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Stress-preconditioning as a novel tool to improve assisted reproductive procedures

Summary of PhD thesis

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2019

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1. INTRODUCTION AND AIMS

Under natural circumstances, mammalian gametes and embryos derive, develop, and function *in vivo*, in the protected environment of the genital organs of the individual. However, development and routine application of several biotechnological procedures during the last fifty years require culture, fertilization, or cryopreservation of these cells and tissues under *in vitro* conditions. Besides the beneficial effects of these procedures (e.g. gene preservation, increasing the number of offspring, fertility preservation), they go hand in hand with harmful effects like cell injuries or cell death, thus resulting in decreased developmental competence. Several studies proved that a properly applied and well-defined sublethal environmental stress, particularly hydrostatic pressure induces general adaptation of cells and makes them more resistant during subsequent interventions such as cryopreservation. The aims of this series of studies were to assess the long-term effects and to find new applications for hydrostatic pressure stress treatment in the field of assisted reproduction.

In the present study the term “stress” or “stress treatment” is used in the initial phase of the experiments, before the cell specific, optimized protocol was defined. After determining the optimal cell-specific stress treatment parameters, the procedure is named as PTAT, based on the term Pressure Triggered Activation of Tolerance, and can be included as one additional step in the routine laboratory protocols.

A potential new application of PTAT is zebrafish embryo cryopreservation or chilled storage. Zebrafish is widely used as model organism in developmental biology, genetics, physiology, toxicology and environmental genomics (Long et al., 2013), thus the number of genetically modified strains grows rapidly. Cryopreservation of zebrafish embryos is still an unsolved problem despite market demand and massive efforts to preserve genetic variation among numerous existing lines. Zebrafish embryos’ highly impermeable chorion, high chilling sensitivity, and different water- and cryoprotectant permeability of various embryo compartments are the most important obstacles that interfere success of cryopreservation. Chilled storage of embryos might be a step towards developing successful cryopreservation, but no methods to date have worked. By the application of PTAT procedure cells can be prepared for an upcoming stress factor (e.g., the ones associated with cryopreservation such as mechanic and osmotic stresses and the toxic effects of the cryoprotectants). The objective of the zebrafish experiments was to investigate whether PTAT treatment improves the chilling tolerance of these embryos in terms of post-hatch survival. We further hypothesized that PTAT-treated chilled embryos can develop into adult fish and that their reproductive performance would be physiological.

Another possible new application of PTAT is improving the success rate of human oocyte vitrification. Sperm and embryo cryopreservation have become routine procedures in human assisted reproduction, however, embryo cryopreservation generated ethical, moral and legal issues, thus some countries have enacted specific laws that restrict or even forbid embryo cryopreservation. As an alternative and in accordance with the legal prohibition of embryo cryopreservation, oocyte cryopreservation had been introduced into routine practice in these countries. Successful cryopreservation of oocytes is essential not only to maximize the safety and efficacy of ovarian stimulation cycles in an *in vitro* fertilization treatment, but also to enable fertility preservation in patients with cancer. Oocyte has a very special structure (i.e., large size, very sensitive to low temperature, extremely fragile, high water content, low surface to volume ratio, presence of the spindle and other cell organelles, unfavourable plasma membrane permeability to cryoprotectants and water, etc.) that leads to complex difficulties associated with its cryopreservation. To resolve these obstacles, several methods of slow freezing and vitrification have been developed, with good, but still improvable results. In the present study mouse model was used to find the optimal PTAT treatment in order to increase success rate following mouse oocyte vitrification.

In the final phase of the experiments we also aimed to elucidate PTAT's mode of action, with the help of gene expression microarray experiments. Initially we performed these investigations on matured oocytes to study their response immediately after the PTAT. Since the embryonic genome activation occurs at the 2-cell stage in mouse embryos, initiating *de novo* RNA synthesis, treatment effects generated in the oocyte may become apparent at the transcriptional level in subsequent stages of embryo development. Therefore, we also analyzed the global gene expression pattern of four-cell stage embryos developed from PTAT treated oocytes after fertilization with intracytoplasmic sperm injection.

