University of Veterinary Medicine
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Stress-preconditioning as a novel tool to improve assisted reproductive procedures

PhD thesis

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ABBREVIATIONS

BT	body temperature
cDNA, cRNA	complementary DNA, RNA
COC	cumulus-oocyte complex
CTRL	control
DMAP	6-dimethylaminopurine
DMSO	dimethyl sulfoxide
dpf	day(s) post fertilization
DS	dilution solution (for Cryotop vitrification)
EG	ethylene glycol
ES	equilibration solution (for Cryotop vitrification)
Exp.	Experiment
GAS	general adaptation syndrome
GV	germinal vesicle (-stage oocyte)
hCG	human chorionic gonadotropin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (buffer)
HMC	hand-made cloning
hpf	hour(s) post fertilization
ICM	inner cell mass
ICSI	intracytoplasmic sperm injection
ip	intraperitoneal
IVC	*in vitro* culture, *in vitro* cultured
IVF	*in vitro* fertilization, *in vitro* fertilized
IVM	*in vitro* maturation, *in vitro* maturated
IU	international unit
KSOM+AA	potassium simplex optimized medium, supplemented with amino acids
MII	metaphase II (-stage oocyte)
MOPS	3-(N-morpholino)propanesulfonic acid (buffer)
MPVA	mannitol-PVA (polyvinyl alcohol) fusion medium
PA	parthenogenetic activation
PMSG	pregnant mare serum gonadotropin
PTAT	pressure triggered activation of tolerance
RIN	RNA integrity number
RT	room temperature
RT-qPCR	quantitative real-time PCR (polymerase chain reaction)

SCNT	enucleation and somatic cell nuclear transfer
SEM	standard error of the mean
TALP	Tyrode's Albumin Lactate Pyruvate
TCMH	Tyrode's complete medium with heparin
TS	thawing solution (for Cryotop vitrification)
VS	vitrification solution (for Cryotop vitrification)
WS	washing solution (for Cryotop vitrification)

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1. SUMMARY

Sublethal stress treatment of cells was reported to temporarily increase their stress tolerance. High hydrostatic pressure stress treatment improved success rates in several experiments with cryopreservation of stem cells, gametes and embryos of different species or somatic cell nuclear transfer. The procedure (named as PTAT, based on the term Pressure Triggered Activation of Tolerance) can be included into the routine laboratory protocols after having determined the optimal cell-specific stress treatment parameters.

In the present work stress tolerance of embryos in certain developmental stages (two-cell stage mouse embryos, *in vitro* and *in vivo* produced mouse blastocysts, zebrafish embryos 4, 24, and 48 hours post-fertilization) and oocytes has been determined, in order to find the optimal cell-specific PTAT treatment. Then, the technology's long-term effects were evaluated on PTAT-treated zebrafish embryos which were cultured for 30 days post-fertilization, and proved to have normal hatching rates and survival with normal morphology. Similarly, 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 too. Further investigations were also performed in the latter phases of the experiments. In these, PTAT's and certain additional procedures' (e.g. chilled storage, vitrification, intracytoplasmic sperm injection) long-term effects were evaluated simultaneously.

PTAT-treated 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). In addition, the PTAT-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 Control embryos died by 30 days post fertilization, PTAT-treated group reached maturity and were able to reproduce, resulting in offspring in normal quantity and quality.

PTAT had comparable results to control in the post-thawing and post-fertilization survival rates of mouse oocytes (80% vs. 76%; and 73% vs. 68% in PTAT vs. Control, respectively), however showed 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 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). Besides this, transferred two-cell stage embryos had a significantly higher chance for

implantation and development to a healthy pup (27% vs. 12% in PTAT vs. Control), proving the improved quality of these embryos.

Gene expression analysis of PTAT-treated and then fertilized oocytes has shown that a significant effect of the treatment became apparent after embryonic genome activation at the four-cell-stage, exhibiting a downregulation of ribosome related genes.

In conclusion, PTAT technology improved the quality of chilled-stored zebrafish embryos and vitrified mouse oocytes. PTAT-treated chilled-stored zebrafish embryos developed normally until maturity, passed on their genetic material successfully, thus 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 provides a solution to synchronize or delay the development for experimental purposes. PTAT-treated vitrified mouse oocytes had a significantly improved developmental competence, which 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 gene expression study 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.

2. 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), these methods 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 (reviewed by Pribenszky et al., 2010b, and Pribenszky and Vajta, 2011). The aims of this series of studies wereto 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 (Hagedorn et al., 1997a and 1997b, Hagedorn and Kleinhans, 2011) 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 (Rienzi et al., 2017). 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 (Konc et al., 2014). 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.

As target objects, oocytes and embryos of two species, in certain developmental stages (two-cell stage mouse embryos, *in vitro* and *in vivo* produced mouse blastocysts, zebrafish embryos 4, 24, and 48 hours post-fertilization, and mouse oocytes) were utilized, to get a wide and comprehensive insight of environmental stress's effects. We also planned to reveal the differences in stress tolerance of the embryos in different developmental stages, to find the optimal time of PTAT treatment, when it will have the best improving effect.

The initial experiments of the study aimed to determine the hydrostatic pressure stress tolerance of mouse oocytes, and mouse and zebrafish embryos in different developmental stages, in order to find the level which may enhance the survival rates following cryopreservation or chilled storage, respectively. Then, long-term effects of the stress treatment were also evaluated on mouse and zebrafish embryos, by investigating the developmental potential and fertilizing ability of the individuals developing from the stress-treated embryos.

In the next phase of the experiment we inserted the defined 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 was also performed, in which the PTAT-treated,

and then chilled-stored embryos were reared until maturity, and their reproducing ability was evaluated.

