Protocol

Artificial Mitochondrial Transfer/Transplant to *Danio rerio* embryos by adapting MitoCeption as a tool to study developmental energetics

Roberto Espinosa-Vallejo 1, 2, 3, Andrés Romero-Carvajal 1, Andrés Caicedo 2, 3.

1 Pontificia Universidad Católica del Ecuador, Escuela de Biología, 17-05-25, Quito, Ecuador

2 Universidad San Francisco de Quito, Colegio de Ciencias de la Salud, Escuela de Medicina, 17-12-841, Quito, Ecuador

3 Sistemas Médicos SIME, Universidad San Francisco de Quito, 17-12-841, Quito, Ecuador

Co-corresponding authors: Andrés Caicedo, acaicedo@usfq.edu; Andrés Romero, maromero@puce.edu.ec andresro40@gmail.com

Keywords

MitoCeption, xenogenic transplant, artificial mitochondrial transfer/transplant (AMT/T), zygotes, embryos

Abstract

Mitochondria are responsible for metabolic balance and homeostasis in cells, regulating energy, cell aging, and apoptosis. By fulfilling such important functions, damaged mitochondria can cause acquired or hereditary pathologies that could compromise the independence or survival of the organism. Given this problem, it is of utmost importance to develop methodologies to understand mitochondrial transplant better and reach new therapeutic techniques, such as mitochondrial replacement therapies (MRT), coincubation, Magnetomitotransfer, and MitoCeption. Within the various existing methodologies, MitoCeption stands out for its simplicity and innovative procedure, which consists of a coincubation modification by incorporating centrifugation and heat shock. This research seeks to adapt Mitoception in a new animal model, *Danio rerio*. Human-zebrafish mitochondrial xenotransplantation was executed because it facilitates obtaining mitochondria and their identification in the recipient organism. *Danio rerio* was selected because it allows constant monitoring thanks to its transparent tissue and short development timeline. First, the optimal conditions for the fundamental parts of MitoCeption were set. For centrifugation: 50, 100, 300, 500, 1000 RCF; for the medium: E2 and RPMI; and heat shock (4ºC-37ºC). Secondly, MitoCeption was carried out in *D. rerio* embryos, at 500 RCF in RPMI, with a thermal shock of 4º C to 37 ºC. Finally, the results were analyzed with fluorescence microscopy. All experiments and analyses were carried out during the last semester of 2019 and the first trimester of 2020. Through our study, we seek to broaden the comprehension of mitochondrial transplantation and foster the development of new methods to achieve it.

1. Introduction

Mitochondria plays a fundamental role in metabolic functions as well as in the regulation of homeostasis in organisms (Herst et al., 2017); they are responsible for energy production and support biological processes in the cell, as key mediators in aging and even in the induction of apoptotic death (Chakrabarty et al., 2018).

Mitochondrial damage can cause various diseases. Leber's hereditary optic neuropathy (LHON), chronic progressive external ophthalmoplegia (CPEO), and mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS) are among the most common mitochondrial hereditary diseases caused by mutations in the mtDNA affecting the respiratory chain complexes (Liufu & Wang, 2020; Radelfahr & Klopstock, 2019). To address the problem of mitochondrial pathologies, the majority of current approaches aim to keep the patient's symptoms controlled but do not repair, eliminate, or compensate for hereditary or acquired alterations in mtDNA, or in non-functional mitochondria. There is a possibility of replacing or enriching the healthy mitochondrial pool to minimize the effects of hereditary mutations in future offspring through the transfer of pronuclei after fertilization of the oocyte and the transfer of the spindle from metaphase II to a healthy enucleated oocyte (Wallace, 2018). This has been the case with neonates with three different genetic heritage, they present the paternal DNA, the maternal DNA, and the DNA of the mitochondria of the egg donor. This infant was born in 2016 and no conditions have been reported throughout his development (Dimond & Stephens, 2018; Zhang et al., 2016).

Currently, most curative treatments lack sufficient clinical validation, available worldwide, for disorders of mitochondrial function. Recently, early evidence of artificial mitochondrial transfer/transplantation (AMT/T) by MitoCeption of human mitochondria to mouse embryos leading to healthy offspring has been provided (Cabrera et al., 2022). This study opened the possibility of performing AMT/T in mammalian embryos;

however, this technique has not been performed in other non-mammalian species, such as fish or amphibian models.

Performing AMT/T in *D. rerio* opens the possibility of tracking mitochondrial distribution and dynamics during embryonic development since zebrafish have transparent embryos that allow their development to be monitored throughout their different stages. As well as the ability of studying advanced genetic and chemical screens. In addition, understanding how exogenous mitochondria interact with endogenous mitochondria or how it affects normal embryo development is key to further exploring the therapeutic value of AMT/T before moving on to different animal models such as pigs, primates, and others. Hence the importance of developing AMT/T in other organisms, since each one provides different advantages when studying mitochondrial dynamics. *Danio rerio* shows a 70% similarity to the human genome (Howe et al., 2013). Other advantages, when compared to mammalian models such as the mouse, are its rapid development, 72h for larval stage, and its numerous oviposition (Gilbert, 1988). It lays between 200 to 300 eggs per week, which are fertilized externally, facilitating its handling and maintenance, as well as performing multiple essays at the same time (Hoo et al., 2016; Spence et al., 2008). There are several examples of xenotransplantation in zebrafish; an example is the human melanoma cells transplant performed in specimens of *D. rerio* (Lee et al., 2005). Despite several studies in cell transplantation, there have been only limited studies in AMT/T (Artificial Mitochondrial Transfer/Transplantation) and, even more, this is the first time, that an attempt is made to transfer mitochondria in zebrafish.

The objective of this research is to establish a methodology based on MitoCeption to perform AMT/T in *D. rerio* embryos. The Mitoception technique was chosen as it does not require specialized equipment, like other methodologies, but only an incubator and a temperature-controlled centrifuge. Additionally, thanks to its simplicity (centrifugation, thermal shock, and coincubation) and effectiveness

(manipulation of a large number of mitochondria) it is optimal for its use to develop new therapeutic techniques to treat mitochondrial dysfunctions. The research was divided into three parts. First PUCE provided the zebrafish embryos to analyze the ability of *D. rerio* to overcome key aspects of their biology against mechanical MitoCeption stress conditions: centrifugation, medium, heat shock, fluorescent dye concentrations, mitochondrial concentration. In the second phase, the MitoCeption process was adapted to the zebrafish model. Finally, in the fourth phase, the results were accessed based on fluorescence microscopy.

2. Methodology

2.1 Animal model

The animal model was granted by the Developmental Laboratory of PUCE. The colony of *D. rerio* origin was from an amazonian zebrafish line.

2.2 Adapting MitoCeptions conditions to *D. rerio*

To carry out the MitoCeption, the “Bioterio para investigación en Pez Cebra” from PUCE granted the embryos of *D. rerio* for the tests of resistance to centrifugation, thermal shock and the medium used; as well as the MitoCeption process.

In an attempt to test if MitoCeption could be applied in *D. rerio* embryos, five essays for centrifugation, and three essays for temperature, and medium were performed to assess the favorable conditions for their development. The resistance of embryos to centrifugation, thermic shock, and medium was tested based on previous publications (Caicedo et al. 2015; Cabrera et al. 2022). All treatments used 1,5 mL conical tubes with 15 embryos each.

Centrifugation

Four settings were used: 50, 300, 500, 1000 (RCF) and control (no centrifugation). Five essays were tested with 15 embryos per treatment seeking to standardize the maximum *g* force without affecting the embryo development. For statistical analysis (One-Way ANOVA test), an average of the five experimental trials was made.

