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Contribution to transplant of haploid cell nucleus either androgenote or gynogenote into zebrafish oocytes.

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ABSTRACT

The existence of the imprinting mechanism in zebrafish may be useful for studying the role this same phenomenon has in mammals. To try to figure out how the imprinting occurs in zebrafish, androgenote and gynogenote haploid larvae are built. Gynogenote haploid larvae are produced by fertilization of oocytes with UV light irradiated spermatozoa, and androgenote haploid larvae by UV light irradiated oocyte fertilized with sperm. Differences in the obtaining of these larvae are noted and the effectiveness of the applied radiation may be analysed. With the haploid larvae that survive after 24 hours and diploid larvae, cell cultures were performed. Using cultures derived from such larvae, somatic cell nuclear transplant (NT) experiments make it possible to assess the capacity of the oocyte to reprogram the nucleus from somatic haploid cells from already differentiated larvae. The results showed that there are significant differences between fertilization and obtainment of androgenote and gynogenote larvae, in favour of these last. The oocyte radiation can cause the destruction of cytoplasmic components as mDNA and mRNA required to obtain viable larvae, which converges in a low number of haploid androgenote larvae obtained. Regarding the cultures, contamination problems were found, but still the NT technique could be carried out with cells derived from some of these cultures. From the assays performed in NT, very few showed some cell division. For a better analysis of NT more tests and more time to master the technique would be required.

Keywords: cell culture, haploid, nuclear transplant, reprogramming, zebrafish

RESUMEN

La existencia del mecanismo de imprinting en pez cebra puede ser útil para estudiar el papel que cumple este mismo fenómeno en mamíferos. Para intentar averiguar cómo tiene lugar el imprinting en el pez cebra, se construyen larvas haploides ginogenotas y androgenotas. Las larvas haploides ginogenotas se producen por fertilización de ovocitos con espermatozoides radiados con luz UV, y las larvas haploides androgenotas por fertilización de ovocitos radiados con luz UV con espermatozoides. Se observan diferencias en la obtención de estas larvas y se podrá analizar la eficacia de la radiación aplicada. Con las larvas haploides que sobrevivan tras 24 horas y con larvas diploides se realizan cultivos celulares. El uso de los cultivos derivados de tales larvas en experimentos de transplante nuclear (TN) de células somáticas, permitirá evaluar mejor la capacidad del ovocito para reprogramar el núcleo de células somáticas haploides procedentes de larvas ya diferenciadas. Los resultados mostraron que existen diferencias significativas entre la fertilización y la obtención de larvas androgenotas y gynogenotas, a favor de estas últimos. La radiación del ovocito puede causar la destrucción de componentes citoplasmáticos como mDNA y mRNA requeridos para obtener larvas viables, que converge en un bajo número de larvas haploides androgenotas obtenido. En cuanto a los cultivos, se encontraron problemas de contaminación, pero todavía la técnica de TN pudo llevarse a cabo con células derivadas de algunos de estos cultivos. A partir de los ensayos realizados en TN, muy pocos mostraron alguna división celular. Para un mejor análisis de TN se necesitarían más pruebas y más tiempo para dominar la técnica.

Palabras clave: cultivo celular, haploide, transplante nuclear, reprogramación, pez cebra

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1. INTRODUCTION

1. 1 Epigenetics

The literal meaning of the term epigenetic is "on top of or in addition to genetics." While the genome defines the complete set of genetic information contained in the DNA, the epigenome refers to the complex modifications associated with genomic DNA, imparting a unique cellular and developmental identity. Epigenetic factors modify, restrict or enhance the potential for genes to be expressed with the consequence that cells may be able to follow a different phenotypic pathway. These series of chemical tags that modify DNA and its associated structures constitute the epigenome, and include any genetic expression modifier independent of the DNA sequence of a gene.

Therefore, the epigenome integrates the information encoded in the genome with all the molecular and chemical indications of cellular, extracellular, and environmental origin. Together they instruct the unique gene expression program of each cell type to define its functional identity during development or disease (Rivera and Ren, 2013). The phenotypic pathways are passed on through either mitosis or meiosis. They may last through cell divisions for the duration of the cell's life, and may also last for multiple generations even though they do not involve changes in the underlying DNA sequence.

The term also refers to the changes themselves. Examples of mechanisms that produce such changes are DNA methylation and histone modification, each of which alters how genes are expressed. Gene expression can be controlled through the action of repressor proteins that attach to silencer regions of the DNA. Specific epigenetic processes include paramutation, bookmarking, imprinting, gene silencing, X chromosome inactivation, position effect, reprogramming, transvection, maternal effects, the progress of carcinogenesis, many effects of teratogens, regulation of histone modifications and heterochromatin, and technical limitations affecting parthenogenesis and cloning.

Examples of epigenetics (Ptashne, 2007):

i. DNA methylation and chromatin remodelling

Chromatin is the complex of DNA and the histone proteins with which it associates. Gene expression depends on how DNA is wrapped around the histones. It is proposed that chromatin remodelling is executed through two main mechanisms:

The first mechanism is through post-translational modification of the amino acids that make up histone proteins. The change in the amino acids could imply a change in the shape of the histone. Since DNA is not completely unwound during replication, it is possible, that the modified histones may be carried into the new copy of the DNA. Here these histones may act as templates, initiating the surrounding new histones to be shaped in the new manner. By altering the shape of the histones around them, these modified histones would ensure that a lineage-specific transcription program is maintained after cell division.
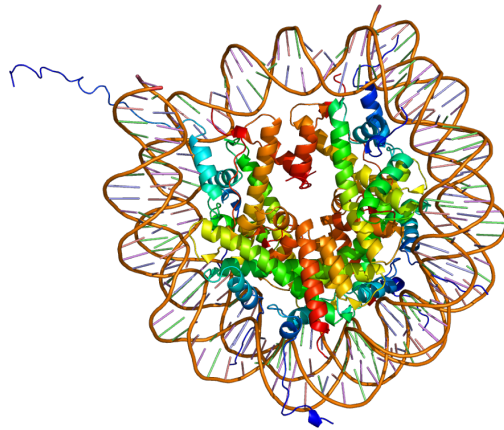
Figure 1: Nucleosome structure, DNA and histones. (Universidad de los Andes. Facultad de Ciencias. May 2014. Nº 16 ISSN 1692-729X).

The second mechanism is the addition of methyl groups to the DNA. This addition takes part mostly at CpG sites, and converts cytosine to 5-methylcytosine. 5-Methylcytosine is very similar to normal cytosine and still pairs with a guanine in double-stranded DNA. However, highly methylated areas tend to be less transcriptionally active. Methylation of cytosines can also persist from the germ line of one of the parents into the zygote, marking the chromosome as being inherited from one parent or the other (genetic imprinting).
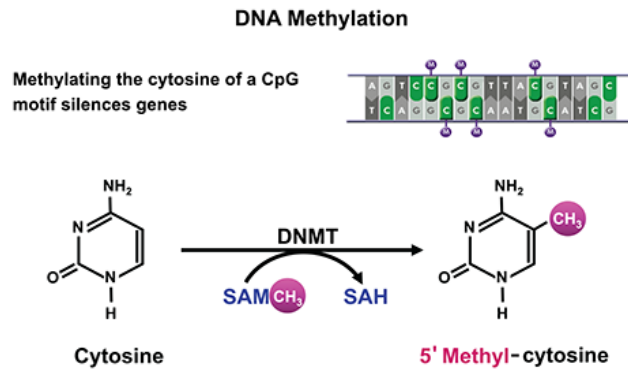
Figure 2: DNA methylation mechanism. (Samir Zakhari, Alcohol Metabolism and Epigenetics Changes).

Mechanisms of heritability of histone state are not well understood. In the other hand, much is known about the mechanism of heritability of DNA methylation state during cell division and differentiation. Heritability of methylation state depends on certain enzymes (such as DNMT1) that have a higher affinity for 5-methylcytosine than for cytosine. If this enzyme reaches a "hemimethylated" part of DNA (where 5-methylcytosine is in only one of the two DNA strands) the enzyme will methylate the other half.

Although histone modifications occur throughout the entire sequence, the unstructured N-termini of histones (called histone tails) are particularly highly modified. These modifications include acetylation, methylation, ubiquitylation, phosphorylation, sumoylation and ribosylation.

ii. RNA transcripts and their encoded proteins

RNA signalling includes differential recruitment of a hierarchy of generic chromatin modifying complexes and DNA methyltransferases to specific loci by RNAs during differentiation and development (Mattick *et al*. 2009). Other epigenetic changes are mediated by the production of different splice forms of RNA, or by formation of double-stranded RNA. Descendants of the cell in which the gene was turned on will inherit this activity, even if the original stimulus for gene-activation is no longer present. These genes are often turned on or off by signal transduction, although in some systems, where syncytia or gap junctions are important, RNA may spread directly to other cells or nuclei by diffusion. The mother contributes a large amount of RNA and protein to the zygote during oogenesis or via nurse cells, resulting in maternal effect phenotypes. A smaller quantity of sperm RNA is transmitted from the father, but this epigenetic information can lead to visible changes in several generations of offspring.

iii. MicroRNAs

Many miRNAs are epigenetically regulated. About 50% of miRNA genes are associated with CpG islands, (Bernal *et al*. 2012) which may be repressed by epigenetic methylation. Transcription from methylated CpG islands is strongly and heritably repressed (Goll *et al*. 2005). Other miRNAs are epigenetically regulated by either histone modifications or by combined DNA methylation and histone modification. It appears that about 60% of human protein coding genes are regulated by miRNAs.

