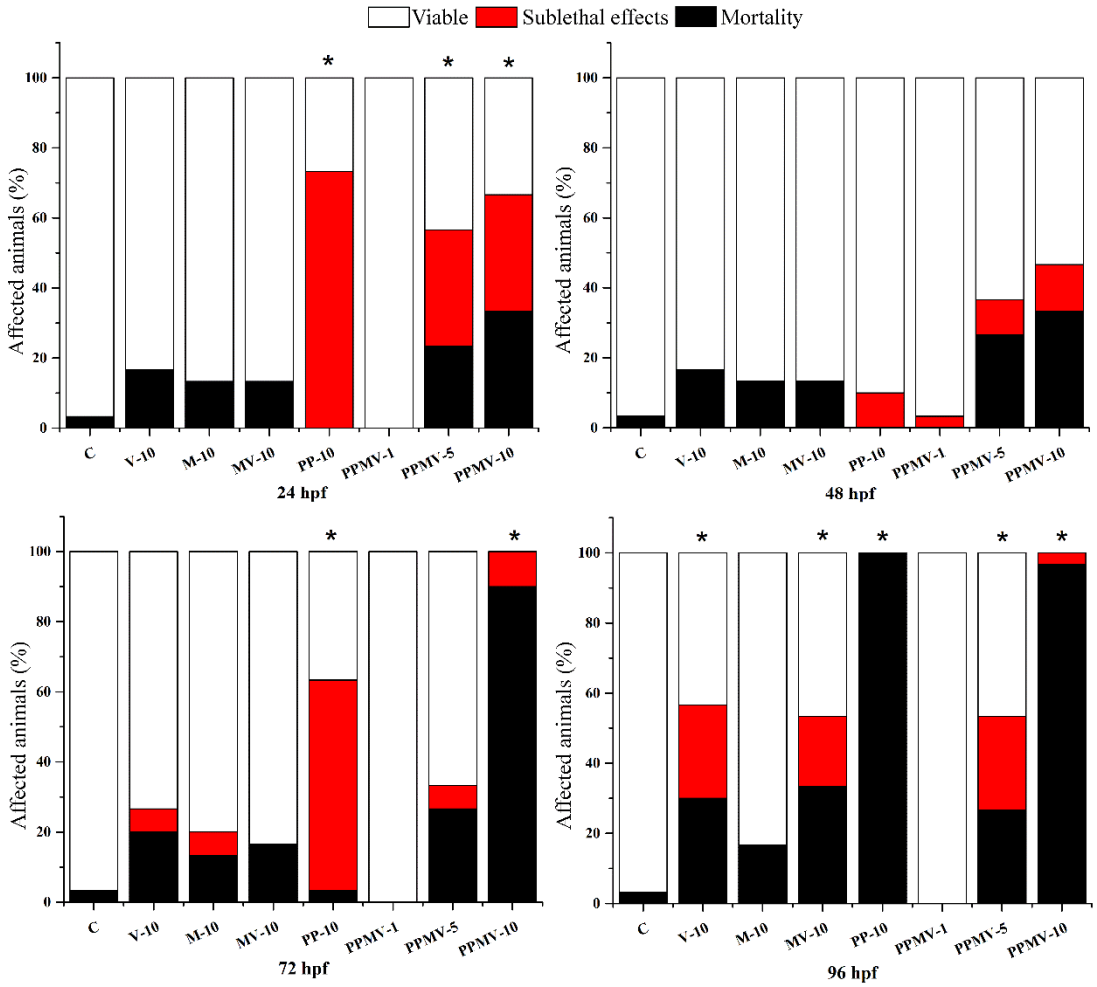

Fig. 2. Frequency of affected animals in the period of 24-96 hpf after exposure to free antidiabetic drugs metformin and sodium metavanadate, white polycaprolactone (PCL) nanoparticles, and the different concentrations of metformin and sodium metavanadate co-encapsulated in PCL nanoparticles. The increase in the frequency of affected animals was considered significant when * = $p < 0.05$ by Tukey's test in relation to the Control group. Each experimental group was compared with the control group by *one-way* ANOVA in the periods of 24 hpf ($F(7.23) = 12.41$, $p < 0.001$), 48 hpf ($F(7.23) = 2.62$, $p = 0.05$), 72 hpf ($F(7.23) = 18.36$, $p < 0.001$) and 96 hpf ($F(7.23) = 23.68$, $p < 0.001$). Acronyms: C – Control; V – Sodium metavanadate; M – Metformin; MV – Sodium metavanadate associated with Metformin; PP – Polycaprolactone Nanoparticles containing Poloxamer 188; PPMV - Polycaprolactone nanoparticles

containing Poloxamer 188 with association of co-encapsulated sodium methavanadate and metformin.