Thermic shock

Three settings were used: the first consisted of a range of 4 °C (for 5 minutes) to 37 °C (for one hour); the second at 28 °C, optimal temperature for embryonic development, for 65 minutes, and the third at 20 °C, room temperature control, for 65 minutes. Three essays were tested with 15 embryos per treatment. For statistical analysis (One-Way ANOVA test), an average of the three experimental trials was made.

Medium

Three settings were used for medium testing by accessing embryo viability or death: Gibco Roswell Park Memorial Institute (RPMI), Phosphate-buffered saline (PBS) and Embryo medium (E2) (control), which is a specific medium for fish eggs. Three essays were tested with 15 embryos per treatment. For statistical analysis (One-Way ANOVA test), an average of the three experimental trials was made.

After all treatments (centrifugation, thermic shock, and medium) the embryos were incubated at 28 ºC for 72 h, and then the surviving rate was monitored at 24-36 HPF (hours post fertilization).

2.3 MitoCeption

2.3.1 Ethics evaluation

Mitochondrial isolation from human donor peripheral mononuclear cells (PBMC) was carried out according to the parameters of the Human Research Ethics Committee or with human samples (CEISH-USFQ) of the Institutional Review Committee of the Universidad San Francisco de Quito (USFQ).

Blood isolation was performed in accordance with the guidelines and regulations of the USFQ Ethics Committee for mitochondrial studies (2017-026IN).

2.3.2 PBMCs Isolation

Mitochondria were isolated from PBMCs as it is represented in Figure 1. PBMCs are monocytes and lymphocytes (B cells, T cells, and NK cells) which were separated from multinuclei granulocytes (basophils, eosinophils, and neutrophils) and erythrocytes and platelets (which lack nuclei).

For PBMC isolation, Ficoll-Paque PLUS (GE Healthcare Life Sciences; MA, US) was used, as it is a neutral hydrophilic polysaccharide that facilitates the separation of the different blood cells, working as a sieve.

The blood was extracted from four individuals for each time the MitoCeption was performed - four samples each (between 15 and 20 mL of blood per person) using BD Vacutainer tubes with EDTA as storage.

In four 50 mL Falcon tubes (one for each person), 15 mL of Ficoll-Paque PLUS (GE Healthcare Life Sciences; MA, US) were poured and placed in the freezer at -18 ºC.

Blood of each individual (one tube per person) was poured into four 50 mL Falcon tubes, and standardized to a total volume of 30 mL using PBS (phosphate buffer saline), and homogenized.

Falcon-Ficoll tubes were taken from the freezer and placed inside the biological safety cabinet. Each blood-PBS mixture was slowly poured into a 50 mL Falcon with Ficoll (one for each sample) avoiding the mixture of blood and Ficoll.

Falcon tubes were centrifuged at 400 g for 45 min, without acceleration or braking, at room temperature (20 °C). After centrifugation, a gradient was obtained (from the bottom to the surface of the Falcon): granulocytes, erythrocytes, Ficoll, cloudy layer (where the PBMCs are found) and plasma. The PBMCs were gently removed, using a 1000 µL pipette, avoiding taking in Ficoll or disturbing the gradient. The PBMCs

were placed in another 15 mL Falcon tube (one for each individual) and standardized to 14 mL with PBS. Finally, the PBMCs were centrifuged at 1500 g for 20 min, with acceleration and brake of 9 and a temperature of 4 ºC to obtain a pellet where all PBMCs will be concentrated.

Storage:

The supernatant was discarded and the pellet was resuspended in 2 mL of PBS and then stored in a 2 mL “Eppendorf” tube at 4 °C for maximum 1 day.

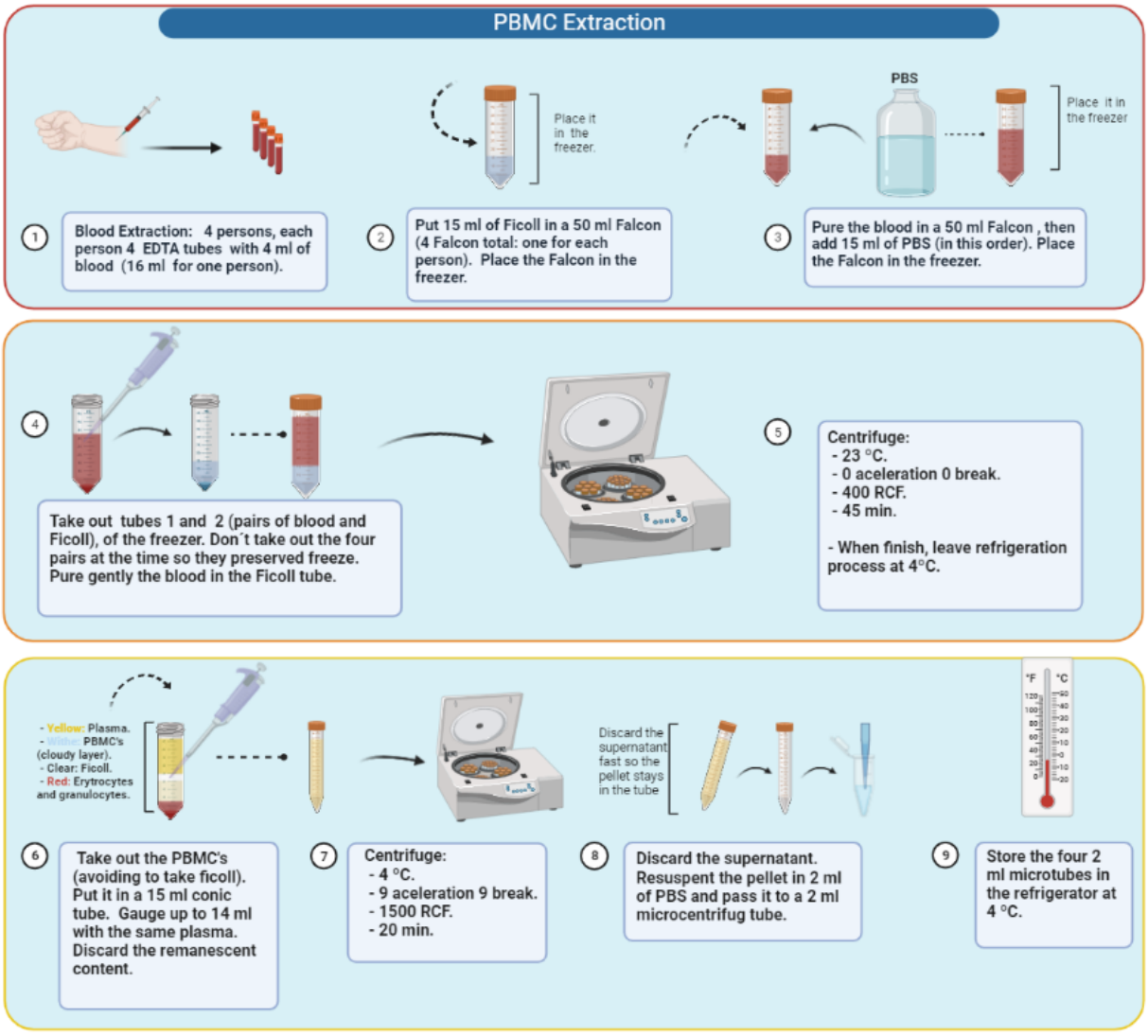


Figure 1: PBMCs extraction process.