Similarly, 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 long-term effects 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.

In the final experiment, a comprehensive gene expression analysis was performed on PTAT-treated oocytes following the treatment at oocyte stage, and on four-cell embryos developing from these oocytes, to elucidate PTAT's mode of action.

Experimental design of the study and interconnection of the experiments is shown on **Figure 1.**

General scheme of the experiments – how are the procedures and results interconnected?

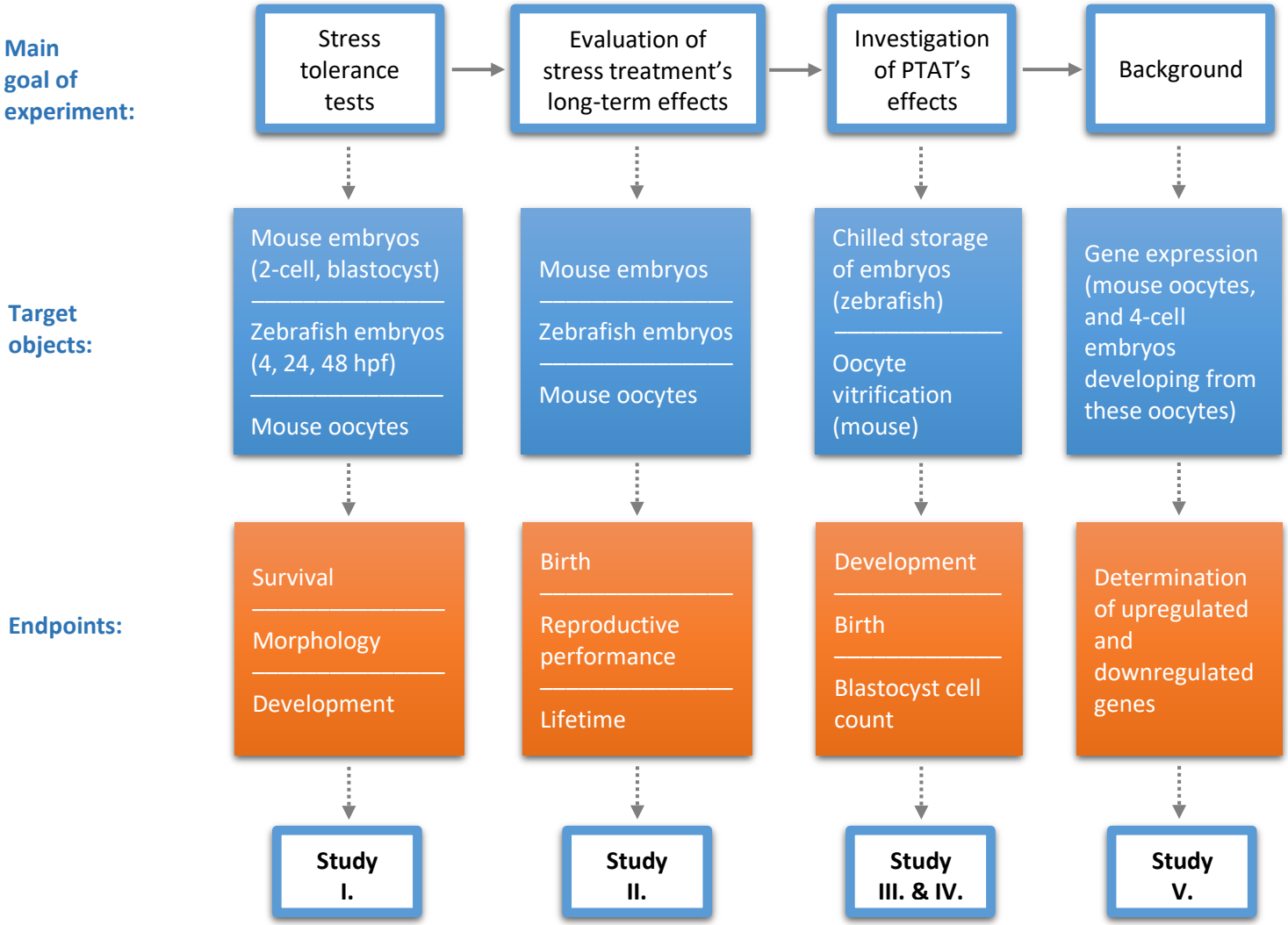

Figure 1. Overview of the experiments

3. REVIEW OF THE RELATED LITERATURE

3.1. Oocyte and embryo cryopreservation

Parallel with the development of *in vitro* laboratory procedures, the demand for cryopreservation of gametes and embryos not only emerged, but gradually became attainable. First efforts to cryopreserve cells date back to the early 1900s, however, these attempts were not successful before the identification of dehydrating properties of sugars (Luyet and Hodapp, 1938), and the cryoprotective effects of glycerol (Polge et al., 1949). After successful cryopreservation of spermatozoa of several species including the human (Bunge et al., 1954), the use of glycerol has also been explored in cryopreservation of mouse embryos (Whittingham et al., 1972). Although initial attempts with mature oocytes had failed, with the application of dimethylsulphoxide (DMSO) Whittingham (1977) achieved the first female gamete cryopreservation. Following the demonstration of good cryosurvival, comparable fertilization rates and the birth of live offspring reported by Whittingham, the procedure was subsequently used on oocytes from rat (Kasai et al., 1979, Parkening and Chang, 1977), hamster (Critser et al., 1986, Parkening and Chang, 1977), rabbit (Diedrich et al., 1986, Siebzehnruebl et al., 1989) and primate (DeMayo et al., 1985) (Gook, 2011). Despite these successes, differences in the ability to store male and female gametes limit the possibilities in animal breeding and preservation. In the human, this creates a distinct difference in reproductive choice between men and women, providing extreme flexibility for males and severe, in some situations tragic, restrictions for females (Vajta and Nagy, 2006b). However the widespread of vitrification fitted to the needs of the oocytes has revolutionized oocyte freezing providing fertility results equivalent to fresh eggs (Kuwayama, 2007, Argyle et al., 2016).