3.1.3Effects of metformin, sodium metavanadate, white PCL nanoparticles, and different concentrations of antidiabetic drugs co-encapsulated in PCL on zebrafish's heart rate

In relation to the effect of nanoparticles on heart rate, the results can be seen in Fig. 3. An increase in heart rate is expected over time, as it can be seen in the Group C. This was also observed in the V-10 and M-10 groups, indicating that these isolated compounds did not affect the heart rate of the animals. However, in the MV-10 group, there was an increase in heart rate, indicating that the interaction between the compounds can cause some toxicity. In the PPMV-1 and PPMV-5 groups, there was an increase in heart rate in relation to time up to 72 hpf, with a decrease of 96 hpf. In the PPMV-10 and PP-10 groups, a reduction in heart rate occurred from 72 hpf. When compared with each other, the reduction in heart rate in the PPMV-10 group is significant in relation to the PP-10 group ($p = 0.0001$) at 48 hpf (Fig. 3), with PPMV10 being the most toxic among all.

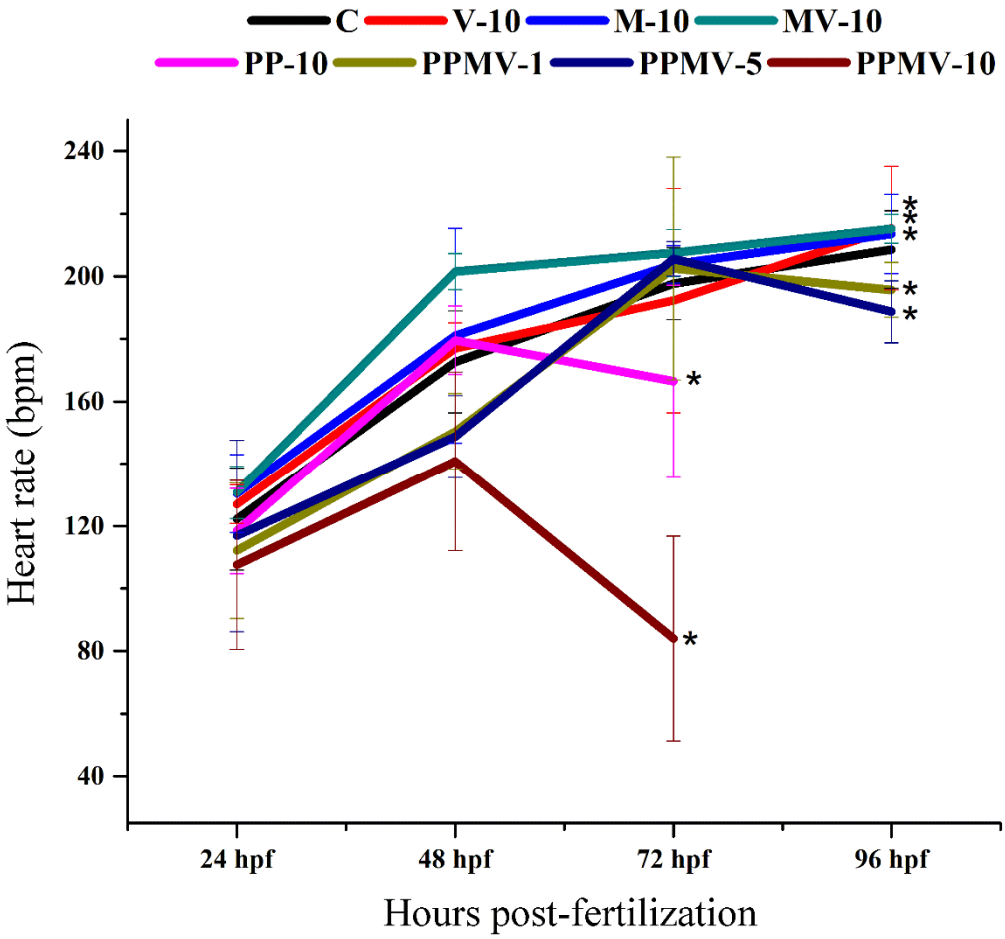

Fig. 3. Comparison of the heart rate of the groups in the period of 24-96 hpfafter exposure to the antidiabetic drugs metformin and sodium metavanadate, the white polycaprolactone (PCL) nanoparticles and the different concentrations of metformin

and sodium metavanadate co-encapsulated in PCL nanoparticles by *two-way* ANOVA in the periods of 24 hpf (F(7.26) = 5.37, p < 0.001), 48 hpf (F(7.26) = 32.45, p < 0.001), 72 hpf (F(7.24) = 22.32, p < 0.001) and 96 hpf (F(6.20) = 21.98, p < 0.001) followed by Tukey's test (p < 0.05).

3.2 Discussion

Toxicity assessment using *D. rerio* is a simple, effective, and ethical method to analyze the acute effects of exposure to nanoparticles and their long-term impact during embryonic development, providing important information on sublethal and lethal effects that may occur in the organism (da Silva et al., 2023). However, few studies address the effects of pharmaceutical excipients used for the formulation of nanoparticles for veterinary use. In this study, we provide evidence that PCL nanoparticles can increase mortality and morbidity in D*. rerio embryos and* eleuthero-embryos, causing malformations such as spine deviation and pericardial and yolk sac edema.