2.3.3 Mitochondrial isolation

Mitochondria were isolated from PBMCs by using the ThermoScientific Mitochondria Isolation Tissue Kit, 89801 following manufacturer's instructions. After isolation, mitochondria were centrifuged at 3000 RFS for 15 min to remove any reagents left from the kit. After that, the mitochondria pellet was resuspended in RPMI and quantified according to Pierce™ Coomassie Plus (Bradford) Assay Kit from ThermoFisher Scientific. Finally, isolated mitochondria were stored at 4 ºC until MitoCeption.

2.3.4 Staining of Mitochondria

Staining was performed as follows: 1% RPMI-FCS medium was prepared by dissolving 10% RPMI (previously prepared with 500 mL of RPMI, 50 mL of FCS and 5 mL of PenStrep) with plain RPMI (Pen.Strep 1% without FCS). Then, the PBMCs were incubated with MitoTracker™ Red (MTR) from ThermoScientific in 1% RPMI medium at 37 ºC for 30 min with 12,5, 25, 50 and 100 ng/ul mitochondria concentration. To eliminate the excess of the fluorescent dye, the mitochondria were gently washed with 1 mL of PBS after their isolation from PBMCs.

2.3.5 Staining of embryos

1% RPMI-FCS medium was prepared by dissolving 10% RPMI (previously prepared with 500 mL of RPMI, 50 mL of FCS and 5 mL of PenStrep) with plain RPMI (Pen.Strep 1% without FCS). Then, the embryos were incubated with CellTracker™ Green (CTG) from ThermoScientific in 1% RPMI medium at 37 ºC for 30 min with 25, 50 ng/ul mitochondria concentration. To eliminate the excess of the fluorescent dye, the embryos were gently washed with 1 mL of PBS.

2.3.6 MitoCeption settings for *D. rerio* embryos:

Isolated mitochondria were placed in coculture with 15 embryos in 1,5 mL conical tubes. The solution carrying the mitochondria consisted of 250 µL with 1% Fetal Calf Serum (FCS) RPMI and mixed in the vortex for five seconds. Then,

embryos-mitochondria conical tubes were centrifuged at 4 ºC, 500 RCF with maximum acceleration and break. The cells were incubated at 37 ºC for 1 h, then they were washed and the supernatant was discarded, followed by a resuspension of the cells in E2 medium.

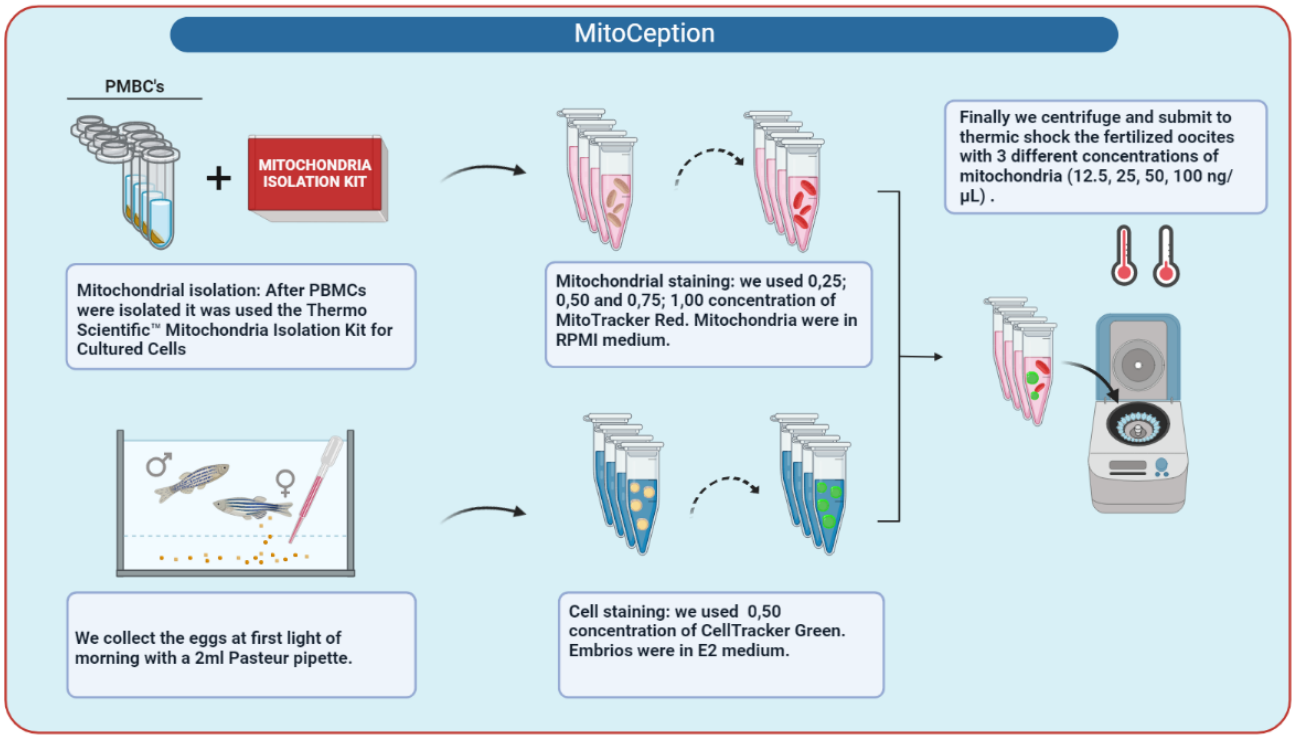


Figure 2: MitoCeption process of zebrafish embryos. After the extraction of PBMCs, recollection of zygotes was held. Then mitochondria were stained with MTR, and cells with CTG. The stained embryos and mitochondria were placed in a 1,5 ml eppendorf tube in RPMI medium. The eppendorf tubes were then placed in the centrifuge for 5 minutes at 4ºC and then incubated for one hour at 37ºC. Finally, they were placed at 28 ºC for observation.

2.3.6 Fluorescence microscopy

Mitocepted embryos were accessed based on fluorescence microscopy by using the ECLIPSE Ts2R inverted research microscope and the software Nikon NIS-Elements 2022.

2.4 Statistical analyses

The data was analyzed using a One-Way ANOVA test to estimate if there was no difference in the embryos survival means among the groups (centrifugation, thermic shock and medium). An alpha value of 0,05 was used to determine statistical significance.