3.1.1. Cell damage due to cryopreservation

Vajta and Nagy (2006) distinguish three types of damage according to the different temperature ranges the cells pass through during cooling. At relatively high temperatures between +15 and −5°C, the chilling injury is the major factor, damaging predominantly the cytoplasmic lipid droplets and microtubules including the meiotic spindle (Aman and Parks, 1994; Leibo et al., 1996; Martino et al., 1996; Zenzes et al., 2001). While the latter damage may be reversible, the former is always irreversible and contributes to much of the death of cryopreserved lipid-rich oocytes and embryos of some species. Between −5 and −80°C, extracellular or, predominantly, intracellular ice crystal formation is the main source of injury, while between −50 and −150°C fracture damage to the zona pellucida or the cytoplasm (Rall and Meyer, 1989) are postulated to occur. Storage below −150°C (typically in liquid nitrogen, at −196°C) is probably the least dangerous phase of the cryopreservation procedure, however,

accidental warming is probably the most frequent form of injury. At warming, the same types of injuries may occur as at cooling, obviously in inverse order (Vajta and Nagy, 2006).

Cryopreservation strategies are based on two factors: cryoprotectants and cooling−warming rates. The common feature of cryoprotectants is the ability to decrease cryoinjuries. A wide range of materials fit this definition, including simple, defined, low molecular weight solvents such as ethanol; or complex, partially undefined biological compounds like sera or egg yolk (Vajta and Nagy, 2006). Exposure to cryoprotectants supports the dehydration of the cell and reduces intracellular ice formation. Among others, Konc et al. (2014) divides cryoprotectants into two groups: intracellular/membrane-permeating (i.e., propylene glycol, DMSO, glycerol, and ethylene glycol) and extracellular/membrane-nonpermeating compounds (i.e., sucrose, trehalose, glucose, amid, ficoll, proteins, and lipoproteins). The permeable cryoprotectants displace water via an osmotic gradient and partly occupy the place of the intracellular water, while the extracellular cryoprotectants increase the extracellular osmolarity generating an osmotic gradient across the cell membrane supporting the dehydration of the cell before cryopreservation. At the same time, they prevent the rapid entry of water into the cell after thawing during rehydration/dilution out of the permeating cryoprotectants (Fuller et al., 2004; Mazur, 2004; Leibo and Songsasen, 2002; Fahy and Rall, 2007). However, both permeable and nonpermeable cryoprotectants may also have additional protective mechanisms, for example to stabilize intracellular structures and the cell membrane. Unfortunately, most cryoprotectants have some negative effects, including toxicity and osmotic injuries. Toxicity is usually proportional to the concentration of the substance and to the time of exposure (at physiological temperatures) (Vajta and Nagy, 2006).

Cooling rates may vary according to the applied method, from moderate or stepwise cooling between the physiological temperature to −4°C, highly controlled cooling rates to −40 or −80°C, followed by plunging to liquid nitrogen, to either rapid (around 200°C/min) or ultrarapid (up to 20,000– 100,000°C/min) rates throughout the whole temperature range (Rall, 2001; Kasai and Mukaida, 2004). Warming may be also performed stepwise, with highly controlled or just slightly delayed increase of the temperature, or (more typically) rapidly, including the commonly achievable highest rates of temperature change described above. Cryoprotectant composition, addition, concentration and removal, as well as warming rates, are more or less determined by the selected cooling rates (Vajta and Nagy, 2006).

3.1.2. Properties of slow freezing and vitrification

As reviewed by Rienzi (2017), slow-freezing allows for cryopreservation to occur at a sufficiently slow rate to permit adequate cellular dehydration while minimizing intracellular ice formation. The first successful protocol applied in 1972 for mammalian embryo

cryopreservation required a cooling rate of ~1°C/min to −70°C (Whittingham et al., 1972). Embryo cooling performed this way is referred to as equilibrium freezing (Mazur, 1990). Subsequently, slow-rate cooling was only applied to around −30°C (Willadsen, 1977). With this approach, intracellular water content was converted into small intracellular ice crystals or into a glass. To avoid extensive crystallization, a very rapid warming was required.

Vajta and Nagy (2006) interpret slow freezing as an attempt to create a delicate balance between various damaging factors including ice crystal formation, fracture, toxic and osmotic damage. Toxic and osmotic damage caused by the relatively low concentration of cryoprotectant solutions may not be too serious in slow freezing. However, this concentration is insufficient to avoid ice crystal formation; therefore, an additional manipulation is required to minimize the damage. It is the slow cooling and seeding that result in controlled growth of ice in the extracellular solution; consequently, a considerable increase of the concentration of ions, macromolecules and other components, including cryoprotectants, occurs in the remaining fluid. The slow rate of the procedure allows solution exchange between the extracellular and intracellular fluids without serious osmotic effects and deformation of the cells (this fact is reflected in the other name of the procedure: equilibrium freezing; Mazur, 1990). Although the concentration of these components, especially of cryoprotectants, seems to be dangerously high at the final phases, it happens at low temperatures, where the real toxic effect is minimal.