2. MATERIALS AND METHODS

2.1. Oocyte and embryo production

For mouse oocyte and embryo production female mice were superovulated by intraperitoneal injections of PMSG and hCG, with a 48-hours interval. For mouse oocyte production females were kept in their cage without a male mouse after the hCG injection, and on the next morning their oviducts were flushed for oocyte harvesting. For embryo harvesting females were mated with fertile males in monogamous pairs after hCG administration; females with a vaginal plug – as a proof of copulation – were used for embryo harvesting. For the two-cell stage and *in vitro* developed blastocyst production one-cell stage embryos were harvested from the oviducts of copulated females on the day when the vaginal plug was observed. Zygotes were freed from cumulus cells and then were cultured for 24 hours until they reached the two-cell stage and then further on till the expanded blastocyst stage.

In vivo developed blastocyst-stage embryos were derived from compacted morulae or early blastocyst-stage embryos harvested from the uterus of pregnant females at Day 3.5 post-hCG administration. Embryos were cultured for 4 to 24 hours until the expanded blastocyst stage.

For zebrafish embryo production parents were placed into double breeding tanks 15–16 hours prior to spawning, with males and females separated by a transparent removable wall. Immediately before spawning, the wall was removed, and the parents were allowed to spawn. Following spawning, the parents were removed with the inner tank, and the embryos were collected from the outer tank by filtering the water of the tank.

2.2. Embryo culture

Mouse embryos of each developmental stages were cultured in the incubator in 30-µl KSOM+AA (EmbryoMax, Millipore, USA) under mineral oil (Ovoil, Vitrolife, Sweden), at 37°C, with 6% CO_2 and 90% humidity in air. Embryo development was recorded daily under stereomicroscope.

Zebrafish embryos were cultured in system water, at 26°C. From the 5-day stage larvae were fed once a day with a mixture of banana worms and SDS 100 feed dissolved in system water. Larval development was monitored daily under a stereomicroscope; mortalities and possible developmental defects were recorded. Groups of larvae were placed into 3.5-L culture tanks in the recirculating housing system on 15 dpf (days post-fertilization).

2.3. PTAT treatment

Before the PTAT treatment mouse oocytes and embryos were aspirated into 0.25-ml artificial straws (IMV, France) in G-MOPS Plus (Vitrolife, Sweden), while zebrafish embryos were

placed into 2-ml luer lock syringes (B.Braun, Germany) in system water. Straws and syringes were closed without air bubbles, by plastic plugs or luer-lock caps, respectively.

PTAT treatments were performed by a computer controlled hydrostatic pressure device GBOX 2010 (Applied Cell Technology Ltd., Hungary). The pressure chamber of the device was previously filled with distilled water and heated up to the required temperature. After closing the pressure chamber, the machine executes the pressure program according to the set parameters.

2.4. Chilled storage of zebrafish embryos

Chilling was performed according to the modified protocol of Desai et al. (2015). Briefly, embryos were placed into 50-ml screw-cap centrifuge tubes containing 10 ml of chilling medium (system water supplied with 1 M methanol and 0.1 M sucrose as cryoprotectants) and placed on ice for 24 hours. After the 24-hour exposure, embryos were transferred to a fine mesh dip net and washed with system water for approximately 30 sec. The embryos were then placed into 10-cm Petri-dishes, and further rearing was performed as described previously.

2.5. Spawning of adult fish developed from the PTAT-treated and chilled embryos

Animals were spawned separately in spawning tanks with Leopard danio (*Danio rerio var. frankei*) individuals (three Leopard danio to one experimental fish) to keep track of the zebrafish during experiments. Following spawning, fish were removed from the spawning tanks, and embryos were cultured until 10 dpf, as described previously.

2.6. Vitrification and warming of mouse oocytes

The mouse oocytes were vitrified by the Cryotop method, according to the protocol of Kitazato Biopharma Co. (Kuwayama, 2005).

2.7. *In vitro* fertilization of mouse oocytes

To evaluate their developmental competence, mouse oocytes were fertilized by the intracytoplasmic sperm injection (ICSI) method, according to the protocol of Kuretake et al. (1996). After the injection with mouse sperm heads, mouse oocytes were incubated in KSOM+AA for 6–8 hours. Embryos showing two pronuclei and a second polar body were cultured further in KSOM+AA medium.

2.8. Parthenogenetic activation of mouse oocytes

In certain experiments parthenogenetic activation was used as an alternative to verify the functionality of mouse oocytes instead of *in vitro* fertilization. For parthenogenetic activation

oocytes, harvested 17 hours after hCG injection, were placed into droplets of 10 μM Calcimycin dissolved in G-MOPS Plus, then were incubated in 5 mM DMAP solution under mineral oil for 3 hours. After the treatment oocytes were cultured *in vitro* as described previously.