1.1.1 Imprinting

Genomic imprinting is an epigenetic process that involves DNA methylation and histone methylation in order to achieve the expression of only one allele without altering the genetic sequence. It is a regulatory mechanism that causes parent-of-origin-specific gene expression and has been demonstrated in fungi, plants and animals. The epigenetic marks are established ("imprinted") in the germ line (sperm or egg cells) of the parents and are maintained through mitotic cell divisions in the somatic cells of an organism.

It was first discovered in plants in 1970, following the analysis of the unusual maternal effect of the R gene responsible for pigmentation of the seed endosperm in maize. In mice, imprinting was discovered following the unexpected outcome of nuclear transplantation experiments. Mouse embryos that had only maternal genomes (gynogenotes) or only paternal genomes (androgenotes) were enormously abnormal and did not develop beyond mid-gestation. These experiments established that a maternal and a paternal genome are both required to achieve normal development. In mice and humans, some 80 genes are now known to be imprinted, such that their expression depends on whether they are inherited from the mother or the father (Feil, Berger, 2007).

Several theories have been proposed to explain the role that genomic imprinting has played of mammalian evolution, but at present it is not clear if a single hypothesis can fully account for the diversity of roles that imprinted genes play (Wood and Oakey, 2006). Here are four evolutionary hypotheses that seem plausible: genetic conflict, the ovarian time bomb, x-linked sex-specific selection and sexually antagonistic selection.

i. Genetic conflict hypothesis (GCH): Because of multiple paternity, a mother's offspring are equally related to her but can be less related to each other. A mother's genetic interests are best served by keeping control over the distribution of her resources to these offspring, sharing it equally among them. Mothers can retain such control by inactivating fetal growth-enhancing genes that they pass on to their offspring. A father's fitness is enhanced, however, by enabling his offspring to obtain as much of this resource as maximizes their survival, even at the expense of half-brothers and the mother. Inactivating fetal growth inhibitors in his offspring serves this purpose.

ii. Ovarian time bomb hypothesis (OTH): The spontaneous development of an unfertilized egg in an ovary is a form of ovarian trophoblastic disease, essentially ovarian cancer. Inactivating the only (maternal) copy of early-acting growth enhancers lowers this risk, as does upregulating any growth inhibitors. This second change could leave the fetus with too much inhibitor, an imbalance that can be corrected by downregulating the paternal copy. Thus, both the GCH and the OTH predict that fetal growth-affecting genes are likely to be targets of imprinting, and that growth enhancers should be maternally attenuated and growth inhibitors, paternally so.

iii. X-linked sex-specific selection hypothesis (XSSH): Early in development, female eutherians randomly inactivate most of one of the two X chromosomes in each cell. Consequently, they are a mosaic of tissues with active paternal or maternal X chromosomes. Males, however, have only a maternal X, which is always active. Thus changes to the level of expression of genes on the paternal X will affect females only; changes to the maternal X will affect males more than females. Thus any selection pressure that differs between sexes would be augmented by imprinting. In particular, selection for larger males (common in mammals) could be assisted by inactivating maternal X-linked growth inhibitors and paternal X-linked growth enhancers, the opposite predictions of the GCH and the OTH.

iv. Sexually antagonistic selection hypothesis (SASH): This idea is an extension of the XSSH to autosomal loci and suggests that loci with different levels of optimal expression in males and females are likely to be imprinted. Imprinting will be favoured if the benefits to offspring of one sex outweigh the costs to those of the other. Moreover, the SASH suggests that (provided some molecular mechanism exists) loci could be subject to sex-specific imprinting (e.g. being maternally silenced in sons but not daughters).

<u>1.1.2 Reprogramming</u>

Self-renewal and pluripotency are properties that define embryonic stem cells (ESCs). They refer, respectively, to the ability of proliferate indefinitely without differentiating in vitro and the ability to differentiate into different cell lineages corresponding to the 3 layers which form an animal embryo (Gonzalez *et al*. 2011). ESCs can be derived from the inner cell mass of blastocyst stage embryos (Yamanaka, 2008). However, for obtaining in vitro stem cells (ESC embryonic type) from differentiated cells, nuclear reprogramming of such cells is required. Cell reprogramming is a term that describes the induced activation of nuclear genes, previously distinct and characteristic in a given cell type (Gurdon and Melton, 2008). Reprogramming refers to erasure and remodelling of epigenetic marks, such as DNA methylation, during development.

The process of cell reprogramming is interesting for several reasons: the first is that it identifies how this process takes place, allowing us to understand how normal expression levels of genes of specialization and cell differentiation remain. The second is that cellular reprogramming is the first important step towards developing cellular regeneration therapies, by which defective cells can be replaced by normal cells of the same or similar type, but derived from a different cell type. The third reason is that nuclear reprogramming allows the cultivation of cell lines from diseased tissue, allowing the study of the prior development of such diseases and search for potential therapeutic compounds.

The first person to successfully demonstrate reprogramming was John Gurdon, who in 1962 demonstrated that differentiated somatic cells could be reprogrammed back into an embryonic state. He managed to obtain swimming tadpoles after the transfer of differentiated intestinal epithelial cells into enucleated frog eggs (Gurdon JB, 1962). For this achievement he received the 2012 Nobel Prize in Medicine alongside Shinya Yamanaka. Dr. Yamanaka was the first to demonstrate that this somatic cell nuclear transfer or oocyte-based reprogramming process, that Dr. Gurdon discovered, could be recapitulated by defined factors (Oct4, Sox2, Klf4, and c-Myc) to generate induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006).

Although direct reprogramming, ie without requiring nuclear transplantation, just by altering the epigenetic control of gene expression by different strategies, is technical and conceptually simple, in vitro is currently an extremely slow and inefficient process that is influenced, moreover, by various variables that affect efficiency, reproducibility

and quality of the resulting iPSCs (Gurdon and Melton, 2008). The generation of iPSC from adult human dermal fibroblasts has been possible (Yamanaka, 2008), but further studies are essential to determine whether iPSCs can replace human ESCs in medical applications.

The gene-expression program of pluripotent cells is a product of regulation by specific transcription factors, chromatin-modifying enzymes, regulatory RNA molecules, and signal-transduction pathways. In vitro reprogramming pluripotency is regulated by factors both internal and external.

i. Internal reprogramming factors

Internal factors are usually transcription factors essential for maintaining the proliferation and pluripotency. The best-studied transcription factors are Oct3/4, Sox2, c-Myc, Klf4 and Nanog, which have been shown to play an essential role in the expression of pluripotency.

Oct3/4 (POU5F1): is a transcription factor of the POU family specifically expressed in ESCs, early embryos and germ cells. It was originally named as Oct3 (Okamoto *et al*. 1990) and Oct4 (Scholer *et al*. 1989). It is strongly involved in the maintenance of self-renewal of pluripotent cells. Genome-wide studies in human and mouse revealed a large list of target genes with Oct-regulatory elements and many targets have frequently been implicated in ESC signalling. A large number of these genes possess regulatory elements for the transcription of Sox2 and Nanog (Boyer *et al*. 2005, Loh *et al.* 2006). A number of putative regulatory factors for Oct3/4 have been identified (Niwa, 2007).

Sox2: (SRY-type high mobility group box 2) is part of a large family of 20 proteins that share a similar HMG box DNA-binding motif. So far, it is the only Sox protein found to have a crucial function in sustenance of ESC pluripotency. Another protein of Sox family, Sox15, is also highly expressed in ESCs, but its role is unclear. Sox2 is expressed in ESCs, early embryos, stem cells and neural stem cells (Koopman *et al*. 2004). Downregulation of Sox2 in murine ESCs promotes ESC differentiation (Ivanova *et al*. 2006). Sox2 regulatory elements in gene promoter regions are often found in close proximity to Oct3/4 and Nanog binding sites (Boyer *et al*. 2005). Several genes specific to ESCs are transcriptionally regulated by the combined action of Sox2 and Oct3/4.

c-Myc: is a helix-loop-helix/leucine zipper transcription factor, associated with a number of cellular functions including cell growth, differentiation and proliferation but also with oncogenic transformation. c-Myc has been proposed as a major downstream target for two pathways that support maintenance of pluripotency: the LIF (leukaemia inhibitory factor)/STAT3 and the Wnt signalling cascades. The first pathway, LIF signalling, is routinely used in murine ESC culture (Cartwright *et al*. 2005) and triggers a signalling cascade by binding to a hetero-dimeric LIF-receptor, resulting in activation and nuclear translocation of the transcription factor STAT3. STAT3, when overexpressed, is sufficient for the continued self-renewal of mouse ESCs even in absence of LIF (Matsuda *et al*. 1999). c-Myc has many binding sites throughout the genome (Fernandez *et al*. 2003, Li *et al*. 2003, Cawley *et al*. 2004), and is thought to alter the structure of chromatin and activate the expression of some miRNAs (Knoepfler *et al*. 2006).