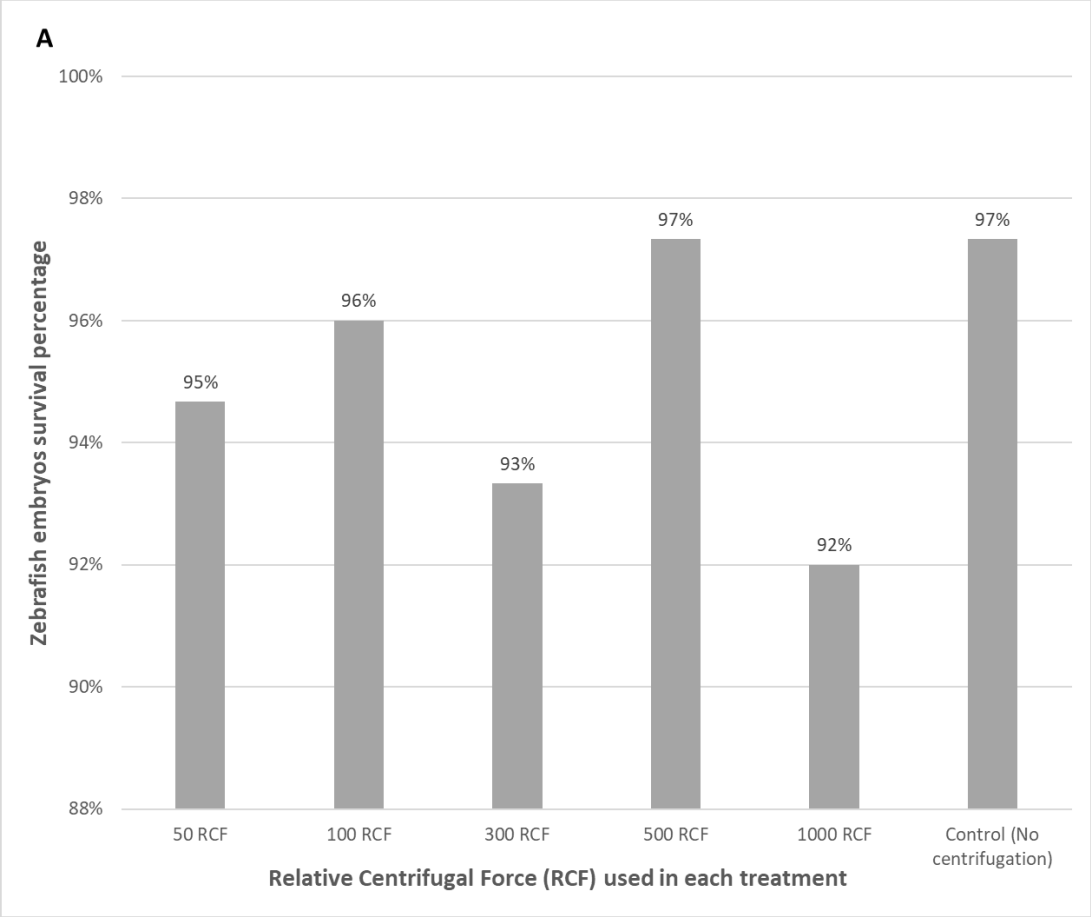
3. Results

3.1 Adapting MitoCeptions conditions to *D. rerio*

Throughout the adaptation of MitoCeption, it was needed a constant modification of the variables in all treatments: centrifugation, thermic shock and medium. Embryos in all treatments were checked for survival at 24-36 HPF.

Centrifugation

The zebrafish embryos were resistant to centrifugation in view of its size (around 0,7- 0,9 mm) (Kimmel et al., 1995), which is considerably bigger than other animal embryos such as the mice embryos (0,08 mm) or the human embryos (0,1- 0,2 mm) (VanPutte et al., 2019). Thus, over 90% of zebrafish embryos survived to the five treatments 50 RCF, 100 RCF, 300 RCF, 500 RCF, and even at 1000 RCF, as observed in Figure 3.


A
100%
98%
96%
94%
92%
90%
88%
Zebrafish embryos survival percentage
95%
96%
93%
97%
92%
97%
50 RCF
100 RCF
300 RCF
500 RCF
1000 RCF
Control (No centrifugation)
Relative Centrifugal Force (RCF) used in each treatment

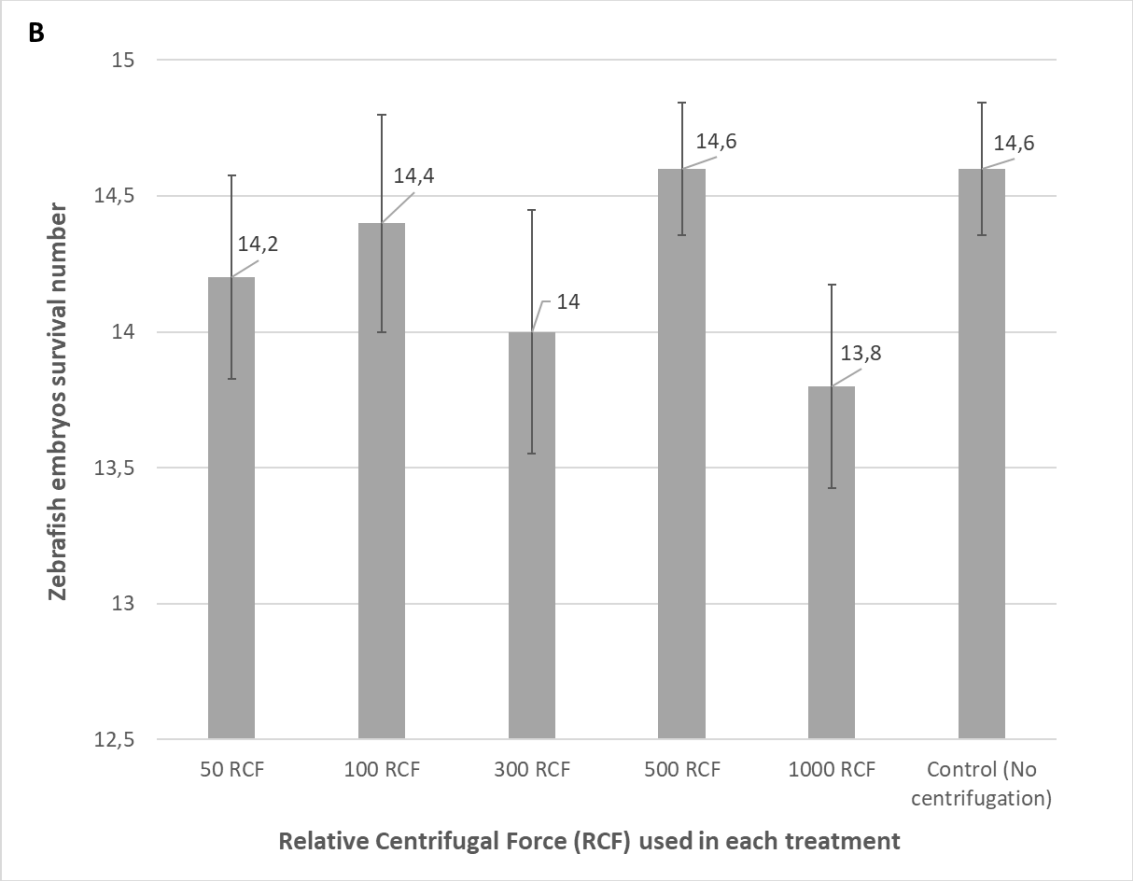
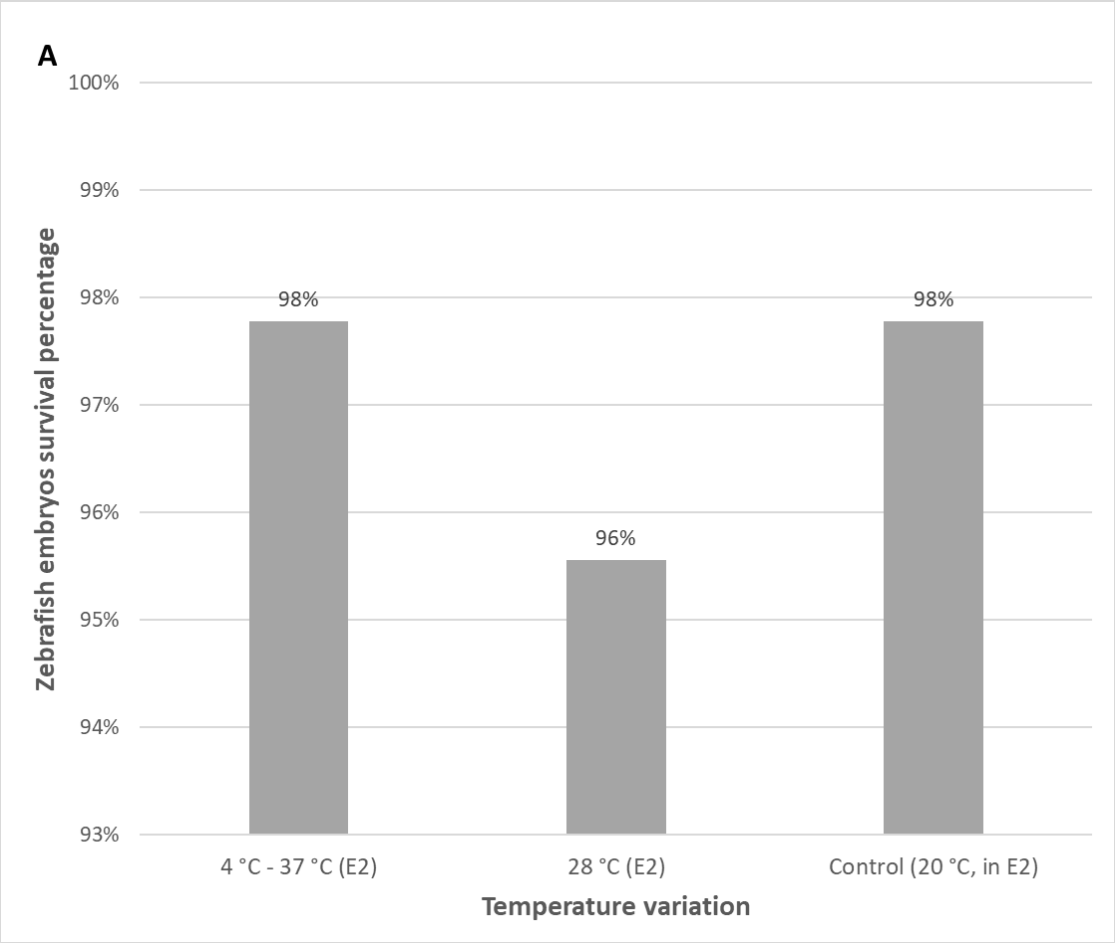