2.9. Determination of blastocyst cell number

Blastocysts' total cell number and inner cell mass (ICM) cell number have been determined with whole-mount immunostaining. For ICM cells, Anti-OCT4 primary antibodies (Polyclonal Rabbit 19081, Santa Cruz Biotechnology) and Alexa Fluor 594 anti-rabbit secondary antibodies (Molecular Probes, USA) were used. Then blastocysts were mounted on the slides and covered with Vectashield (Vector Laboratories, USA) containing DAPI (Sigma-Aldrich, USA).

The assessment for cell number counting was made using digital images on different focal planes. ICM cells were identified by their red (Alexa fluor 594), total cells by their blue (DAPI staining) fluorescence.

2.10. Mouse embryo transfer

The pseudopregnant recipient females used for embryo transfer were obtained by natural mating with vasectomized males. Females with a vaginal plug were anesthetized, then the ovary, oviduct, and a small portion of the upper uterus were carefully pulled out of the abdominal cavity. The oviduct was punctured with a 27-gauge needle, and embryos were gently blown into the uterus through a tiny glass pipette. After removing the pipette from the uterus, the oviduct and ovary were placed back, and the abdominal cavity was closed.

2.11. Gene expression investigations

Total RNA was isolated using the RNeasy Plus Micro Kit (Qiagen, Hilden, Germany). A two-round RNA amplification procedure was utilized to amplify an appropriate quantity of complementary RNA. A CyDye Post-Labelling Reactive Dye Pack (GE Healthcare, Waukesha, WI, USA) was used to generate labelled cRNA target, which was hybridized to an Agilent 4 X 44K whole mouse genome chip (GPL4134; Agilent Technologies).

Isolated RNA was reverse transcribed using the SuperScript VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Following primer optimisation, twelve selected genes for the oocyte investigations, eight selected genes for the 4-cell embryos, and the three previously validated reference genes were used for real-time polymerase chain reaction verification.

3. RESULTS

3.1. Stress tolerance tests

In these experiments our goal was to determine the stress-tolerance limit; the pressure level after the application of which embryos and oocytes could survive without irreversible damage. For this purpose, target objects (mouse oocytes, mouse two-cell stage, *in-vitro*- and *in-vivo*-developed blastocyst stage embryos, and zebrafish embryos 4-, 24-, and 48-hours post-fertilization) were exposed to various magnitudes of hydrostatic pressure for various durations, and their morphology and developmental potential were measured. These experiments defined the treatment parameters that were used in the forthcoming experiments where PTAT treatment was followed by a routine intervention (e.g. vitrification, fertilization, chilled storage).

Results showed that stress tolerance of the embryos depends on the developmental stage of the embryo, and may come together with reversible morphological alterations including the collapse of the blastocoel and reduction of the size of the blastomeres. In each target cell or embryo type the optimal magnitude, duration, and temperature of stress treatment have been determined successfully. This optimal treatment defined in the first phase was used further on for the long-term effects studies, then, for increasing the resistance and improving the survival rate following chilled storage or vitrification, and finally, to assess the biochemical background of the treatment's beneficial effects.

3.2. Evaluation of stress treatment's long-term effects

In this phase of the experiments, stress treatment's effects on long-term viability were evaluated. For this purpose, continued *in vitro* development, implantation, lifetime, and ability to produce healthy offspring was tested in PTAT-treated zebrafish embryos and in PTAT-treated and then transferred mouse blastocysts.

Zebrafish embryos exposed to sublethal stress had normal hatching rates and survival with normal morphology until 30 days post fertilization. Transfer of sublethal stress treated mouse blastocysts resulted in normal offspring with normal reproductive functions. Progeny of these mice had normal health status and lifespan. Similar safety tests were repeated in the latter phases of the experiments, together with additional procedures – e.g. chilled storage, vitrification, intracytoplasmic sperm injection, etc. –, thus the stress treatment's safety has also been evaluated.