In contrast to slow-freezing, vitrification is a cryopreservation method that allows solidification of the cell(s) and the extracellular milieu into a glass-like state without the formation of ice. The most widely used vitrification method for mammalian embryos requires the use of high initial concentrations of cryoprotectants, low volumes and ultra-rapid cooling-warming rates. This approach was first introduced in human embryology for cleavage-stage embryos (Mukaida et al., 1998) and then for oocytes (Kuleshova et al., 1999) and pronuclear-stage embryos (Jelinkova et al., 2002; Selman and El-Danasouri, 2002). In the last 15 years several vitrification protocols have been described, which differ from one another in the type of cryoprotectants, equilibration and dilution parameters, the carrier tools and the cooling, storage and warming methods (Rienzi et al., 2017).

Vajta and Nagy (2006) regards vitrification as a radical approach, as one of the main sources of injuries, ice crystal formation, is entirely eliminated. However, a negative consequence of this strategy is the increased probability of nearly all forms of injury except for those caused by ice crystal formation. To achieve vitrification of solutions, a substantial increase of both the cooling rates and the concentration of cryoprotectants is required. The higher the cooling rate, the lower the required cryoprotectant concentration is, and vice versa. With the extreme increase of cooling rate (to approximately 10^7 °C/s), vitrification could also be achieved in pure water, but the usual limits in embryology are far below this rate (Rall, 1987). The balance

required in vitrification is between (i) establishment of a safe system for maximal and reliable cooling (and warming) rates while avoiding consequent damage including fracture of the zona pellucida or the cells, and (ii) elimination or minimization of the toxic and osmotic effects of high cryoprotectant concentrations needed to obtain and maintain the glasslike solidification. Cell shrinkage caused by non-permeable cryoprotectants and the incomplete penetration of permeable components may cause a relative increase of intracellular concentration of macromolecules that is enough to hamper intracellular ice formation. Accordingly, vitrification belongs to the group of non-equilibrium cryopreservation methods (Vajta and Nagy, 2006).

3.1.3. Properties of oocyte and embryo cryopreservation

Generally, the earlier the development stage (starting from the germinal vesicle stage), the more sensitive oocytes and embryos are. In the case of the oocytes, the low surface/volume ratio may negatively influence the rate of penetration of the cryoprotectant (Vajta and Nagy, 2006). However, although there is only a minimal difference between the size and shape, the immature oocytes are usually more sensitive to cryopreservation than mature (metaphase II /MII/) oocytes (Leibo et al., 1996; Men et al., 2002; Ghetler et al., 2005). As described by Konc et al. (2014), the MII 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, unfavourably low plasma membrane permeability to cryoprotectants and water, etc.) that leads to complex difficulties associated with its cryopreservation. The spindle is crucial for the events following fertilization in the completion of meiosis, second polar body formation, migration of the pronuclei, and formation of the first mitotic spindle. The damage (depolymerization) and/or absence of the spindle due to cryoinjury compromise the ability of the oocyte to fertilize and undergo normal preimplantation development. In addition, hardening of the zona pellucida − which is a consequence of cryopreservation − can adversely affect the normal fertilization process.

The increased chilling sensitivity of membranes also needs to be taken into consideration: the lipid phase transition at room temperature storage in human germinal vesicle and MII stage oocytes is 10 times higher than that of human pronuclear embryos (Ghetler et al., 2005). In cattle and in pigs, the higher the cell number of the developing zona intact embryo is, the higher the survival rates, as expected (Vajta et al., 1997; Berthelot et al., 2001). In human, the survival rates after slow freezing are not significantly different between zygotes, cleavage stage embryos and blastocysts (between 75 and 80% for each; Veeck, 2003; Pool and Leibo, 2004).

3.1.4. Properties of fish gamete and embryo cryopreservation

Conservation of biological resources by the cryopreservation of gametes and embryos has successfully been applied in various areas, including assisted human reproduction (Magli et al, 2008; Lambertini et al., 2016, Solomon and Schattman, 2015), livestock breeding (Mara et al., 2013) and preservation of various species (Pampai et al., 2016, Kikuchi et al, 2016). However, cryopreservation of zebrafish embryos remains unsuccessful to date. Many obstacles prevent successful zebrafish embryo cryopreservation: highly impermeable chorion, high chilling sensitivity, and different water- and cryoprotectant permeability of various embryo compartments (Hagedorn et al., 1997a, 1997b, and Hagedorn and Kleinhans, 2011). 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 (Hagedorn et al., 2004). 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 (Robles et al., 2004 and 2005, Cabrita et al., 2003, Harvey et al., 1983).

Most studies focus on the high chilling sensitivity of fish embryos as one of the main obstacles for a successful cryopreservation protocol. Various methods were used to reduce fish embryo chilling injuries including embryonic dechorionation to facilitate cryoprotectant penetration into embryos (Harvey et al., 1983), selection of advanced embryonic stages that are more likely to survive (Hagedorn et al, 1997a, Valdez et al., 2005, Lahnsteiner, 2009), using of cryoprotectants to increase chilling tolerance (Ahammad et al, 1998, 2002, 2003a and 2003b, Desai et al., 2015a,b), or partial removal of the yolk from advanced-stage embryos (Liu et al, 1999), but none were successful. The major factors affecting survival of chilled embryos include duration and temperature of exposure (Lahnsteiner et al., 2009).

Over recent decades, zebrafish have gained prominence as an important model organism across disciplines such as developmental biology, genetics, physiology, toxicology and environmental genomics (Long et al., 2013). Additionally, comparative genomics between zebrafish and humans has revealed a considerable amount of genetic homology. The high degree of similarity with the human genome has propelled zebrafish as an important model organism for human disease (Barbazuk et al., 2000). Consequently, the number of genetically modified zebrafish lines is rapidly growing. However, the preservation of the numerous genetic variants is still the major problem.