Figure 3: a) In the relationship between RCF applied to zebrafish embryos and the survival percentage, after an average of the 5 repetitions and a total of 75 embryos (each trial with 15 embryos), values greater than 90% of individuals alive are observed at 24- 36 HPF. Thus, we can say that in the present study centrifugation did not represent a factor that had an impact on the development of *D. rerio*.b) Comparison of RCF applied (50, 100, 300, 500, 1000 RCF) and alive embryos means of 5 treatments and control group in 5 experimental essays. Statistical analysis: One-Way ANOVA test using an alpha value of 0,05 for statistical significance. The error bars describe the standard error for each compared category in the One-Way ANOVA test calculation.

Thermic Shock

Thermic shock applied to zebrafish embryos was established at 4 ºC for 5 minutes to 37 ºC for one hour, at 28 ºC for 65 minutes (optimal temperature for embryos

development), and control (room temperature 20°C, for 65 minutes); all in E2 medium. The results suggest there is not a considerable variation between alive-dead fish embryos at 4ºC to 37 ºC compared to control treatment at 20ºC (Figure 4).



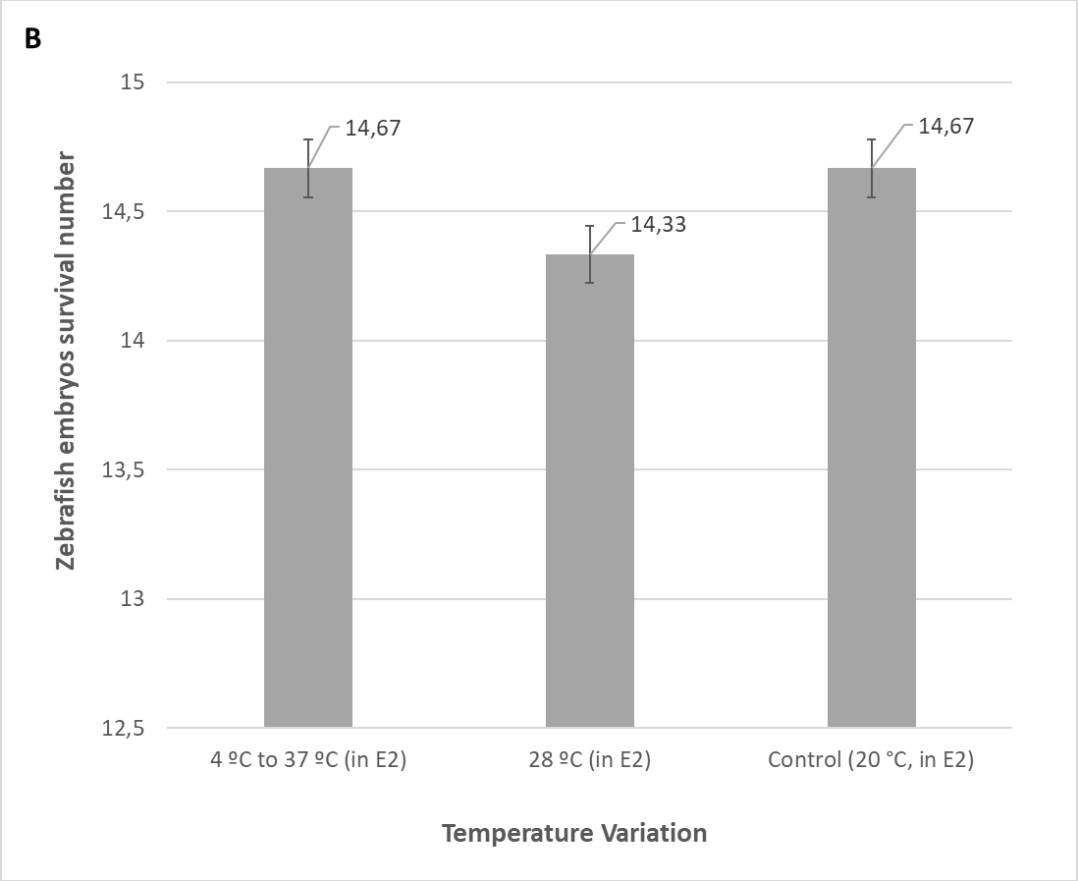
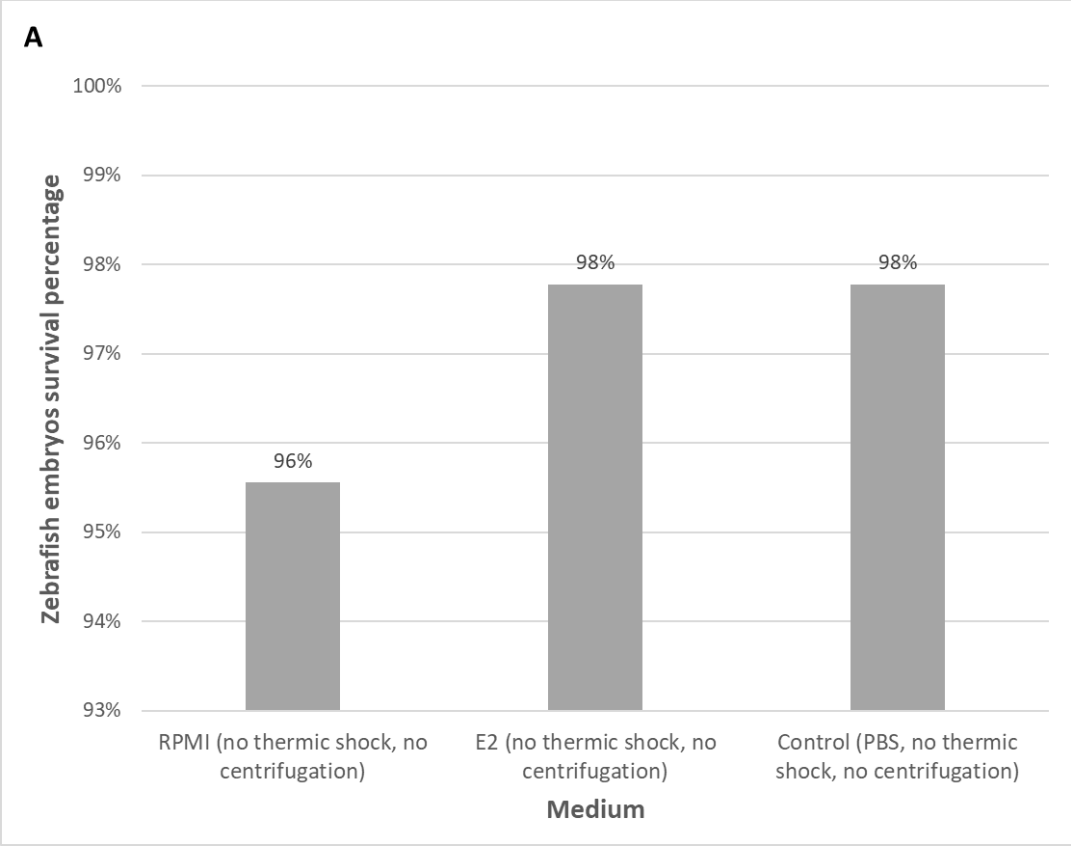