3.3. Effect of PTAT on developmental potential of chilled-stored zebrafish embryos

As a continuation of the previously described experiments, we inserted the defined stress treatment – or in other words PTAT (Pressure Triggered Activation of Tolerance) treatment –

into the procedure of zebrafish embryo chilling, in which the embryos were kept on 0°C for 24 hours. Survival rate, developmental competence, and embryo morphology were used as endpoints in the evaluation of PTAT's efficacy. In addition, a further test on long-term effects was also performed, in which the PTAT-treated, and then chilled-stored embryos were reared until maturity, and their reproducing ability was evaluated.

Zebrafish embryos had a significantly higher survival and better developmental rate following chilled storage on 0°C for 24 hours (hatching rate on 6 dpf (days post fertilization) 37.6 ± 3.4% vs. 23.0±3.8%; heartbeat rate on 10 dpf 17.1 ± 3.5% vs. 4.3 ± 1.7% PTAT vs. Control, respectively), moreover the treated group had a higher ratio of normal morphology during continued development (42.5 ± 23.7% vs. 22.1 ± 14.3% in PTAT vs. Control). While all controls from chilled embryos died by 30 days post fertilization, the treated group reached maturity (~90–120 days) and were able to reproduce, resulting in offspring in expected quantity and quality.

3.4. Effect of PTAT on developmental potential of vitrified mouse oocytes

In the next phase of experiments, the predefined PTAT treatment was introduced into the laboratory protocol of mouse oocyte vitrification. Following the treatment, oocytes were vitrified and warmed, fertilized by intracytoplasmic sperm injection, and cultured until blastocyst stage. Post-vitrification and post-fertilization survival rates, developmental competence, and blastocyst cell number were the endpoints of this experiment. Moreover, we also tested the safety of PTAT integrated into the additional laboratory procedure: PTAT-treated, then vitrified, *in vitro* fertilized and cultured embryos were transferred, the offspring was mated, and the next generation was checked for possible malformations, degenerative diseases and lifespan.

Results showed that PTAT increased the post-thawing and post-fertilization survival rates of treated mouse oocytes (80% vs. 76%; and 73% vs. 68% in PTAT vs. Control, respectively), and moreover, had long-term beneficial effects on the embryos developing from these cells. As a result of the treatment, rate of embryos reaching two-cell stage and blastocysts stage were significantly higher (73% vs. 57%; and 60% vs. 50% in PTAT vs. Control, respectively). In addition, blastocysts developing from PTAT-treated oocytes had significantly higher total cell numbers and ICM (inner cell mass) cell numbers (50 vs. 45; and 21 vs. 17, in PTAT vs. Control, respectively). Moreover, transferred two-cell stage embryos had a significantly higher chance for implantation and development to a healthy pup, demonstrating the improved viability of these embryos.

3.5. Investigation of PTAT's effects on gene expression

The aim of this experiment was to analyze the impact of PTAT treatment during mouse early preimplantation development using gene expression microarrays. Firstly, the differences between the gene-expression profiles were examined on untreated and PTAT-treated mouse oocytes. Then, PTAT's effects were also investigated on 4-cell embryos derived from untreated and treated oocytes following fertilization by intracytoplasmic sperm injection, to evaluate the PTAT-induced transcriptional response after embryonic genome activation.

Detailed analysis has shown that the transcriptome of the oocytes was not perturbed directly by the PTAT treatment. An indirect but significant effect of the treatment became apparent after embryonic genome activation at the 4-cell stage, exhibiting a downregulation of ribosome related genes, thus revealing a transcriptional footprint of PTAT-induced genes. These results suggest a potential mechanism for how PTAT preconditions the cells and improves cell survival and function, but subsequent investigations are necessary to elucidate the complete mechanism underlying the effect of PTAT.

4. DISCUSSION

As a general statement from the pioneering studies, PTAT treatment positively affects efficiency of assisted reproductive technologies when applied throughout preimplantation development, from the gamete to the blastocyst stage. The present experiments confirmed these findings and showed that PTAT treatment improved the quality of the treated gametes or embryos, and altered the expression of several genes.

Sublethal stress treatment of oocytes, spermatozoa, embryos or embryonic stem cells were reported to increase cells' general resistance that resulted in increased survival and developmental competence after cryopreservation. During these procedures a cell specific PTAT treatment program was used to treat cells. The given program was based on a multiple step process aimed at finding the sublethal treatment parameters at each of the cell types. In the present stress tolerance studies we determined the stress-tolerance limit of the target cells; the pressure level after the application of which embryos and oocytes could survive without irreversible damage. Supporting previous experiences the results have shown that the species and the stage of development affects the stress tolerance to hydrostatic pressure treatment.