As, 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 stress 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.

3.2. Stress and PTAT – what doesn't kill you makes you stronger

Under natural circumstances, mammalian gametes and embryos develop and function *in vivo*, in safe and optimal conditions, without leaving the organism during their entire lifespan. However, parallel with the development of several scientific fields, culturing and manipulating cells and tissues *in vitro* occurred as a new demand.

Sidney Ringer was the first physiologist who made efforts towards culturing organs *in vitro* in the 1880's, observing the effects of adding albumin, potassium, and calcium into the sodium chloride solution in which he cultured the isolated frog heart. (Ringer and Sainsbury, 1882). Almost a decade later Walter Heape retrieved embryos from the uterus of a donor rabbit, which have been successfully transferred to the recipient's uterus (Heape, 1891). In 1934 *in vitro* fertilization has been executed by Gregory Pincus (Pincus, 1936), and, as a result of the improvements of several research teams (Hammond, 1949, Whitten, 1956, and Adams, 1956) the first basic embryo culture media have been developed and adjusted towards the needs of the embryos.

From this time, until recent days, these techniques have been improved and fine-tuned, from allowing embryo culture until blastocyst stage or gamete and embryo cryopreservation, till complicated and sophisticated manipulations such as intracytoplasmic sperm injection (ICSI), blastomere biopsy, parthenogenetic activation (PA), enucleation and somatic cell nuclear transfer (SCNT), with high success rate.

During these improvements, the artificial environment provided by the developed culture media and incubators and laboratory techniques always tried to simulate the *in vivo* conditions, and to eliminate the possibly harmful factors during the *in vitro* phase of the processes. Therefore, temperature, pH, osmotic conditions, composition of the culture media were aiming to satisfy passively the needs of the gametes and embryos. Contrarily, a recently emerged concept utilizes a controlled environmental impact as a stress pretreatment for cells and tissues aims to improve neither the *in vitro* conditions nor the procedures, but actively, the cell itself (Pribenszky et al., 2012).

3.2.1. Stress and its utilization as stress preconditioning

Sublethal stress in details has been first investigated by Cannon, in the beginning of the 20th century. He described the fight-or-flight response (also called the acute stress response), which is a physiological reaction that occurs in response to a perceived harmful event, attack, or threat to survival (Cannon, 1915). This phenomenon can be observed in different levels of life, from bacteria to multicellular organisms including humans. This response was later recognised as the first stage of a general adaptation syndrome (GAS) that regulates stress responses among vertebrates and other organisms (Selye, 1936).

Selye showed that if the organism is severely damaged by acute non-specific harmful agents such as exposure to cold, surgical injury, production of spinal shock, excessive muscular exercise, or intoxications with sublethal doses of diverse drugs, a typical syndrome appears, the symptoms of which are independent of the nature of the damaging agent. A fully-developed GAS consists of three stages: the alarm reaction, the stage of resistance and the stage of exhaustion (**Figure 2**).

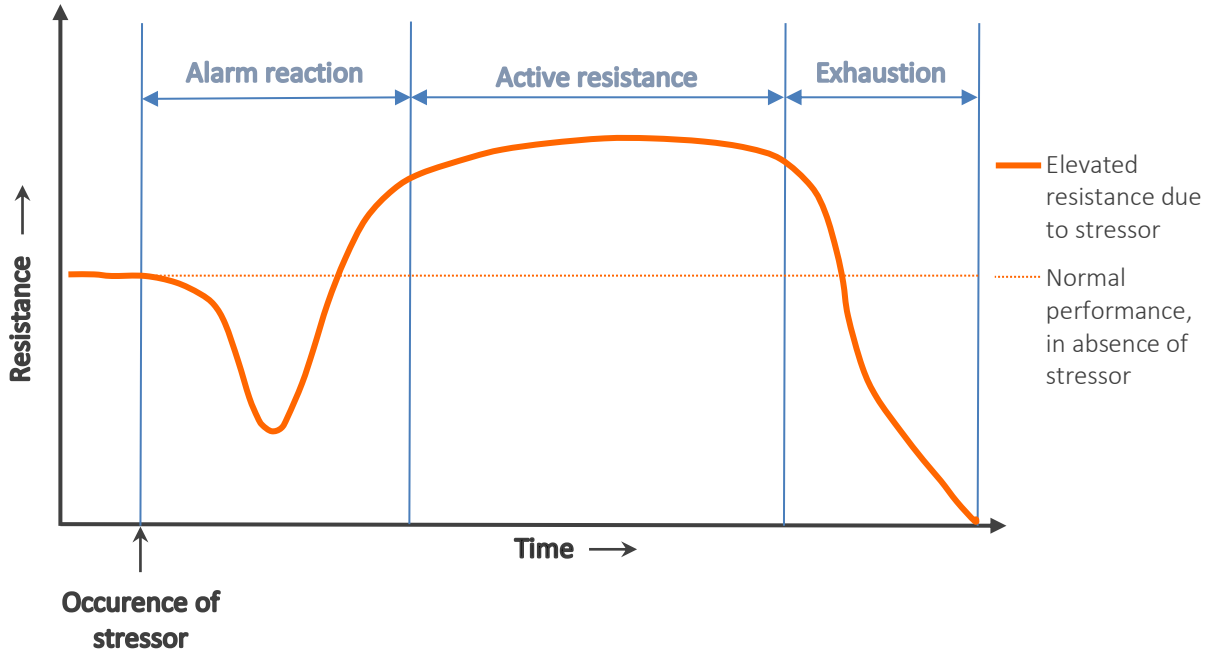

Figure 2. The three stages of general adaptation syndrome: alarm reaction, active resistance, exhaustion, and the effect of a stressor on the performance of the organism.