Klf4: is a Kruepel-tupe zinc-finger transcription factor, and like c-Myc it is a downstream target of activated STAT3 in LIF-induced ESCs. Its overexpression leads to sustained expression of Oct3/4 and inhibition of differentiation in ESCs (Lin *et al*. 2005). Similar to Sox2, Klf4 can also act as a cofactor for Oct3/4-mediated regulation of gene transcription. However, this seems to apply only to a very limited number of genes, including Klf4 itself and Lefty1 (Nakatake *et al*. 2006). So far there is no evidence that Klf4 is required to exert a similar function for other target genes of Oct3/4 or Sox2. Klf4 may be indirectly involved in the upregulation of Nanog protein by repressing p53 (Rowland *et al*. 2005), a negative regulator of Nanog (Lin *et al*. 2005).
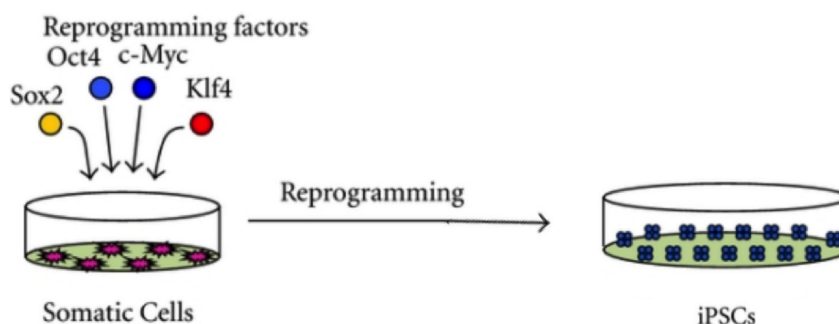

Figure 3: Reprogramming. (Based on: Sun G, Fu C, Shen C, Shi Y. Histone Deacetylases in Neural Stem Cells and Induced Pluripotent Stem Cells. J. Biomed. Biotechnol. 2011).

Nanog: is a homeobox protein specifically expressed in pluripotent cells and inner cell mass of blastocyst stage embryos (Chambers *et al*. 2003, Mitsui *et al*. 2003). The overexpression of Nanog in mouse ESCs allows cells self-renewal in the absence of LIF. Similarly, overexpression of this gene in human ESCs allows growth without co-culture with feeder cells (Darr *et al*. 2006). In addition, Nanog overexpressing ESCs show a markedly higher reprogramming capacity when fused to somatic cells (Silva *et al*. 2006). The expression of Nanog by Oct3 / 4 and Sox2 (Kuroda *et al.* 2005, Rodda *et al.* 2005) is activated and is suppressed by p53 (Lin *et al*. 2005), GCNF (Gu *et al*. 2005) and TCF3 (Pereira *et al*. 2006). Analysis by precipitation of the complete genome shows that Oct3 / 4, Sox2 and Nanog share many target genes in mouse and human ESCs (Boyer *et al*. 2005, Loh *et al*. 2006).

Epigenetic regulation: the DNA methylation pattern is one of the most important regulation factors in the transcription of a cell, so the reprogramming of a differentiated cell must involve demethylation of methylated genes. In addition to the processes of methylation, epigenetics regulation of pluripotency also includes promoters of transcription factors, chromatin-modifying enzymes, and proteins of the Polycomb group (PcG) which are epigenetic regulators that facilitate maintenance of cell state through gene silencing (Bernstein *et al*. 2006, Boyer *et al*. 2006, Lee *et al*. 2006).

ii. External reprogramming factors

External factors such as signalling pathways of growth factors are very important for the regulation of cellular self-renewal. So isolated ESCs maintained in culture need different signals from the feeder cells to maintain their undifferentiated state. External factors necessary for the regulation of pluripotency are different in mouse and human, depending on the growth factor receptors, which are expressed in every species. For example mESCs require LIF that activates STAT3 when it binds to its receptor, whereas hESCs require FGF2 to proliferate maintaining a dedifferentiated state (Noisa and parnpai, 2011).

So the process of dedifferentiation of somatic cells to a pluripotent state, by which the cell adopts characteristics of ESC or embryo cells, which is what is called cellular reprogramming can be achieved in vitro by different techniques: cellular fusion with ESCs, cellular extracts exposition, nuclear transplant and ectopic expression of transcription factors, resulting in the latter case to iPS.

Strategies to induce epigenetic reprogramming

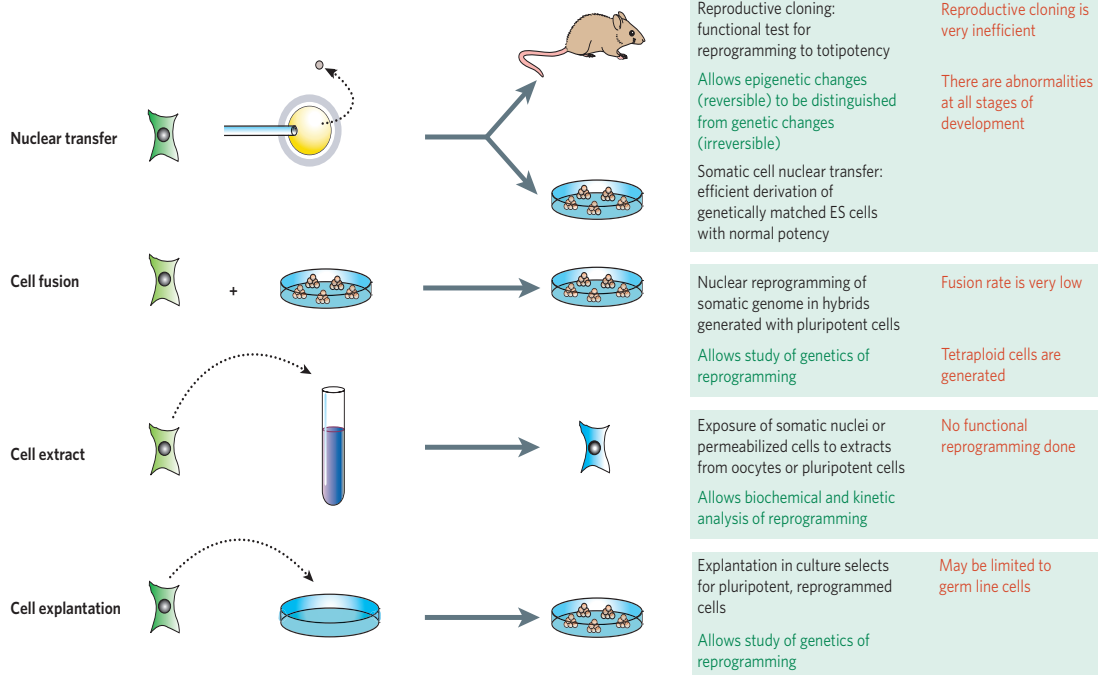|	Experimental approach / Mechanistic insights	Limitations
Nuclear transfer	Reproductive cloning: functional test for reprogramming to totipotency	Reproductive cloning is very inefficient
	Allows epigenetic changes (reversible) to be distinguished from genetic changes (irreversible)	There are abnormalities at all stages of development
	Somatic cell nuclear transfer: efficient derivation of genetically matched ES cells with normal potency	
Cell fusion	Nuclear reprogramming of somatic genome in hybrids generated with pluripotent cells	Fusion rate is very low
	Allows study of genetics of reprogramming	Tetraploid cells are generated
Cell extract	Exposure of somatic nuclei or permeabilized cells to extracts from oocytes or pluripotent cells	No functional reprogramming done
	Allows biochemical and kinetic analysis of reprogramming	
Cell explantation	Explantation in culture selects for pluripotent, reprogrammed cells	May be limited to germ line cells
	Allows study of genetics of reprogramming	

Figure 4: Different approaches for studying Nuclear reprogramming. First, nuclear transfer involves the injection of a somatic nucleus into an enucleated oocyte, which, upon transfer into a surrogate mother, can give rise to a clone (reproductive cloning), or, upon explantation in culture, can give rise to genetically matched ES cells (somatic cell nuclear transplant). Second, cell fusion of differentiated cells with pluripotent ES cells results in the generation of hybrids that show all features of pluripotent ES cells. Third, exposure of somatic cells or nuclei to cell extracts fro oocytes or ES cells recapitulates early biochemical events of reprogramming without stably changing cell fate. Fourth, explantation of germ cells in culture selects for immortal cell lines that have regained pluripotency. (Konrad Hochedlinger and Rudolf Jaenisch. Nuclear reprogramming and pluripotency. Nature vol 441 29 june 2006).

The fusion NT is characteristic of mammals, in contrast other animal models, such as zebrafish, the microinjection NT technique is used. This is also the technique that is used to reprogram and obtaining viable embryos.

<u>1.1.2.1 Nuclear transplant (NT)</u>

After normal fertilization, zygotes can form complete and fertile individuals. Even when not fertilized, in many species, parthenogenetically activated oocytes, especially if they are diploid, can develop complete individuals, but not in all cases are able to develop germ line. On the other hand, somatic cells lack the ability to generate a complete organism, so the nucleus of a cell of this type has to be reprogrammed to participate in the normal development of an individual (Gurdon and Wilmut, 2011).

The NT of the nucleus of a fully differentiated cell to an oocyte or enucleated egg, allows the reprogramming of a cell in differentiated state to a totipotent state, so that it participates in the development of an entire organism (Gurdon and Byrne, 2003). During NT, the oocyte turns off tissue specific genes in the somatic cell nucleus and turns back on embryonic specific genes. This implies the elimination of gene expression patterns and epigenetic state in the donor nucleus, followed by a reestablishment of totipotency in the transplanted embryos.