Figure 4: a) In the relationship between Temperature Variation, 4 ºC - 37 ºC (in E2), 28 ºC (in E2) and 20 °C (control in room temperature, in E2), applied to zebrafish embryos and the survival percentage, after an average of the 3 repetitions and a total of 45 embryos (each trial with 15 embryos), values greater than 90% of individuals alive are observed at 24- 36 HPF. Thus, we can say that in the present study thermic shock did not represent a factor that had an impact on the development of *D. rerio.* b) Comparison of temperature variation per treatment (4 ºC - 37 ºC (in E2), 28 ºC (in E2)) and 20°C (control at room temperature in E2) with alive embryos means of 2 treatments in 3 experimental essays. Statistical analysis: One-Way ANOVA test using an alpha value of 0,05 for statistical significance.The error bars describe the standard error for each compared category in the One-Way ANOVA test calculation.

Medium

The Medium treatments applied to zebrafish embryos were RPMI, E2 and PBS (control). When compared RPMI, E2, and PBS did not suggest a significant difference between the *D. rerio* embryos survival rate means, as evidenced in Figure 5.



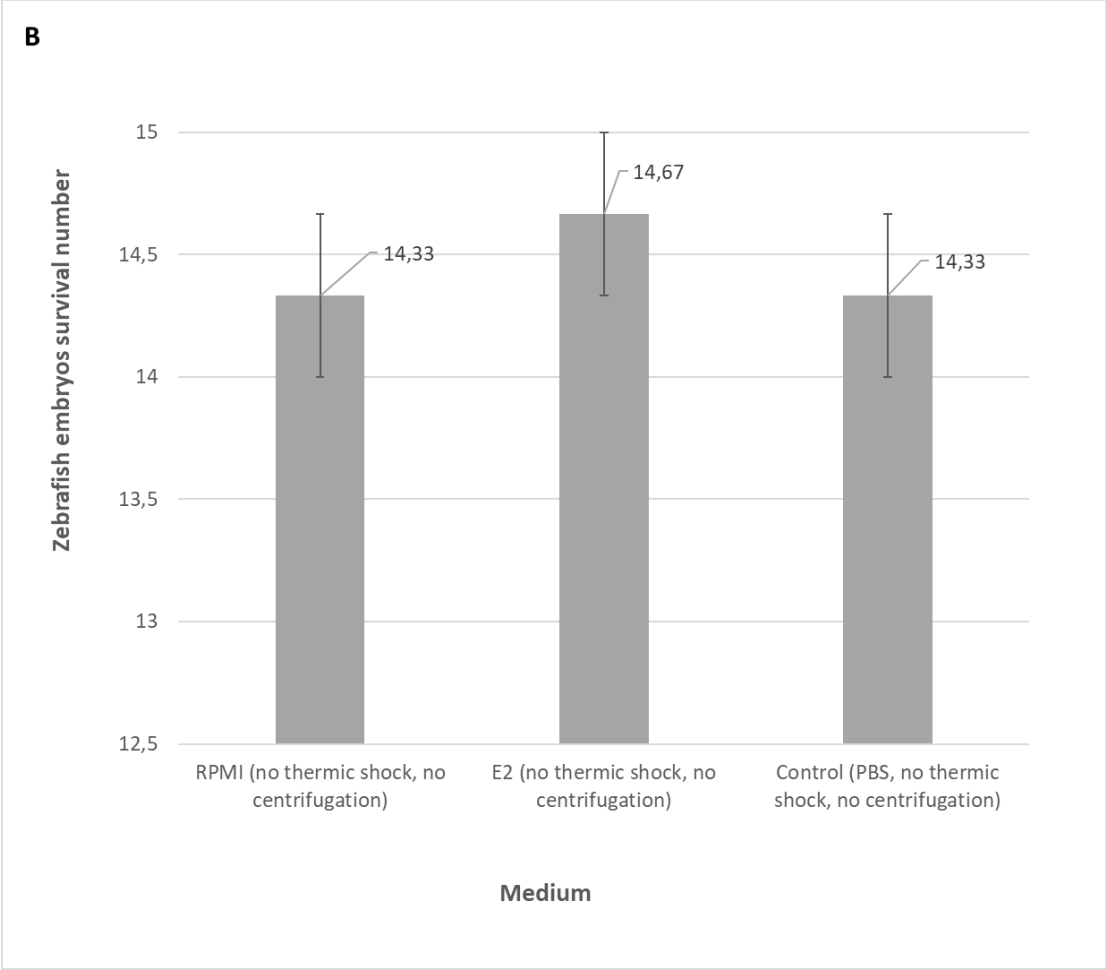


Figure 5: a) In the relationship between Medium, RPMI (no thermic shock, no centrifugation), E2 (no thermic shock, no centrifugation) and control (PBS and no thermic shock, no centrifugation), used with zebrafish embryos and the survival percentage, after an average of the 3 repetitions and a total of 45 embryos (each trial with 15 embryos), values greater than 90% of individuals alive are observed at 24- 36 HPF. Thus, we can say that in the present study thermic shock did not represent a factor that had an impact on the development of *D. rerio.* b) Comparison of alive embryos means 2 treatments and control group. Statistical analysis: One-Way ANOVA test using an alpha value of 0,05 for statistical significance. The error bars describe the standard error for each compared category in the One-Way ANOVA test calculation.

3.2 Xenotransplant of mitochondria in different concentrations in embryos of *D. rerio*

To establish the optimal concentration of mitochondria MitoCeption at 500 RCF, with thermic shock of 4 ºC-37 ºC, and RPMI medium was performed. Four different concentrations of mitochondria were utilized 12,5; 25; 50; 100 ng/ul including the non mitocepted control. The optimal mitochondrial concentrations were found to be between 50 and 100 ng/ul; we did not observe evident changes of embryo viability between the two concentrations (Figure 6). Mitochondrial interiorization, as indicated by the red dots, was detected in a higher concentration in the blastoderm above the yolk. Additionally, mitocepted embryos were recorded with different mitochondrial concentrations, observing the inner presence of exogenous labeled mitochondria in the embryos.