Kültz (2003) studied the effects of stress on cellular level. Similarly to the response noticeable in an organism, the cellular stress response is a reaction to any form of macromolecular damage that exceeds a set threshold, independent of the underlying cause. It is aimed at temporarily increasing tolerance limits towards macromolecular damage, and this mechanism affords time for a separate set of stressor-specific adaptations, designed to re-establish cellular homeostasis, to take action. (Kültz 2003). Where stress enhances the function it may be

considered as a positive impact, also called eustress (**Figure 2**) (Selye, 1975). However, if the stress level is over the limit of tolerance, programmed cell death (apoptosis) or necrosis occur (Hansen 2007).

The idea of applying a controlled sublethal stress treatment to gametes and embryos purposely, in order to utilize the beneficial effects of this impact and thus to improve their viability, comes, interestingly, from the unexpected lessons of former food microbiology studies (Pribenszky et al., 2010b).

Sublethal stress was originally used in food industry to apply a sequence of mild treatments to foodstuff to reduce microbial load, but at the same time preserve food quality. Hite (1899) reported reduced germ load and longer storage life for milk and fruits if they were pretreated with 400-600 MPa hydrostatic pressure, while the quality of these foodstuff was less damaged compared to other conservation methods. As large-scale application of such high pressure was demanding for the food industry, sequential combination of relatively mild treatments (hurdle technology), including hydrostatic pressure, cooling, and/or heating, has become the widely applied approach. However, in sharp contrast with the expected effect, Wemekamp-Kamphuis et al. (2002) reported that the proliferation of *Listeria monocytogenes* was not decreased but significantly increased as a consequence of sequential treatment with cold shock and hydrostatic pressure. It appears that biological effects of the first sublethal treatment preconditioned the bacteria, protecting them from the detrimental effects of the second sublethal treatment.

This observation eventually motivated research to apply sublethal stress to gametes, embryos, and stem cells, to improve success rates of *in vitro* procedures such as cryopreservation, *in vitro* maturation, *in vitro* culture, somatic cell nuclear transfer, extended *in vitro* storage, or even artificial insemination (Pribenszky et al., 2012).

3.2.2. Environmental stressors – Why hydrostatic pressure?

Everything that surrounds cells could serve as a stressor. Environmental changes such as heat or cold, modifications in the pH, hyper- or hypo-osmosis, presence of oxidative agents, irradiation, light or inappropriate nutrition such as lack of energy, all serve as stress factors and initiate cellular stress reactions. On the cellular level these processes are addressed as heat or cold shock, oxidative stress, nutritional stress, osmotic shock and so on. One of the common features of these impacts is that their effect to the cells is gradual and time-dependent, attacking first the cell membrane, then progressing gradually towards the center of the cell. If the intensity of the stress changes, its intracellular consequences are again gradual and time-dependent (Pribenszky and Vajta, 2011 review).

Unlike these stressors, hydrostatic pressure treatment has unique and outstanding features: (i) acts instantly and uniformly at every point of the cell, (ii) features zero penetration problems or gradient effects, (iii) can be applied with the highest precision, consistency, reliability and safety, (iv) has no or minimal cell-to-cell variation and (v) functions with an extremely high safety margin and wide therapeutic range for the cells (Pribenszky et al., 2012).

Gametes and embryos have surprisingly wide tolerance against hydrostatic pressure. The atmospheric pressure on the sea-level is 1 bar or 0.1 MPa, so this is the pressure value that we, humans and significant proportion of animals, plants, and bacteria are adapted to during the past millions of years on Earth. 10 MPa pressure is equivalent to the hydrostatic pressure measured under 1000m water column. At that depth, there is complete darkness and no mammalian species survive that deep. Hydrostatic pressure in the Marianna Trench is approx. 100 MPa; in this magnitude of pressure none but some barophil organisms can survive. In view of these data, the fact that oocytes, spermatozoa, embryos and stem cells have a hydrostatic pressure tolerance between 30-60 MPa (see details in **Table 1**; details discussed in Chapter 3.3., Former results with PTAT), is quite remarkable (Pribenszky et al., 2010b).

Table 1. Hydrostatic pressure tolerance of different biological materials.

Biological material treated	Optimal pressure range	Optimal treatment duration	Sublethal range
Murine and bovine embryos	40 – 60 MPa	30 – 60 min	≤ 80 MPA
Porcine, murine, or human oocytes	20 MPa	60 – 90 min	≤ 60 MPA
Bull, boar, horse, or rabbit semen	10 – 30 MPa	90 – 120 min	≤ 60 MPA
Embryoid bodies	60 MPa	30 – 60 min	≤ 80 MPA

Our research team started to investigate the effect of defined stress treatment by hydrostatic pressure to cell`s viability fifteen years ago. In order to discover the optimal treatment parameters for a new cell type we have developed a common protocol. In the course of the experiments we determine and describe the stress tolerance of the particular sample, and according to the characteristics of it we establish the cell-specific treatment protocol. This protocol is named as PTAT, based on the term „Pressure Triggered Activation of Tolerance”.

The following paragraphs summarize PTAT effects according to cell types in various assisted reproductive technologies procedures, based on the reviews of Pribenszky et al. (2010b) and Pribenszky and Vajta (2011), with emphasis on oocytes, target cells of the present study.

3.3. Former results with PTAT

The preconditioning protocol is named PTAT, based on the term „Pressure Triggered Activation of Tolerance".

3.3.1. Preimplantation-stage embryos

Experiments were conducted with murine, bovine and ovine embryos by different research groups. The common outcome of the experiments was the significantly increased survival rate and increased speed of the resumption of normal *in vitro* development after cryopreservation and thawing or warming.