Following the stress tolerance studies, the next step was to prove that the defined treatment does not have any harmful effect on the target cells. Embryo transfer of stress-treated mouse blastocysts resulted in normal offspring, and these individuals were able to reproduce normally. 24-hours old zebrafish embryos developed into healthy fish following stress treatment. These findings support other studies, where healthy piglets were born following inseminations with PTAT-treated fresh or PTAT-treated cryopreserved semen. Healthy piglets were born from PTAT-treated, enucleated somatic cell nuclear transferred oocytes as well. These studies confer that treating gametes and embryos with sublethal pressure stress may result in healthy offspring.

Conservation of biological resources by the cryopreservation of gametes and embryos has successfully been applied in various areas, however, cryopreservation of zebrafish embryos remains unsuccessful to date. Several cryopreservation techniques have been tested. Slow freezing has failed as a method because intracellular ice formation was inevitable, regardless of cryoprotectants or the use of aquaporins inserted into embryo membranes. Several studies tested vitrification of embryos from various fish species including zebrafish, however they resulted in either zero or very limited survival, moreover none reported successful continued development passing the larval stage. As zebrafish embryo cryopreservation trials failed, most of the experiments focuses on developing cold- or chilled storage methods, however this area needs major developments as well. Different temperature and exposure times were tested,

but embryos either became nonviable within 6 hours, or had significantly decreades hatching rates.

The results of the present study indicate that PTAT technology improved the quality of chilled-stored zebrafish embryos. PTAT-treated zebrafish embryos had a significantly higher survival and developmental rate following chilled storage on 0°C for 24 hours, and the PTAT-treated group had a higher ratio of normal morphology during continued development. Previous studies reported very limited chilling survival of embryos from zebrafish or other fish species even though they used either hatching embryos or embryos in stages close to hatching. However, PTAT technology enables for the chilled embryos to develop normally until maturity, and to produce healthy offspring as normal, thus passing on their genetic material successfully. Based on these results, it can be concluded that the PTAT preconditioning technology represents a significant improvement in zebrafish embryo chilling tolerance, thus enabling a long-time survival, and providing the potential for application in zebrafish shipment and trade between laboratories as well as gene preservation. Furthermore, as embryonic development is arrested during chilled storage this technology also offers a tool to synchronize or delay the development for experimental purposes.

Human oocyte cryopreservation, as an alternative of embryo cryopreservation has been introduced into the routine practice in several countries for ethical, moral, and legal issues. Moreover, this procedure is essential to maximize the efficacy of ovarian stimulation cycles, and also to enable fertility preservation for cancer patients. In the present study, a series of experiments have been performed using mouse eggs and procedures that are common in the human *in vitro* fertilization laboratory practice. We have expanded the scope by adding further endpoints to examine the safety and efficacy of the PTAT treatment of oocytes before vitrification.

Post-warming survival rate of treated and control mouse oocytes was similar between the groups although numerical differences were seen consistently in favour of the treated group. Observing the endpoints further, cleavage and blastocyst rates, blastocyst inner cell mass cell number, and, most importantly, birth rates were significantly higher in the embryos developing from the oocytes that were PTAT treated before vitrification. We concluded that PTAT treatment improves the quality of the oocytes and the effect is more and more tangible as the development proceeds towards parturition. Following the transfer of 2-cell-stage embryos developed from PTAT-treated or non-treated, vitrified/warmed, fertilised oocytes, the offspring was housed in order to examine their longevity and reproductive performance. We concluded that lifespan, fitness and reproductive performance of the two groups were similar and comparable to the physiological data. Numbers do not allow to make conclusions about

superiority or inferiority, nevertheless trends towards the advantage of the treated group encourages us to continue experiments that is powered to make conclusions about fitness of the offspring. These results may be utilized in fine-tuning the routine human oocyte vitrification protocols, in order to maximize the safety and efficacy of ovarian stimulation cycles in an *in vitro* fertilization treatment, and also to enable fertility preservation.