In mammals the reconstructed embryo can recapitulate embryogenesis. If it is transferred to a receptor animal a full development may occur, resulting in a cloned animal. This process is known as reproductive cloning. The overall rate of obtaining living NT individuals in mammals is between 1-3%.

Furthermore, the ESC can be isolated from the blastocyst in what is known as therapeutic cloning. These cells can be used as tools in biomedical research or as a source of cells for cell therapy. The major application of this technique in humans is the therapeutic cloning, however there are some technical barriers that must be overcome before the cells obtained by nuclear transplant serve as a source for cell therapy (Summer *et al*. 2011). This technique is very restricted in humans, because the access to a source of human oocytes is difficult (you have to use donations) and the ethical constraints at the moment (Noisa and Parnoai, 2011).
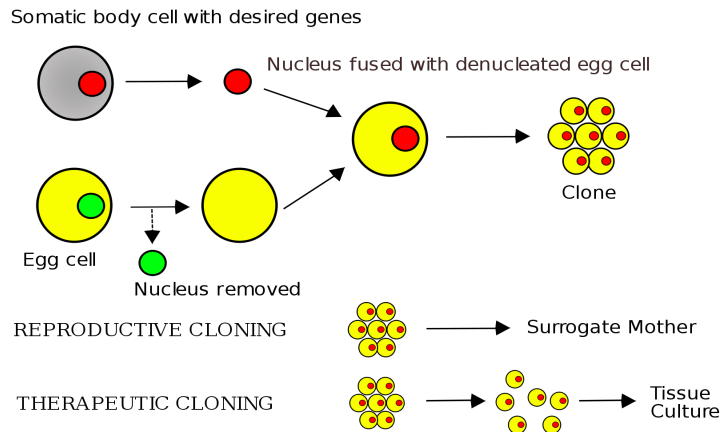
Figure 5: Reproductive and therapeutic cloning. (Wikipedia.org. 5 February 2015).

1.2 How zebrafish is used in these studies

Martin and McGowan (1995) in their study of zebrafish demonstrated the existence of the gametic imprint as in mammals. In this study, it was observed that the methylation of a locus in the female germ line is diminished, while in the male germ line is increased, equivalent to what occurs in mammalian imprinting. The similarity of this process between mammals and zebrafish supports the imprinting conservation throughout evolution. So the existence of the mechanism in zebrafish can be useful to gain an understanding the role of imprinting in mammals.

1.2.1 Zebrafish as a model organism

Zebrafish have been widely used as model organisms for studying various aspects of developmental biology because of their small size, fecundity, and because zebrafish embryos undergo rapid development in vitro, which facilitates morphological monitoring. Over the past decade, these attributes have extended the use of this model fish to a wide variety of fields in biomedical research, including functional genomics, environmental and high-throughput toxicology screening and specific human diseases (Dong, 2007).

The zebrafish has become an excellent genetic model for studying vertebrate development and behaviour, due to their short generation time and high fertility. Researchers from academic institutions and pharmaceutical companies see the

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zebrafish as a powerful new tool in the research and development of new drugs, allowing acceleration in the analysis of biological processes involved. In fact zebrafish has many features that make it a useful model: relatively small as adults (3.4 cm), cheap, with an easy accommodation, and can be kept in large quantities. Besides the embryos are relatively large and transparent, allowing its rapid development to be easily observed through the transparent chorion. Therefore they have a great value in experimental embryology.

Given all these attributes, experiments in zebrafish are a good alternative option to mouse, which is currently one of the main vertebrate models in the development of new drugs. Noted that despite the evolutionary distance, tests in zebrafish allow better understanding of many human pathologies and treatments.

Due to the physical and ethical problems of the realization of experiments on humans, research primarily used animal models to study biological processes conserved between humans and lower vertebrates. The zebrafish, with its special characteristics, has become a popular vertebrate model in different areas of research.

The main scientific areas of the use of zebrafish are: biomedicine, cancer, regenerative medicine, pharmacology and toxicology. A special application is obtaining haploids, since zebrafish has the ability to generate haploid embryos.
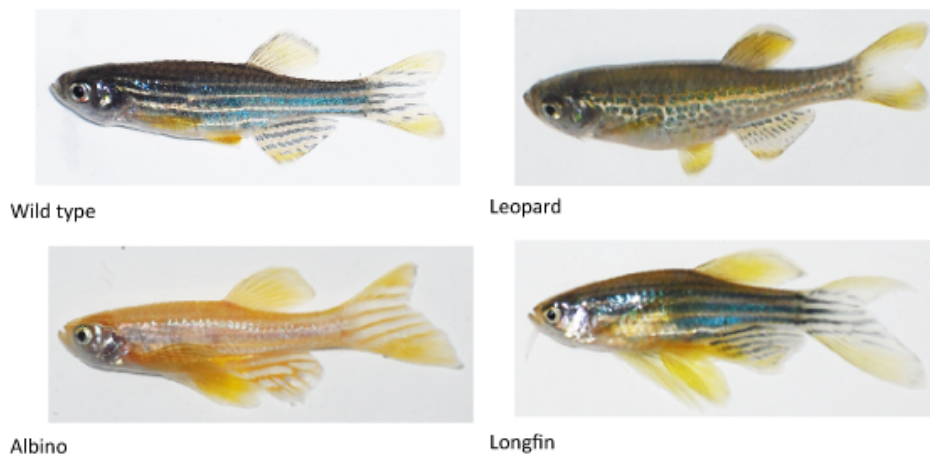

Figure 6: Zebrafish strains. (http://www.kaluefflab.com/zebrafish.html. 5 February 2015).

3. How this work is a continuation of others/Protocol establishment

This study is a continuation of a research line in the biotechnology laboratory of the animal science department. Mireia Pérez and Jose Cardona began this research with their respectives thesis.

Mireia Perez Camps "epigenetic reprogramming of somatic cells by nuclear transplant in zebrafish", 2009.

Jose Cardona Costa "embryological and micromanipulation techniques in zebrafish and pacific oyster", 2010.

Some steps of this research have been refined by Maria del Carmen Santos and Rita Galiana.

Maria del Carmen Santos Merino "Contribucion al establecimiento de un modelo de estudio de imprinting y reprogramacion basado en el cultivo de celulas haploides y transferencia nuclear en pez cebra", 2011.

Rita Galiana Cano "Evaluacion de la capacidad de reprogramacion de celulas somaticas haploides androgenotas y ginogenotas obtenidas a partir de larvas de pez cebra de 24 horas y de 5 o mas dias post-fecundacion utilizadas como pseudogametos mediante trasplante nuclear", 2012.

2. OBJECTIVES

The general objective of this study is refine and evaluate the different stages in the protocols. The model consists in the utilization of haploid cell cultures capable of being reprogrammed and participate in the generation of diploid organisms. Since it is a final degree project it is also intended to acquire practical knowledge and skills of different techniques in the various stages. The steps to be covered are:

 Obtaining gametes and UV irradiation

 IVF

 Obtaining haploid larvae, androgenotes and gynogenotes

 Haploid cell cultures

 The making of microinstruments

 Micromanipulation for NT

After a statistical analysis we will proceed to the evaluation of the following aspects:

 Compare the efficacy of IVF with either irradiated or non-irradiated gametes

 Comparison of androgenote and gynogenote larvae

 Comparison of cell cultures

 NT analysis

3. MATERIALS AND METHODS

To achieve the objectives described above we must carry out the following steps in order to complete the model of utilization.

3.1 Care and maintenance of zebrafish colony

Two different strains of zebrafish (*Danio rerio*) were used: wild and gold. The colonies of these two strains were maintained in separate tanks in an adequate proportion females/males of 3:2 under standard conditions (Westerfield, 2007). Its feeding was based on a commercial granule for tropical fish and was supplemented with egg yolk and shrimp meat, as an alternative to living food (daphnia or brine shrimp nauplii), which are highly recommended for egg production (Francisco-Simão, 2007). Food distribution was automatic with two intakes, in the morning and afternoon, and the supplements were preferably distributed in the morning. The tanks were 20-liter with water filtration, their circadian cycle was adjusted to 14 light hours and 10 dark hours, and were kept at a water temperature of 28.5 ºC.

Figure 7: Maintaining of the adult zebrafish colonies.

3.2 Obtaining inactivated gametes

At the time of the artificial sunrise (8:45 am), those tanks in which fishes showed reproductive behaviour were selected. Immediately males and females were separated to avoid natural reproduction.

Figure 8: Separation of males and females.