The optimal treatment parameters of expanded blastocysts, regardless of the species, were in the range of 40–60 MPa for 30–70 min, with stress-tolerance levels up to 80–90 MPa. The other common feature was the treatment-related, reversible collapse of the blastocoel.

The treatment of *in vivo*-derived mouse blastocysts at 60 MPa for 30 min significantly improved their re-expansion and hatching rates (94% vs. 46%, and 95% vs. 0% in PTAT vs. Control, respectively) after traditional freezing (Pribenszky et al., 2005a).

In vitro-produced bovine blastocysts could tolerate 80 MPa pressure treatment for 45 minutes, and, similarly to the murine embryos their post-freezing survival rate was better (81% vs. 41% for PTAT vs. Control embryos, respectively; Pribenszky et al., 2005b). In another study the treatment of *in vitro*-produced bovine blastocysts at 60 MPa for 1 h before vitrification (open pulled straw (OPS) method as described by Vajta et al., 1998) resulted in higher re-expansion (89% vs. 69% for PTAT vs. Control embryos 24 hours post-freezing), and hatching (76% vs. 63% for PTAT vs. Control embryos 72 hours post-freezing) rates. The strongest effect was observed when vitrification was preceded by 1 h equilibration after the PTAT treatment (Siqueira et al., 2011).

The electron-microscopic ultrastructure of the treated and non-treated bovine embryos was also investigated. Embryos submitted to 60 MPa for 1 h had more microvilli and increased hooded mitochondria compared with control embryos. In the vitrified groups treated embryos had a larger amount of nucleus per area compared with the control group (Siquiera, E.S., Silva, R.C., Pribenszky C., Molnar M., Harnos A., Lucci, C.M. and Rump, R., unpubl. data).

Popovic et al. (2013) investigated the effects of PTAT on *in vitro* produced bovine embryos. On Day 7 of *in vitro* culture, blastocysts-stage embryos were graded according to their

morphological quality (Grade 1 (excellent): appropriate for cryopreservation and transfer; Grade 2 (good): appropriate for fresh transfer but inappropriate for cryopreservation; Grade 3 (poor): not appropriate both for transfer and freezing). Following a 60MPa treatment for 60 minutes, embryos were vitrified and warmed. In Grade 2 embryos a significantly higher proportion (68% vs. 36%) of PTAT treated blastocysts remained of transferable quality after cryopreservation, embryos contained significantly higher percentage of live cells (73% vs. 55% in PTAT vs. control) after vitrification and warming, and, importantly, the *in vivo* developmental potential of these vitrified blastocysts was very similar to fresh controls, up to the Day 65 of gestation at least. (Popovic et al., 2013).

Trigal et al. (2013) also studied *in vitro* produced bovine embryos following a 7-8 days long *in vitro* cultivation. Prior to vitrification the previously described PTAT treatment was used. There was no significant difference in the post-warming survival rate of the embryos, while the PTAT-treated embryos had significantly higher ICM (inner cell mass) cell numbers (50 vs. 39 cells in PTAT vs. Control embryos, Trigal et al., 2013).

Jiang et al. (2016) treated bovine blastocysts with three different PTAT treatments (40, 60 and 80 MPa), in combination with three recovery periods (0, 1 h, 2 h post-PTAT). Re-expansion rates after vitrification and warming were significantly higher following 40 and 60 MPa treatments compared to control, however, 80 MPa treatment decreased the post-vitrification survival rate.

Bogliolo et al. (2010) tested the sublethal hydrostatic pressure stress on the quality of fresh and vitrified ovine blastocysts, and found that 40 MPa, 70 min treatment at 38 °C improved the quality of the embryos by increasing their cell number (161 cells vs. 124 cells in PTAT vs. Control embryos) and reducing the proportion of picnosis (picnotic index: 1.28 vs. 3.84 in PTAT vs. Control embryos) (Bogliolo et al., 2011). Further studies by Ledda et al. (2010) revealed that the speed of re-expansion of the vitrified ovine blastocysts was significantly higher in the PTAT-treated groups (re-expansion rate 3 h after warming was 68.7% vs. 44.3% for the PTAT vs. Control embryos) and cell number of the treated blastocysts was also higher compared with untreated controls (159 vs. 129 in PTAT vs. control).

3.3.2. Semen

Experiments were conducted on bull and boar spermatozoa, and in both species PTAT treatment resulted in increased cell survival after cryopreservation or prolonged *in vitro* storage.

Pribenszky et al. (2009) investigated the effects of PTAT treatment on porcine spermatozoa *in vivo* in routine inseminations and *in vitro* during cooled storage. Semen samples were split to

treated (fixed PTAT protocol) or untreated control groups. The treated and control samples were either used for artificial insemination 4-24 hours later, or stored at 4°C for 12 days with daily motility control using CASA apparatus. Results showed that the reduction of both total (TM) and progressive (PM) motility of the treated samples was significantly lower compared with control samples (on Day 5, the ratio of live (TM) and progressively motile (PM) cells (PTAT vs. Control) was as follows: TM: 64.6 vs. 55.4%, PM: 46.4 vs. 36.6%; on Day 11, the ratio of TM and PM cells was as follows: TM: 53 vs. 43%; PM: 31.4 vs. 27%). In the *in vivo* experiment mean pregnancy rates and litter weights were not different between the two groups (73 v. 74%; 16.34 v. 16.37 kg; PTAT vs. Control, respectively). PTAT treatment significantly increased litter size (12.4 vs. 11.4 in PTAT vs. Control), however, this increase was observable in the gilts only. (Pribenszky et al., 2009).