All exposed groups presented sublethal and/or lethal effects. The mortality presented in the group exposed to metformin (M-10) may be related to the concentration of the drug used since Elizalde-Velázquez *et al*. (2021) used a concentration of ≤ 0.1 mg/mL, which is lower than those used in our study, and they did not see such effects. The sublethal effects observed in the group exposed to sodium metavanadate and the association of this drug with metformin, such as pericardial and yolk sac edema and spinal deformation, may be associated with the presence of the vanadium compound in question, since in Bittencourt *et al*. (2018) these effects were the most evident in relation to this drug and were not observed in the group exposed to metformin only. Additionally, Santos et al. (2021) found that nanoparticles of vanadium at 0.010 mg/mL caused malformation in embryos after 96hpf. Their results were found at a concentration of almost half of ours and at the same time of exposure. The mortality presented in the group exposed to the association of these drugs may be related to a possible synergistic effect between these two compounds, since there was a higher lethality than in the groups exposed to metformin or sodium metavanadate alone.

In the groups exposed to PCL-nanoencapsulated drugs, the sublethal effects, such as pericardial and yolk sac edema, and lethal effects were dependent on the concentration used, indicating that some of the excipients used in the formulation caused such effects. Metformin and sodium methavanadate can be discarded as possible agents that have caused such effects, since in our study there is a group of interactions of these drugs that caused sublethal and lethal effects in the animals, but not to such a high degree. An excipient that can be ruled out as a possible agent of the effects presented is the surfactant known as poloxamer 188 since Hering *et al*. (2020) indicate that only concentrations above those used in our study showed sublethal effects and no lethality.

Poly(ethyleneglycol) (PEG)-b-poly(ε-caprolactone) (PCL), namely PEG-b-PCL, a nano-micelle similar to the PCL nanoparticles used in our study, on the other hand, presented sublethal and lethal effects from 0.06 mg/mL in a dose-dependent mannerin an animal model (Zhou et al., 2016), which corroborates our study, since in the group exposed to the highest concentration of the association of nanoencapsulated metformin and sodium metavanadate, all animals observed presented sublethal effects, with a lethality of about 96%. The group exposed to the white nanoparticle has the same concentration of PCL as the group exposed to the highest concentration of the association of nanoencapsulated metformin and sodium metavanadate, presenting very similar

dynamics of sublethal and lethal effects, demonstrating that the increase in the concentration of PCL is responsible for the lethal and sublethal effects presented in these groups.

The nature of the lethality presented in the animals at 24 hpf may be related to the process of embryonic angiogenesis, as PCL downregulates the expression of the vascular endothelial growth factor (VEGF)after 24h. Furthermore,it was proved that PEG-b-PCL nano-micelles upregulated the expression of apoptotic genes, p53 and AIF, increasing endothelial cell apoptosis and thus inhibiting the process of angiogenesis (Zhou *et al*., 2016). Also, *in vitro*, these micelles showed cell toxicity by increasing the level of tumor necrosis factor (TNF), an inflammatoryfactor, in cells in contact with PEG-b-PCL nano-micelles after 24h (Zhao et al., 2013).At 72 hpf, there is an increase in lethality, notably in groups exposed to PCL nanoparticles. Since these animals are now in the *eleuthero-embryo* stage and do not have chorion, they are more exposed to the compounds without a protective envelope (Duan *et al*., 2020). These results demonstrate that the toxicity of nanoparticles is more related to the *eleuthero-embryo* and the constituents of the nanoparticles than to the metformin and sodium metavanadate.

Regarding heart rate, the association of free drugs caused an increase in the exposed animals, indicating that there is a synergy between the two drugs, which is in line with the administration of these drugs alone, since metformin and sodium metavanadate decrease heart rate (Bittencourt *et al*., 2018; Borg *et al*., 2020). In the case of PCL, the heart rate in the animals decreased, regardless of the concentration used. This change may be a result of the toxic effect caused by this polymer on angiogenesis (Zhou *et al*. 2016).

4. CONCLUSION

The presence of PCL in the formulation of nanoparticles was harmful at the cardiovascular level in embryos and *eleuthero-embryos* of *D. rerio*, affecting their development and heart rate, regardless of their concentration, and may cause sublethal and lethal effects in these animals. This results in greater care regarding the amount of nanomaterial used for the development of nanoparticles. This implies that although they convey the idea that nanomaterials are more bioavailable for veterinary use, they need to undergo careful evaluation of their toxicity in animal models. Therefore, the toxicological evaluation of the nanomaterial used is as important as its therapeutic efficacy, since such results can anticipate possible toxicity problems, which may compromise its veterinary application in the future.Finally, because polycaprolactone can alter nanoparticle toxicity, we recommend that complexes are not treated as free molecule enhancements but rather as novel productsthat should be evaluated individually regarding their toxicity.

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration amongall authors. All authors read and approved the final manuscript.

COMPETING INTERESTS

The authors declare that no competing interests exist.

ETHICAL APPROVAL

All tests involving animals were previously approved by the Ethics Committee for Animal Use of the *Universidade Federal Rural de Pernambuco*, under License nº 071/2019.

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