3.2.1 Obtaining oocytes

Oocytes were only extracted from those females that clearly exhibit reproductive behaviour, and afterwards the quality (coloration, shape, size, opacity, etc.) of the oocytes obtained was evaluated (Santos Merino, 2011). To do so, females were sedated in a solution of clove oil (200 µL in 1 L of dechlorinated and decalcified water: system water (Westerfield, 2007)) and the oocytes were obtained by extrusion of the ovarian cavity. Special care needed to be taken so that the eggs did not come into contact with water, as they would be immediately activated. Successively, eggs were placed on a petri dish with Hank's buffered salt solution (Sigma-Aldrich H8264) with 1.5% (v/v) bovine serum albumin (BSA) and 0.1 g NaCl/ 100 cc Hank's (F1 medium; pH 7.4; osmolarity 310 - 320 mOsm) and were maintained at a temperature of 8 ºC until use (90 minutes at most).
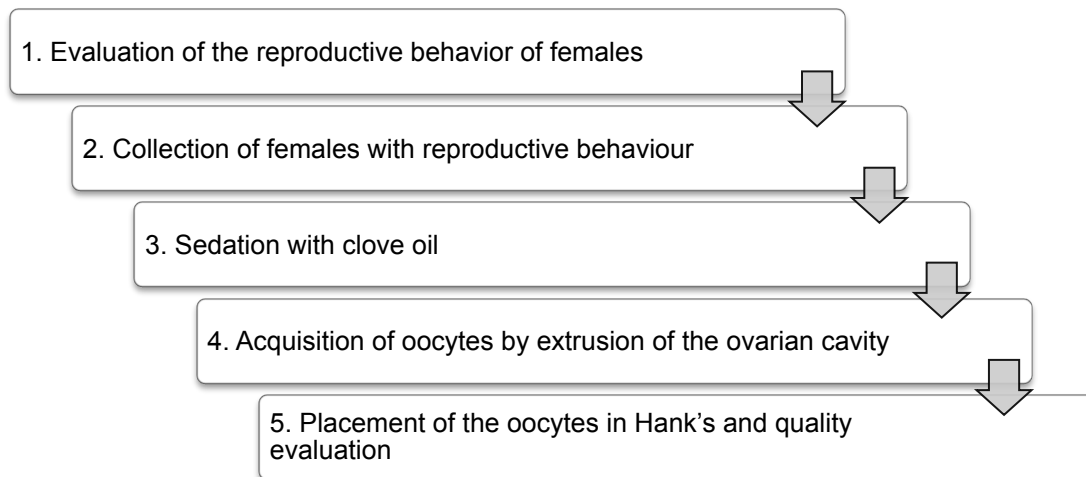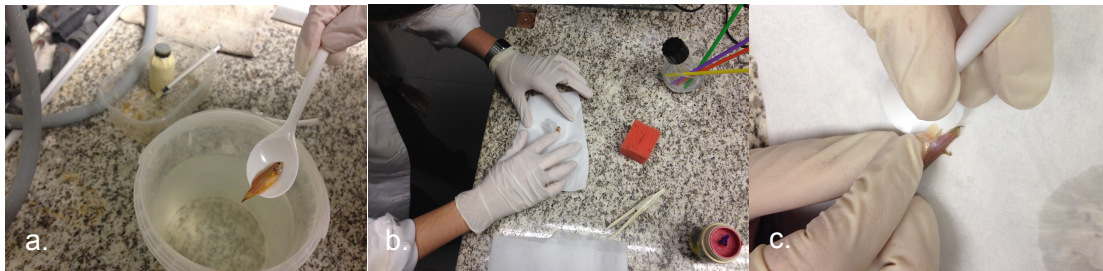
Figure 9: Steps to obtain oocytes.

Figure 10: a. Sedation with clove oil, b. removal of water, c. extrusion of ovarian cavity.

3.2.2 Obtaining sperm

Males that exhibit reproductive behaviour were sedated as described above for females. Following the protocol, the abdominal region was gently rubbed with plastic clamps while semen was collected in a glass microcapillary at the genital pore. The microcapillary (1 x 90 mm, Narishige Scientific Instrument Lab.) was then placed in ice until its use. Afterwards semen belonging to different males (2-3 males, 0.5-2 µL per male) was diluted in 100 µL of F1 medium. This way sperm was maintained inactivated, although a sample was activated in order to evaluate its quality and motility under microscope.
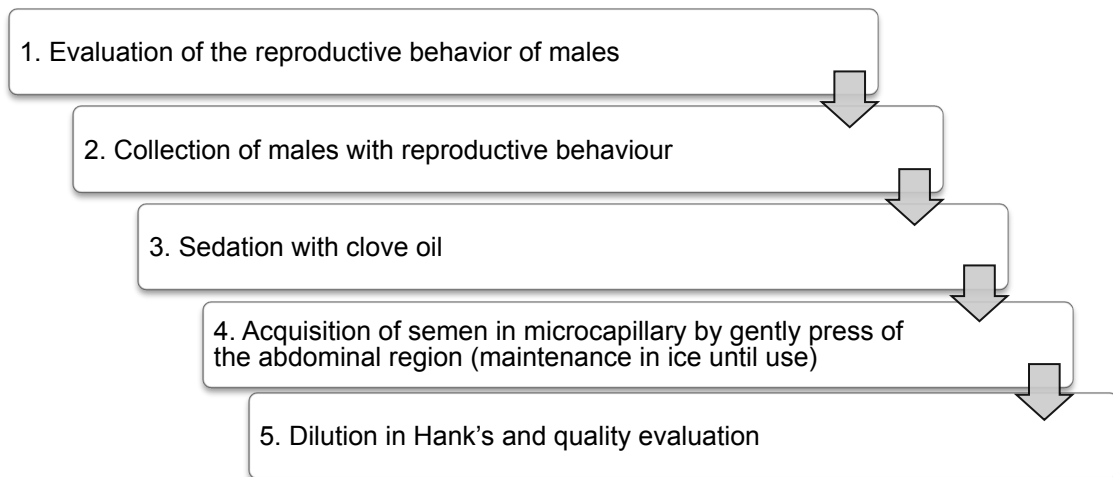
Figure 11: Steps to obtain sperm.

Figure 12: a. Sedation with clove oil, b. extraction of semen, c. maintenance in ice.

3.3 Obtaining haploid cells

Haploid organisms are those which contain just one copy of each chromosome. Achieving haploid organisms is a hard-won labour, principally because of the high death rate of the embryos and larvae. This death rate is due to the abnormalities they present, although in some cases diploidization of the initially haploids helps to overcome this limitation.

Gynogenotes can be developed, naturally or artificially, by a reproductive mechanism in which the genetic information contained in the ovule is activated by a sperm who does not contribute with its genetic information to the development of a new individual. On the other hand, androgenotes are developed from an ovule that only contributes cytoplasmically and a sperm which provides the genetic information for the embryo progress (Carter *et al*. 1991, Purdom, 1969, Nagy, 1987).

3.3.1 Gamete radiation

3.3.1.1 Sperm radiation (obtaining gynogenotes)

Samples of semen diluted in 100 µL of Hank's solution were irradiated using a UV germicide lamp (General Electric, 30 W). The samples were placed 62 cm from the spotlight and a radiation of 0.529 mW/cm2 was applied (measured with USB 400, a miniature optic fibre spectrophotometer; Ocean Optics Inc. First In Photonics, USA). Sample was vortexed at 200 rpm in order to standardize the area of radiation during exposure to UV light (Francisco-Simão et al. 2010). The radiation time was 1 minute 30 seconds.

3.3.1.2 Oocyte radiation (obtaining androgenotes)

The gathered oocytes were irradiated with UV light following the procedure for sperm in a similar manner, but without vortex agitation (to avoid activation) and extending the exposure time to 12 minutes.

3.3.2 In vitro fertilization (IVF)

In zebrafish oocytes rapidly lose their ability to be fertilized (90 minutes at maximum). Moreover, the time lapse between full activation of oocytes and IVF is extremely short (seconds) in this species (Nüssslen-Volhard and Dahm, 2002). Previous to fertilization, sperm samples showing optimal motility (80-100 %) were selected.

The IVF of non-irradiated oocytes with irradiated sperm and vice versa was carried out following the method described by Westerfield (2007). Inactivated oocytes maintained in Hank's were mixed with sperm in 35 mm petri dishes and both gametes were activated by the addition of a few drops of system water. After 2-3 minutes, the plate was filled with system water and placed in an incubator at temperature 28.5 ºC. An hour later, fertilization was assessed, and fertilized oocytes were placed in 90 mm petri dishes. A maximum of 20 embryos were placed per plate to allow a proper development.
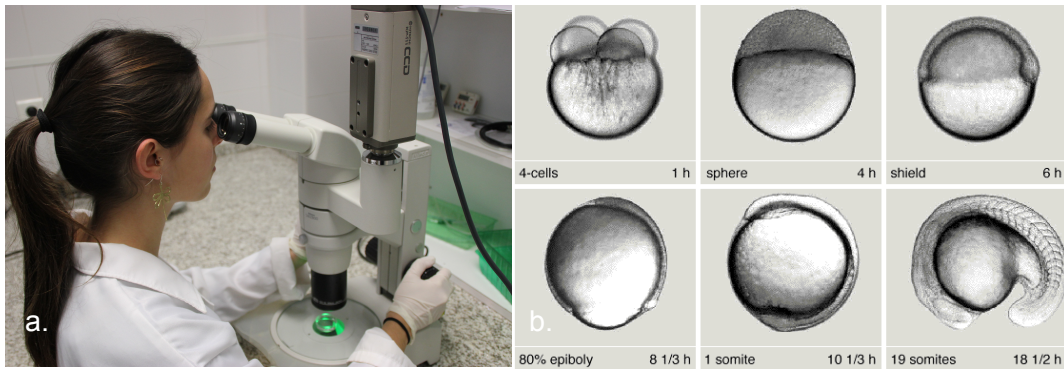
Figure 13: a. Embryo observation after 1 hour, b. embryo stages. (http://people.ucalgary.ca/~browder/why_fish.html 5 February 2015).