The transcription profiling experiments of the PTAT-treated oocytes and four-cell stage embryos that developed from these oocytes showed distinct responses to the applied stress. PTAT treatment did not perturb the transcriptome of oocytes, however, the same treatment did result in a marked effect on transcription at the 4-cell stage, i.e. after the embryonic genome activation. To gain insights into the molecular mechanisms involved, we used functional annotation clustering to identify the genes showing significantly altered expression in 4-cell embryos. A large number of translation related genes were affected by the PTAT treatment, exhibiting massive downregulation. The robust repression suggests that the PTAT stress inhibited ribosome assembly and thus transiently reduced the rate of the protein synthesis during preimplantation development. These results are consistent with the well-known phenomenon in microbes, where high pressure induces ribosomal dissociation. This pressure-induced ribosome disassembly observed in *E. coli* is completely reversible using pressure below 100 MPa; after the pressure is released, protein synthesis is resumed. These results support our previous observations that oocyte developmental competence was not compromised by the precisely adjusted PTAT treatment. Mouse blastocysts developing from PTAT-treated, vitrified, warmed oocytes had a significantly higher cell number compared to those developing from untreated oocytes. These results suggest a potential mechanism for how PTAT preconditions the cells, but subsequent investigations are necessary to elucidate the complete mechanism underlying the PTAT effect.

5. NEW SCIENTIFIC RESULTS

1. We determined the stress-tolerance limit of mouse oocytes, and mouse and zebrafish embryos in different developmental stages.
2. We demonstrated that stress preconditioning had no long-term harmful effects on mouse oocytes, and mouse and zebrafish embryos. The offspring developing from the treated gametes / embryos had normal lifespan, were able to reproduce, and their offspring were similar to that of the Control.
3. We proved that PTAT treatment significantly increased the survival and developmental rate and ratio of normal morphology during continued development of chilled-stored zebrafish embryos. PTAT treatment enabled for the chilled embryos to develop normally until maturity, and to produce healthy offspring as normal.
4. We demonstrated that PTAT increased the post-thawing and post-fertilization survival rates of treated mouse oocytes, and significantly increased the rate of embryos reaching two-cell stage and blastocysts stage. Blastocysts developing from PTAT-treated oocytes had significantly higher total cell numbers and ICM cell numbers. Transferred two-cell stage embryos had a significantly higher chance for implantation and development to a healthy pup. These mice had a normal lifespan and normal reproductive functions.
5. We presented the first comprehensive analysis describing the cellular response of mouse oocytes to PTAT stress. The transcriptome of the oocytes was not perturbed directly by the PTAT treatment. After embryonic genome activation at the 4-cell stage, embryos developing from the PTAT-trated oocytes exhibited a downregulation of ribosome related genes.

6. THE AUTHOR'S PUBLICATIONS

6.1. Publications in peer-reviewed journals related to the thesis

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7. ACKNOWLEDGEMENTS

First of all I would like to express my great appreciation to **Dr. Csaba Pribenszky** for involving me in the development of stress-preconditioning. I am also grateful for him for actuating me on the path of scientific research and proposing me to start to work on this PhD thesis. I also thank him for his valuable and constructive suggestions during the planning and development of this research work, and in writing the dissertation as well. His willingness to give his time, which he takes away from his delightful family, has been very much appreciated.

I would like to offer my special thanks to **Dr. Claudia Stanca** for her persevering assistance in mouse oocyte ICSIs and embryo transfers, to **Dr. István Bock** for his invaluable help in gene expression studies, and to **Dr. András Dinnyés** for his support and for offering me the resources of BioTalentum Ltd. in running the program.

I am particularly grateful for the assistance given by **Dr. Urbányi Béla**, **Dr. Horváth Ákos**, **Dr. Kollár Tímea**, **Dr. Bernáth Gergely** and **Dr. Csenki-Bakos Zsolt**, the staff of Department of Aquaculture, Szent István University, Gödöllő, Hungary, and for **Dr. Cserepes Judit**, **Dr. Faragó Bernadett**, **Dr. Budai Csilla** and **Szabó Katalin**, the staff of Applied Cell Technology Ltd. for giving me the opportunity to work on the zebrafish embryo cryopreservation and chilled storage project.

I would also like to thank **Dr. László Könyves**, Head of Animal Hygiene Department in University of Veterinary Science, Budapest, Hungary, for mildly forcing me to fulfil my PhD dissertation and, besides this, providing the conditions for it to me.

Finally, I wish to thank **my parents** and my partner **Péter** for their support, understanding, and encouragement throughout my study.