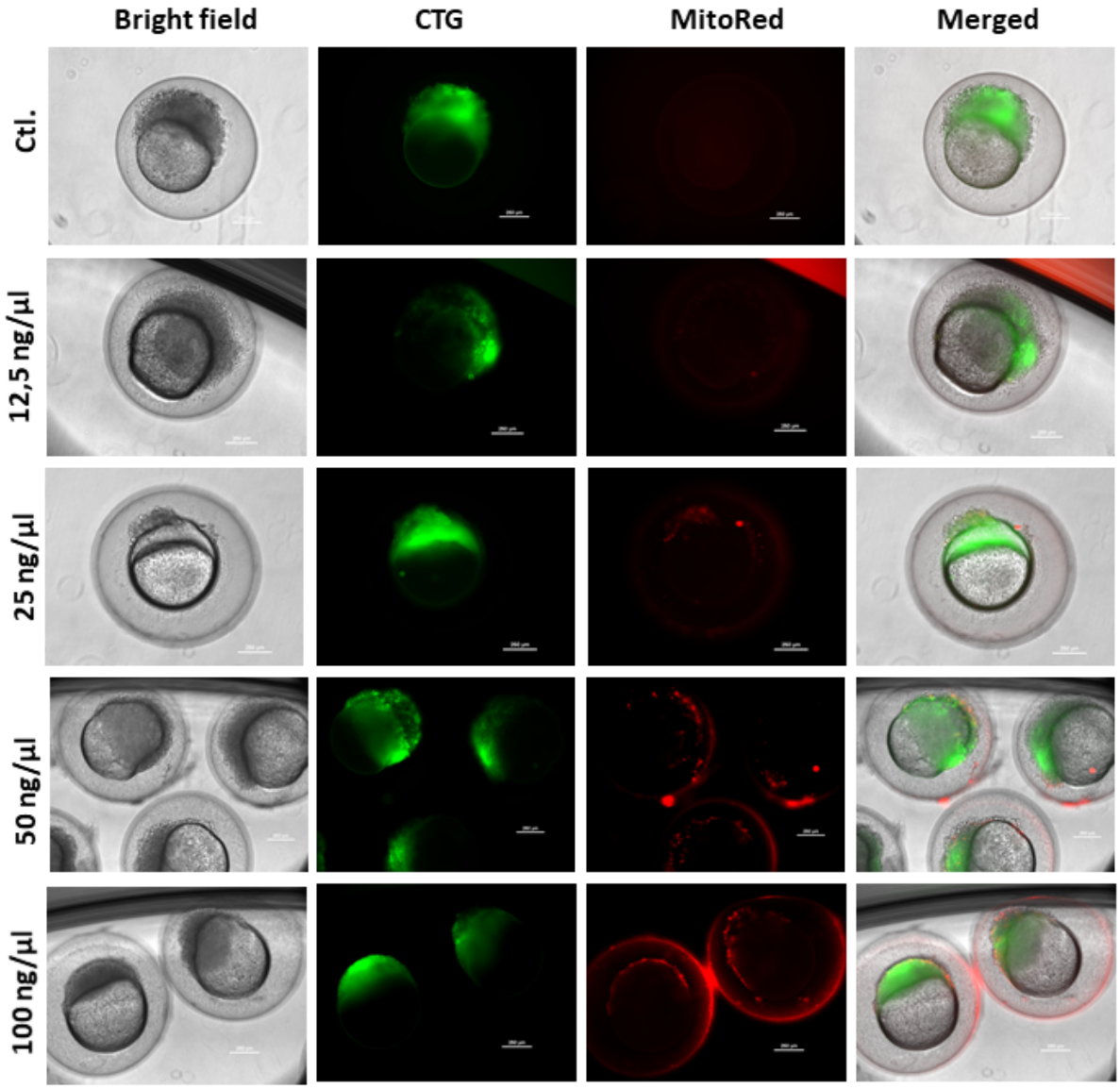


Figure 6: Effect of mitochondrial concentration: 12,5; 25; 50; 100 ng/ul on MitoCeption in *D. rerio* embryos. Stained embryos with cell tracker green and human mitochondria with mitotracker red.

4. Discussion

Previous work showed that mitochondria can be released and transferred from multipotent stem cells to other cells damaged by pathologies or stressful conditions (Andreux et al., 2013; Liufu & Wang, 2020; Miliotis et al., 2019; Radelfahr & Klopstock,

2019). This process has been observed in *in-vitro* and *in-vivo* conditions and it has been linked to a new cell-to-cell way of communication intended to repair cell damage or enrich mitochondrial content. This process inspired scientist to develop therapies based on the stem cells capacity to transfer mitochondria naturally and replicate this process artificially (Caicedo et al., 2017; Miliotis et al., 2019; Newell et al., 2018; Paliwal et al., 2018; Rodríguez-Nuevo et al., 2018). It has been observed that mitochondria are internalized by mammalian oocytes and embryos (Cabrera et al. 2022). Authors remarked in their discussion that the transfer is possible and could be mediated by AMT/T. Additionally, it is important to note that the membrane of *D. rerio* embryos have 0,5–0,7 µm pore size (Bhagat et al., 2020) and human mitochondria is estimated to be between 0,5 and 3 µm (Shami et al., 2021) which makes xenotransplantation feasible.

In the first part, to define the optimal conditions for MitoCeption, three conditions were considered: centrifugation, thermal shock and medium.

Five settings were applied for centrifugation: 50, 100, 300, 500 and 1000 RCF. This was based on the study by Cabrera et al 2022., in which 500 RCF was used to MitoCept mouse embryos (Cabrera et al., 2022). For this reason, a minimum centrifugation force of 50 RCF was used to observe if there was any alteration and at the same time 1000 RCF to verify the resistance of the embryos. Five essays were carried out each with 15 embryos of the 5 settings, in which it was observed, when compared the average, that there was a percentage greater than 90% survival in all essays. The results of the One-Way ANOVA test shows there is not enough evidence to reject the null hypothesis, which states that: there is no difference between the mean of the 6 groups (considering the P value was 0,53). The data obtained suggest that there is not a considerable variation per setting.

The thermal shock was carried out by exposing the embryos to 4 ºC for 5 minutes and then at 37 ºC for one hour to compare with a treatment at 28 ºC for 65

minutes and one at room temperature (20 °C) for 65 minutos. If the percentage of the means is compared, there is a survival greater than 90%, and if we contrast with the One-Way ANOVA test based on the p value, which confirms the null hypothesis, which states that there is no difference between the mean of the three groups (considering the P value was 0,73). Based on this it is possible to suggest that there is no significant relationship between heat shock and the death of *D. rerio* embryos.

For the medium we used E2, RPMI, and PBS as control. PBS is a neutral salt medium that does not alter mitochondria or embryos. On the other hand, E2 is the optimal medium used for incubation of zebrafish embryos. However, we sought to use RPMI as it is the medium applied for mitochondrial processes during mitochondrial isolation (Caicedo et al., 2015). For this reason, it is more useful to apply RPMI medium to facilitate MitoCeption and the manipulation of zebrafish embryos and mitochondria, since it does not affect zebrafish embryos.

When comparing the results of the 3 trials, each with 15 embryos, no major difference was observed in the average survival of the embryos between the used media, with more than 90% survival in all media. When reviewing the One-Way ANOVA test based on the p value, the null hypothesis is not rejected, which mentions that there is no difference between the means of the three groups (considering the P value was 0,73). In this way, it can be stated that since there is no considerable difference between the media, it is recommended to use RPMI to manipulate *D. rerio* embryos.