After 24 hours, the larvae exhibiting the so called "haploid syndrome" were selected for in vitro culture. Haploid syndrome in zebrafish is expressed with the following physical characteristics: haploid larvae are shorter than the diploid, the pericardial cavity is swollen, come into MBT (mid blastula transition stage) earlier, cells are smaller and more advanced in the development, at 48 hours they are more pigmented, and may have more than one otic cavity. Of them all it was decided to assess the presence of multiple "ears".
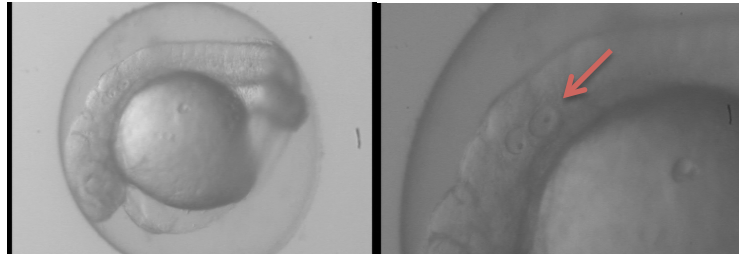

Figure 14: Haploid larvae with multiple ears.

3.3.3 Haploid larvae culture

After 24 hours, the haploid larvae were mechanically dechorionated, disinfected with 0.2 % v/ v bleach/ system water and washed in Hank's and culture medium (L15). Androgenotes and gynogenotes larvae were grown separately in Leibovitz medium supplemented with 20 % FBS and 0.036 g/ 100 ml of glutamine (L15-FBS) in Petri dishes of 90 mm at a temperature of 28.5 ° C (Westerfield, 2007). Besides being dechorionated, the yolk was also removed and the larvae were disaggregated in the culture plate for fixation to substrate. The culture was settled if there were enough haploid larvae, since a minimum of 8 larvae per plate were required to initiate the culture.
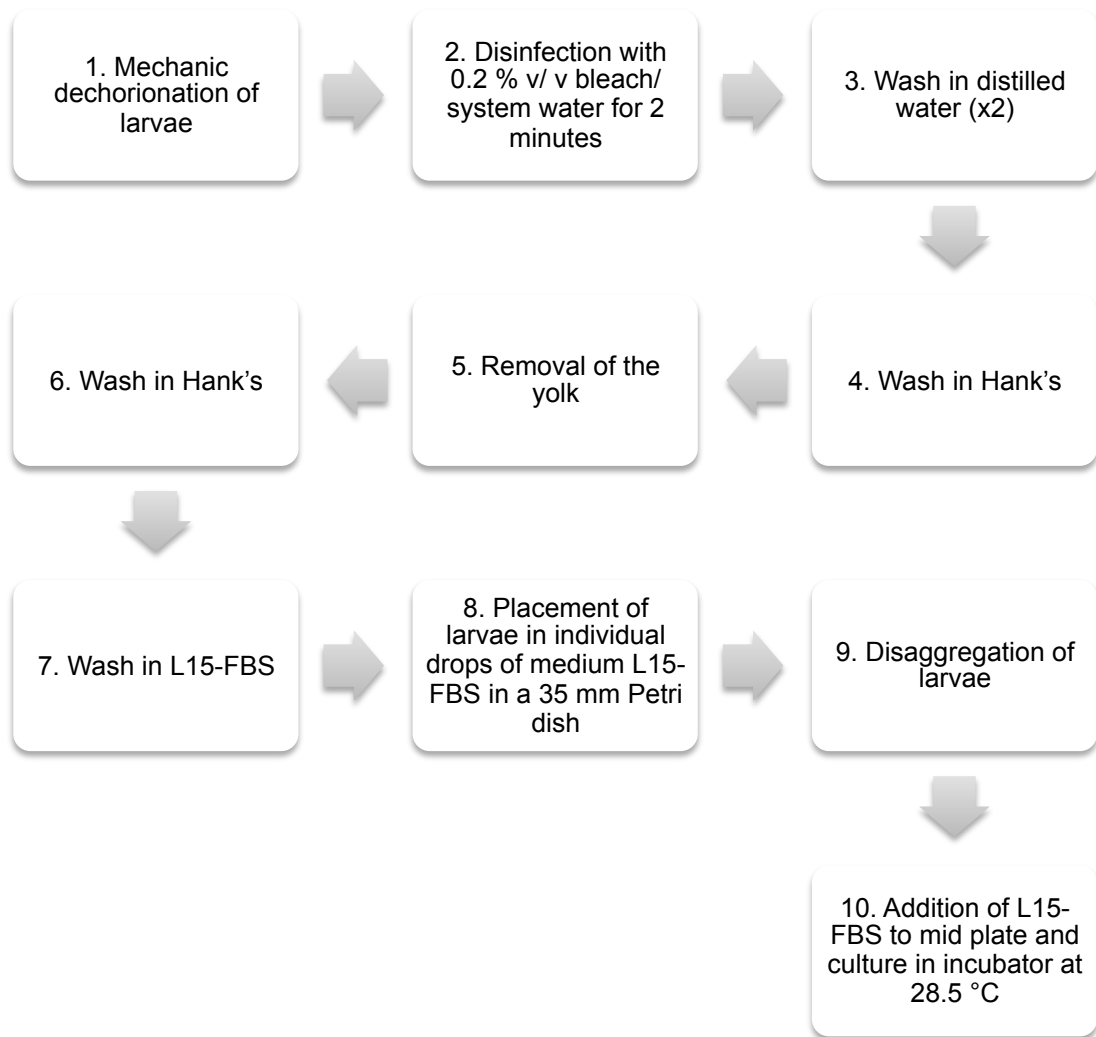
Figure 15: Steps to prepare larvae culture.
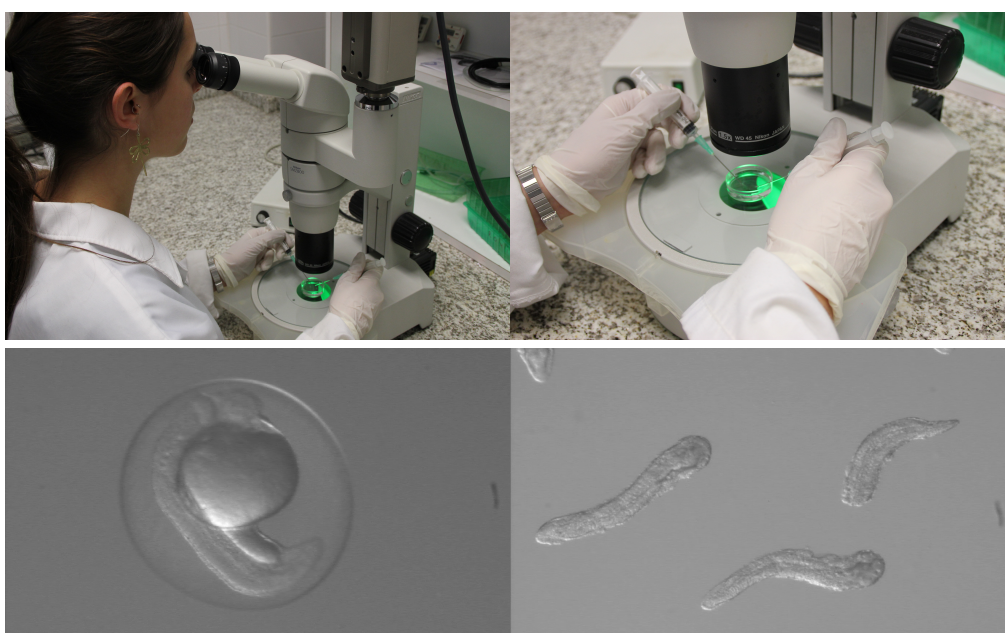
Figure 16: Dechoronation of larvae and yolk removal.

Figure 17: Preparation of larvae culture.

3.4 Obtaining diploid cells (culture control group)

This way, development of haploid cell cultures could be compared with diploid cell cultures.

To achieve diploid cultures (culture control group), the gametes are not radiated. Fertilization was performed with the same procedure and after 24 hours normal diploid larvae were obtained. The same protocol for the cell culture from larvae was carried out. This way, development of haploid cell cultures could be compared with diploid cell cultures.

Figure 18: Proceding of culture larvae steps and incubation.

3.5 Nuclear transplant (NT)

For nuclear transplant a Nikon inverted microscope equipped with two Leitz micromanipulators was used. During the manipulation process, the non-dechorionated eggs were held with a 260 µm outer diameter holding pipette and the cells were picked, lysed and injected into the eggs by means of a 10-12 µm inner diameter microinjection pipette. The microinjection pipette was fire polished, beveled and sharpened.
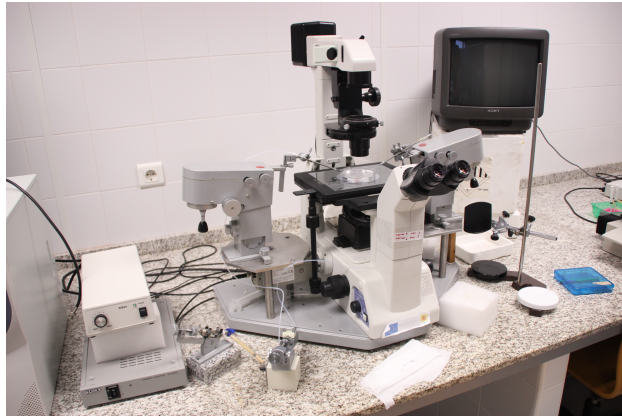
Figure 19: Micromanipulator equipment.

To perform the nuclear transplant, two separated drops were deposited in a Petri dish (90 mm) and covered by mineral oil. One of them contained the donor cells and was composed by L15 medium. The other drop was the handling drop, which means the place where the nuclear transplant was performed, so the medium was system water. The nuclear transplant was simultaneous to the egg activation since they were activated by the water drop. The donor cell was picked up and lysed by aspiration with the injection micropipette before injection. The egg was injected in the incipient animal pole.
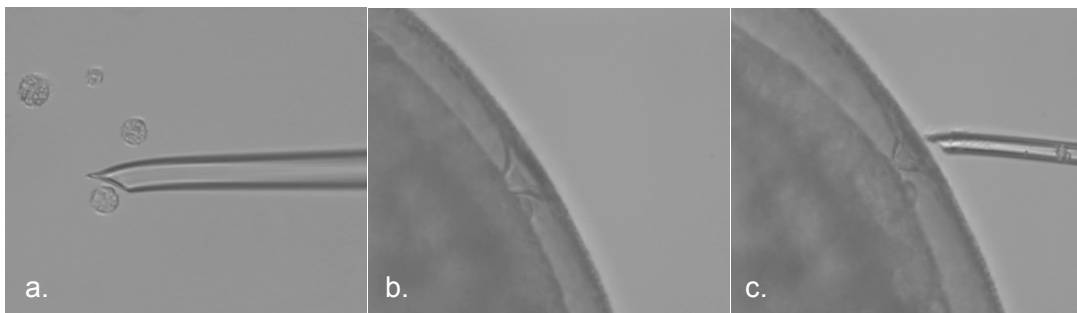
Figure 20: a. Picking of the donor cell, b. animal pole localised, c. microinjection at animal pole.
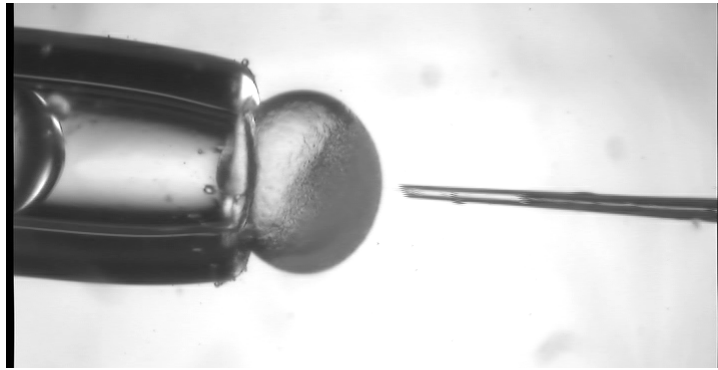
Figure 21: Microinjection of donor cell into oocyte.

Overall, the length of each experimental session did not exceed 90 minutes in any case.
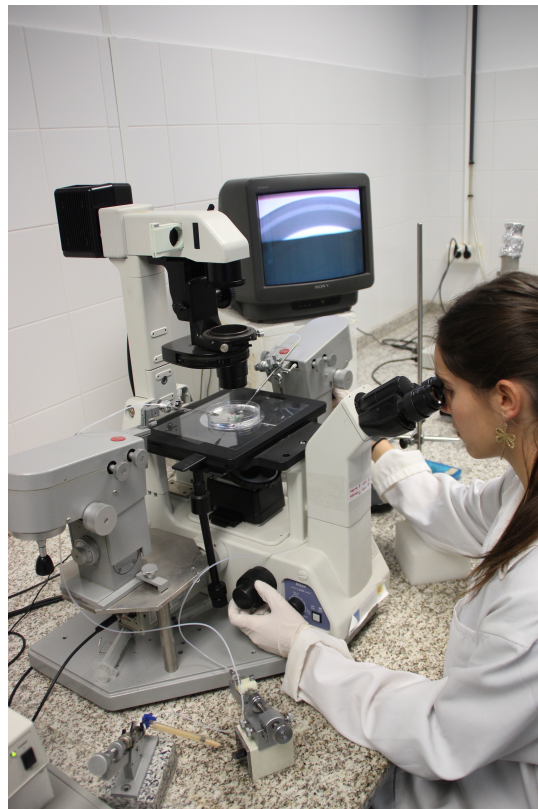
Figure 22: Procedure of NT.

3.6 Statistical analysis of the results

To process the obtained data a statistical analysis was carried out. Since in the experiments percentages are being compared, they were analyzed using a chi-square test. When the analysis showed a single degree of freedom Yates correction for continuity was applied.

4. RESULTS AND DISCUSSION

As mentioned in materials and methods there are different stages, the results of which we will discuss below.

4.1 In vitro fertilization of gametes after being irradiated with UV light

This experiment allows us to assess if the ability of fertilization of irradiated gametes is maintained and if this is regardless of type of radiated gamete. To do so, the percentage of embryos that survive three hours postfertilization (stage MBT) was compared between 3 experimental groups previously established: diploid, androgenote and gynogenote. The diploid group was used as a control and fertilization took place without any irradiated gamete.

Table 1: Fertilization rate.

Diploid	Androgenote	Gynogenote
297/474	314/3389	648/2953
(62.66 %)[a]	(9.27 %)[b]	(21.94 %)[c]

Data in columns with different superscripts are statistically different ($P<0.05$)

The results show that there are significant differences between the 3 groups. As expected, the diploid group has the higher fertilization rate, and between the irradiated gametes the gynogenotes presented a slightly higher rate. This could indicate that the UV radiation affects differentially the fertilizing capacity of oocytes and sperm. According to these results we can say that the radiation with UV light itself affects the fertilizing capacity of gametes, being the oocytes most damaged.

4.2 Obtaining haploid larvae

As well as evaluating the influence of UV radiation on the ability of gametes to perform fertilization, also its effectiveness in the production of haploid larvae was evaluated. The percentage of larvae that had the so called haploid syndrome was assessed after 24 hours of fertilization. Androgenote and gynogenote larvae were distinguished.

Table 2: Haploid larvae rate.

Androgenote	Gynogenote
38/134	83/180
(28.36 %)[a]	(46.11 %)[b]

Data in columns with different superscripts are statistically different ($P<0.05$).

These differences in the efficiencies of haploid larvae production, much higher in gynogenote than androgenote, could be explained considering the long-term effect of UV irradiation on the gamete, greater on oocyte components.

The development of an embryo in the early stages post-fertilization is very dependent on some maternal factors present in the oocyte like mRNA and proteins, provided by the mother during oogenesis. The development period affected by components of maternal origin (up to MZT, in fish corresponds to the MBT), is a period of extraordinary cytological and nuclear transitions, in which they participate (Matzuk and Burns, 2012). The oocyte is also the gamete that provides most of mitochondrial DNA and cytoplasm, and therefore, most of cellular components such as mitochondria, centrioles and so on, which may also be affected by radiation (Corley-Smith *et al*. 1996).

Many maternal genes are translated into factors related to the viability of the zygote in its early stages, such as TAp73 gene. This is a related transcription factor involved in the regulation of cell proliferation, survival and differentiation, as well as p53 and p63 (Rentzsch, 2003). Therefore to irradiate UV light can induce mutations in the DNA that prevent the correct transcription of these genes and thus not fully active.

We must take into account that there are significant differences in the expression of the zygote genome depending on the paternal or maternal origin of the copy of the genome (gametic imprinting). So many functions of maternal genes may be not fully compensated by the expression of the paternal copy, if the first are damaged by UV radiation. Which is the case of androgenotes, and may justify its lower number of haploid larvae obtained. Although zebrafish experimental results suggest that such genes, genetically imprinted, are not completely indispensable to the survival of the embryo (Corley-Smith *et al*. 1996).

Also the inactivation of the maternal genetic material is more difficult than the paternal genome inactivation by UV radiation. In fact the radiation level established in our laboratory is different and higher for oocyte than sperm. It should be taken into account that the oocyte radiation, depending on the applied dose, as we have said can cause the destruction of other cytoplasmic components as mDNA and mRNA required to obtain viable larvae (Lozano *et al*. 1987, Thorgaard, 1983). So maybe there has been excess in the radiation dose of UV light applied to oocytes, that ends/which converges in a low number of haploid androgenote larvae. Maybe a change in the protocol could be proposed and test different doses of radiation to see if the efficiency in obtaining larvae is increased.

All this would explain that the final rate of obtaining haploid larvae is less in the experimental group androgenote, since many of the supposed haploid embryos affected by UV light do not develop into haploid larvae. Another aspect to mention is that many of the larvae obtained were diploid. This could indicate that the gametes had not been greatly affected by exposure to UV light, and that the diploid condition itself would make the larvae survive further. For future experiments a possible increase in the dose of radiation to be applied could be evaluated, although this implies lower viability. There may be biological variables that affect the effectiveness of UV irradiation, since not every day the same quality of gametes are obtained, and these variables we can not control.

This low number of haploid larvae obtained will be a limitation in the following stages of the planned experimental model.

4.3 Cell culture establishment

In this third phase, the ability of in vitro cell growth of the different experimental cell types and their potential for prolonged cultivation was assessed. Cells from these cultures were used as donors in the nuclear transplant technique. Due to the low number of haploid larvae, diploid larvae derived from irradiated gametes were also cultured. We called these experimental groups androgenote control or gynogenote control according to the irradiated gamete. A total of 5 experimental groups were raised and their growth ability in culture was evaluated. These data were analysed based on the number of plates placed in culture.