It was evidenced by the range of tolerance to centrifugation, temperature and medium, that the embryos survived all conditions and are able to be mitocepted. However, more research and data will be needed to analyze and conclude the impact of these procedures.

For MitoCeption, a mitochondrial concentration gradient of 12,5, 25, 50 and 100 ng/μL was carried out. A mitochondrial xenotransplantation between human and zebrafish was done as this facilitates MitoCeption analyses.

Thus blood samples, from a fish, for the extraction of PBMCs and subsequent isolation of mitochondria would be very small, contrary to the samples in humans, where it is possible to obtain several ml (in the case of our study 16 ml per individual) in a single extraction. Finally, through fluorescence microscopy we found the presence of stained human mitochondria inside the zebrafish embryos. According to the gradient of concentration, we can determine that the 50 and 100 ng/μL were the most efficient.

As the methodology of this study has not been performed before, there were different challenges to overcome, from the establishment of the parameters to the successful observation of the mitochondria inside fish embryos.

Due to COVID-19 pandemic disease several limitation confronted this study. Different essays such as identification of mtDNA by qPCR, implantation and dynamics of exogenous mitochondria in zebrafish embryos, and monitoring embryos survival until adult stages after MitoCeption were not carried out.

This work highlights the possibility to perform AMT/T in *D. rerio* embryos, which can be used to address the questions of whether it is possible to xenotransplant human mitochondria and how it interacts with the recipient organism. Mitochondria involvement in different pathologies implicates a ground-standing field of study to better comprehend their dynamics and develop possible therapies. Thus, this research aims to establish a model organism, *D. rerio*, using MitoCeption methodology to broaden this field of study.

Funding

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Author contributions

REV and AC wrote the manuscript, funding acquisition, investigation; REV data curation, formal analysis; REV, AR, AC reviewed the manuscript for final changes and approval; REV is the main responsible for the performed assays and all the data optained; AC support the performance of the assays of REV work. AR and AC supervised and mentored the present work. AC conceptualice the research.

Declaration of Competing Interest

The authors declare that they have no financial interest or personal ties that could influence this scientific article.

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Appendix

- ## Settings Data Tables
  - ### Centrifugation

Number of Zebrafish Survival by RCF applied						
	50 RCF	100 RCF	300 RCF	500 RCF	1000 RCF	Control (No centrifugation)
Alive	14	15	13	15	13	15
	15	14	15	15	14	14
	13	15	13	15	13	14
	14	15	15	14	14	15
	15	13	14	14	15	15
Dead	1	0	2	0	2	0
	0	1	0	0	1	1
	2	0	2	0	2	1
	1	0	0	1	1	0
	0	2	1	1	0	0

Average of the Number of Zebrafish Survival by RCF applied		
	Alive	Dead
50 RCF	14,2	0,8
100 RCF	14,4	0,6
300 RCF	14	1
500 RCF	14,6	0,4
1000 RCF	13,8	1,2
Control (No centrifugation)	14,6	0,4

Percentage of the Number of Zebrafish Survival by RCF applied		
	Alive	Dead
50 RCF	95%	5%
100 RCF	96%	4%
300 RCF	93%	7%
500 RCF	97%	3%
1000 RCF	92%	8%
Control (No centrifugation)	97%	3%

One-Way ANOVA Test (Centrifugation)

Descriptive Statistics					
Groups	*No. Essay*	*No. Individuals*	*Average*	*Variance*	*Standard Error*
50 RCF	5	71	14,2	0,7	0,37
100 RCF	5	72	14,4	0,8	0,40
300 RCF	5	70	14	1	0,45
500 RCF	5	73	14,6	0,3	0,24
1000 RCF	5	69	13,8	0,7	0,37
Control (No centrifugation)	5	73	14,6	0,3	0,24

One-Way ANOVA test						
Source of Variation	*Sum of squares (SS)*	*Degrees Freedom (df)*	*Mean Squares (MS)*	*F value*	*P-Value*	*Critical Value of F*
Between Groups	2,67	5	0,53	0,84	0,53	2,62
Within Groups	15,2	24	0,63			
Total	17,87	29				

- **Thermic Shock**

Number of Zebrafish Survival by Thermic Shock			
	4 °C - 37 °C (in E2)	**28 °C (in E2)**	**Control (20 °C, in E2)**
Alive	15	15	15
	14	14	14
	15	14	15
Dead	0	0	0
	1	1	1
	0	1	0

Average of the Number of Zebrafish Survival by Thermic Shock		
	Alive	Dead
4 ºC - 37 ºC (in E2)	14,66666667	0,333333333
27 ºC - 27 ºC (in E2)	14,33333333	0,666666667
Control (20 °C, in E2)	14,66666667	0,333333333

Percentage of the Number of Zebrafish Survival by Thermic Shock		
	Alive	Dead
4 °C - 37 °C (E2)	98%	2%
28 °C (E2)	96%	4%
Control (20 °C, in E2)	98%	2%

One-Way ANOVA Test (Thermic Shock)

Descriptive Statistics					
Groups	*No. Essay*	*No. Individuals*	*Average*	*Variance*	*Standard Error*
4 ºC to 37 ºC (in E2)	3	44	14,67	0,33	0,33
28 ºC (in E2)	3	43	14,33	0,33	0,33
Control (20 °C, in E2)	3	44	14,67	0,33	0,33

One-Way ANOVA test						
Source of Variation	*Sum of squares (SS)*	*Degrees Freedom (df)*	*Mean Squares (MS)*	*F value*	*P-Value*	*Critical Value of F*
Between	0,222222222	2	0,11111111	0,33333333	0,729	5,14325285
Within Groups	2	6	0,33333333			
Total	2,222222222	8				

- **Medium**

Number of Zebrafish Survival by Medium			
	RPMI (no thermic shock, no centrifugation)	E2 (no thermic shock, no centrifugation)	Control (PBS, no thermic shock, no centrifugation)
Alive	15	15	15
	14	14	14
	14	15	14
Dead	0	0	0
	1	1	1
	1	0	1

Average of the Number of Zebrafish Survival by Medium		
	Alive	Dead
RPMI (no thermic shock, no centrifugation)	14,33333333	0,666666667
E2 (no thermic shock, no centrifugation)	14,66666667	0,333333333
Control (PBS, no thermic shock, no centrifugation)	14,66666667	0,666666667

Percentage of the Number of Zebrafish Survival by RCF applied		
	Alive	Dead
RPMI (no thermic shock, no centrifugation)	96%	4%
E2 (no thermic shock, no centrifugation)	98%	2%
Control (PBS, no thermic shock, no centrifugation)	98%	4%

One-Way ANOVA Test (Medium)

Descriptive Statistics					
Groups	*No. Essay*	*No. Individuals*	*Average*	*Variance*	*Standard Error*
RPMI (no thermic shock, no centrifugation)	3	43	14,333333	0,3333333	0,333333333
E2 (no thermic shock, no centrifugation)	3	44	14,666667	0,3333333	0,333333333
Control (PBS, no thermic shock, no centrifugation)	3	43	14,333333	0,3333333	0,333333333

One-Way ANOVA test						
Source of Variation	*Sum of squares (SS)*	*Degrees Freedom (df)*	*Mean Squares (MS)*	*F value*	*P-Value*	*Critical Value of F*
Between Groups	0,222222	2	0,1111111	0,3333333	0,729	5,1432528
Within Groups	2	6	0,3333333			
Total	2,222222	8				

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