Table 3: Culture results.

	haploid androgenote	haploid gynogenote	control (dipoid)	control androgenote	control gynogenote
plates	3	13	2	8	3
larvae	29	106	20	74	26
fixation	67 %	92 %	100 %	100 %	100 %
colonization	67 %	85 %	100 %	100 %	100 %
proliferation	67 %	69 %	0 %	63 %	33 %
contamination	33 %	23 %	100 %	63 %	100 %
cellular death	0 %	69 %	0 %	0 %	0 %

We know that the initial number of larvae placed in culture influences the ability of colonization and proliferation of cell culture, so instead of placing more plates with fewer larvae we established a medium number of 10 larvae per plate. This allows us to compare the plates between them since the number of larvae per plate is no more a variable.

Observing the number of plates available for each group we can appreciate that the gynogenotes have the most (13), whereas androgenotes have very few (3). This is due to the low number of haploid androgenote larvae obtained. In first place only cultures of androgenote and gynogenote haploid larvae were going to be set, but viewing the large number of diploid larvae the two last control groups were set. These allowed us to complete following experimental stages as well as detecting whether it is the haploid condition or the fact of being radiated what limits culture growth.

When you look at the androgenotes we can see that besides the few plates and larvae, its fixation rate is the lowest of all the experimental groups and therefore the cultures could not continue with the colonization and proliferation. In the gynogenotes nearly all the plates were fixated and continued the growth and expansion process, but they were stopped by cell death. This indicates that the viability of haploid cultures is lower than normal diploid cell cultures, because it does not reach the subconfluency state due to prior cell death.

The control group had no problem with fixation or posterior growth but all plates were contaminated at the proliferation stage. The radiated control groups suffered the same

fate as the control group. They began with good growth forecast but were stopped by contamination and could not proliferate. It is worth noting that radiated control groups were stopped by contamination and not by cell death as in the case of haploid groups, indicating a difference in cultures of diploid and haploid cells.

We can propose that the cultures went not too well because of various reasons: the low number of haploid larvae, contamination problems and possibly because of the haploid condition itself. The results suggest that the cause of poor culture growth is actually due to the haploid condition and not the fact of being radiated, because radiated control groups did grow well. A plausible explanation for this could be that the cells from haploid larvae may have different culture requirements than diploid cells, possibly due to the existence of genomic imprinting (Tsalavouta *et al*. 2009, Jiang *et al*. 2010). We could try growing these cells in other culture media or with some type of supplementation such as serum embryonic extract.

Regarding contamination in the laboratory, it was really problematic and slowed the progress of the experiment. Hygienic and protective measures were carried out but still contamination emerged. For future experiments more laboratory cleanings and environmental sterilization should be periodically made, as well as extreme caution in the handling of cultures.

4.4 NT results

In this final stage, nuclear transplant, using as recipients gold oocytes and as donor the nucleus of wild culture cells, was performed. This allowed evaluation of the ability of non-enucleated egg to reprogram somatic nuclei haploid chromosome based on their endowment and its degree of prior differentiation.

In view of establishing a model of utilization, despite complications found we reached the final stage of the experiment. At first we did not have culture haploid cells to perform NT, so cells derived from trypsinized larvae were used. This way we were able to practice this complex technique. Even though with larvae trypsinized cells (diploid) we obtained triploid organisms and this was not our goal, the data permits to evaluate technically the survival of NT. No data from haploid gynogenote cells was evaluated because of the cell death of these cultures. We were able to use androgenote and androgenote control, as well as diploid cells, to carry out the experiments.

Table 4: NT results.

	trypsinized larvae	control androgenote	androgenote
NT	10	11	8
1ˢᵗ cell division	3	3	0
	30 %	27 %	0 %

From the assays performed, in only 6 some cell division was observed, so the effectiveness of the technique is very low. None of the experiments reached the MBT stage. With these results we cannot tell if there is any difference between NT with diploid or haploid cells. But these 6 trials turned out to be the last trials we performed, suggesting that a greater ability performing the NT trials led to more success. For a better analysis of NT more tests and more time to master the technique would be required.

In this last phase it was expected to find a greater efficiency of NT with haploid than diploid cells. This difference would be caused by restoring the diploid condition when transplanting haploid cells, compared with a triploid condition embryo resulting of transplant with diploid cells. Although in zebrafish the condition of triploidy is not lethal and can reach the adult stage, even resulting in fertile specimens, it can penalize in some degree the development of the embryo.

Since triploidy affects the number of copies of a gene (though not its sequence of nucleotides), consequences related abnormal gene dose are likely to occur. These would alter gene product relative concentrations, interfering with normal development and resulting, in some frequency, in lethality (Pierce, 2010). For certain genes, two copies can modify the development and even produce opposite effects, depending of the origin of the copy (Feil, 2009).

On the other hand, the size of the kinetochore and pre-anaphase spindle length do not increase with ploidy level, so a triploidy condition can interfere with geometric relationships between the key components of the machinery used in the segregation of chromosomes (Otto, 2007).

Likewise, since there are three homologous chromosomes in a triploid organism, the cell divisions that the embryo will suffer could be affected in terms of chromosome segregation. Perhaps one of the homologous chromosomes does not align with the other two and segregate randomly. Even more, if the three chromosomes align two must go into a cell and one to the other. Fate determines which daughter cell will receive the extra chromosome. Sometimes the presence of a third chromosome interferes with the normal alignment and the three chromosomes are segregated into the same daughter cell.

Based on all of the above, the triploid zygotes may develop as mosaics (with diploid, triploid haploid, and without chromosomes cells). Some authors suggest that these mosaics could be caused by a cell cycle asynchrony between the transplanted nucleus (somatic) and the recipient oocyte nucleus (Kaftanovskaya *et al*. 2007).

Beyond this, changes in the structure of the genome have immediate effects on the phenotype of the zygote (Otto, 2007). These genomes, moreover, are genetically less stable and more prone/susceptible to mutations. Therefore they suffer, more than the diploid organisms, recurrent deleterious mutations and may even experience gene inactivation (delete function mutations are much more frequent than gain of function mutations).

The triploid genomes quickly redesign (by mechanisms of ploidy correction) and may decrease its genomic size losing chromosomes and even, sometimes, a complete set of chromosomes, thus restoring the diploid condition. This is possibly caused by an automatic degeneration of the pronucleus of non-enucleated oocyte, so that only the genome of the donor cell will take part in the development (Li *et al*. 2003). Studies in medaka describe the acquisition of diploid and fertile individuals by a transfer of a somatic nucleus on an oocyte without enucleation. These can be developed from transplanted somatic nucleus or from the recipient oocyte nucleus (although the mechanisms of diploidization of this nucleus are unknown). Even tetraploid organisms were obtained by the fusion of two nuclei (somatic nucleus and nucleus of the diploidized oocyte) (Bubenshchikova *et al*. 2005).

The ploidy reduction was observed in fused mouse hepatocytes. The fusion of two hepatocytes results in a tetraploid condition, but culminates in two diploid daughter cells (Duncan *et al*. 2009).

There is a limitation in the ability of the oocyte haploid genome to reprogram the diploid genome transplanted (greater than reprogramming a haploid genome). In any case we must not forget that the triploids obtained by this NT method are double somatic triploids, i.e. the extra genome is a somatic genome. Therefore the reprogramming effort required by the oocyte is even greater than for normal triploid.

We should also see differences in survival efficacy of the embryo between the transplant of an androgenote or a gynogenote cell. A higher rate of efficacy would be expected in NT androgenotes cells, in which a zygote with a genome that comes from a female and a genome (somatic) coming from a male is formed.

Optimal development in many species requires the presence of a maternal and a paternal genome. The organized growth and full development is more difficult to achieve if the chromosome complement of a parent is lost, since the maternal and paternal genomes are not equivalent because of gametic imprinting (Feil, 2009).

The ICR (imprinting control regions) in the genome are completely resistant to chromatin remodelling and demethylation waves that follow fertilization. They do not acquire a new pattern of methylation during or after implantation of the embryo in the uterus (in mammals), they acquire their epigenetic pattern in pre-meiotic spermatogenic cells and will maintain it even after their differentiation as somatic cells. Later in the development, they induce the expression of specific alleles of genes close to these areas. The ICR in the paternal and maternal genome are different and because of this their genomes are functionally different. The absence in the genome of paternally imprinted genes domains (in gynogenote cells) or its aberrant expression may lead to failures in development.

We were not able to come upon results to prove the above because of the limitations in the use of this technique, which are: the complexity of NT technology, the high degree of skill needed, lack of haploid cells, and little practice time. Still, an effort was done to perform all stages of the experiment and complete the model of utilization.

CONCLUSIONS

The general conclusions of this study are the following:

The type of irradiated gamete (oocyte or sperm) has an influence in the fertilization and survival rates.

The highest percentage of haploid larvae was obtained when sperm was irradiated (gynogenotes), suggesting that inactivation of genetic material by UV radiation is less effective in the maternal than the paternal genome.

One of the main problems that arises in establishing haploid cell cultures is the appearance of multiple contamination, which is very difficult to control. Particularly by fungi, which will require the addition of a specific antifungal.

The results of the NT technique are affected by the experience of the manipulator. So a training period of a few months is not sufficient for a manipulator with no previous experience to obtain unbiased results.

Therefore the ultimate goal, which was the NT, could not be completely achieved. This was principally due to low efficacy stages: obtaining haploid larvae, cultures. The NT technique has been performed although the degree of training has been low, primarily due to limitation of time.

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