The temperature of the PTAT treatment has to be adjusted to the state-of-the-art temperature management of the cryopreservation processes (e.g. if a semen sample was cooled down to room temperature, then PTAT treatment should not be done at 37°C) (Pribenszky et al., 2006). Huang et al. (2009) used 20, 30, and 40 MPa PTAT treatment for 90 minutes prior to cryopreservation of porcine semen and demonstrated significantly increased post-thaw motility ($P=0.02$) in each treatment groups (52%, 56% and 56% vs. 46%; following 20, 30, and 40 MPa treatments vs. Control, respectively) (Huang et al., 2009a). The insemination with frozen–thawed treated semen did not affect pregnancy or farrowing rates, but a substantial increase in litter size was achieved (live born and total litter size in the sows inseminated with PTAT treated semen: 9.4 ± 1.0 and 9.7 ±1.0, Control: 4.4 ± 1.1 and 4.6 ± 1.1, respectively; Kuo et al., 2007).

Horváth et al. (2016) investigated the effects of 40 MPa PTAT treatment for 80 minutes prior to cryopreservation on post-thawing motility of boar semen, and in addition, fertility of the PTAT-treated semen was also studied following routine artificial insemination, in non-synchronized sows. Post-thawing motility was found to be significantly higher in the PTAT treated semen (TM: 60.2% vs. 43.7%; PM: 25.6% vs. 18.9% in PTAT vs. Control samples, respectively). In the *in vivo* experiment, rates of non-return to oestrus and pregnancy (86.2% vs. 64.7%, and 82.3% vs. 60.8% in PTAT vs. Control, respectively), and total numbers of piglets and live piglets born were significantly higher (10.8 vs. 8.0, and 9.4 vs. 7.3 in PTAT vs. Control, respectively) in the PTAT-treated group. There was no significant difference in birth weight, weaning weight, and pre-weaning losses among piglets in the PTAT and Control groups.

Increased post-thaw motility and membrane integrity was achieved by the 30 MPa / 90 minutes PTAT treatment of bull spermatozoa. A population of 50 bulls was investigated in Austria and Hungary. Motility of the thawed semen was assessed immediately, 24 and 48 h after thawing,

and membrane integrity was checked by Kovacs-Foote staining (Kovács and Foote, 1992), providing information on the membrane of the acrosome, head and tail. Motility immediately after thawing, as well as 24 hours and 48 hours later (52% vs. 42%, 38% vs. 29%, and 33% vs. 24% in PTAT and Control, respectively) was significantly higher in the PTAT-treated samples. Proportion of sperm cells with intact head, tail, and acrosome was also significantly higher (50% vs. 41%) in the PTAT samples. Representative inseminations revealed a higher non-return rate in the treated groups, though more data are necessary to support this finding (Pribenszky et al., 2007).

Kútvölgyi et al. (2008) also described a significant increase in total and progressive motility of frozen-thawed bull semen using the same PTAT treatment (total motility: 62.3% vs. 54.8%; progressive motility: 47.6% vs. 42.2 %; Control vs. PTAT treated, respectively).

3.3.3. Oocytes

The optimal treatment range of mammalian oocytes was 10–20 MPa hydrostatic pressure for 30–60 min (Pribenszky et al., 2008, 2010a; Mátyás et al., 2010). Experiments revealed significantly increased cryotolerance of porcine, murine and human eggs after PTAT treatment as well as improvements in somatic cell nuclear transfer (SCNT) and *in vitro* culture. The results of oocyte experiments are discussed based on the review of Pribenszky et al. (2012).

3.3.3.1. Stress tolerance and protocol setup

The treatment protocol for porcine oocytes was set up by Pribenszky et al. (2008) and Du et al. (2008a,b,c) and was also applied to murine and human eggs, with minor modifications. In brief, oocytes (immature, in germinal vesicle (GV) stage or *in vitro* matured, in metaphase II. (MII) stage) were aspirated into 0.5 ml artificial straws (IMV, France) in media used to manipulate cells outside the incubator. For porcine oocytes, TCM-199 medium supplemented with Hepes (TCMH) was used, whereas for mouse or human oocytes, G-MOPS (Vitrolife, Sweden) was used.

For stress tolerance studies, porcine cumulus–oocyte complexes were randomly distributed among 12 treatment groups and controls. Treatments were done simultaneously, testing a matrix of treatment parameters including pressures of 20, 40, 60 and 80 MPa, and treatment times of 30, 60 and 120 min, both at room and body temperatures. Oocytes were then partenogenetically activated. Presumptive zygotes were then cultured *in vitro* until 7 days after PTAT treatment.

Oocytes treated in the range of 60–80 MPa did not even cleave after activation, whereas those treated with 20 MPa / 60 min resulted in a 46% blastocyst rate compared with the control group of 22% ($p < 0.05$) (Pribenszky et al., 2008).

In the subsequent step, the effect of recovery time was assessed. The PTAT treatments were followed by different equilibration periods. Equilibration meant that after the treatment oocytes were removed from the instrument performing the PTAT, and placed back into the incubator in *in vitro* maturation medium for 0–2 hours. When the equilibration time was complete, vitrification or enucleation followed. The optimal treatment that resulted the highest improvement in oocyte competence was treatment of cumulus-oocyte complexes (COCs) at 37°C with 20 MPa for 60 min, followed by 1 or 2 hours of equilibration before vitrification or enucleation, respectively (Du et al., 2008a,b,c; Pribenszky et al., 2008).

3.3.3.2. PTAT treatment to improve *in vitro* maturation and *in vitro* embryo production

Nuclear and cytoplasmic maturation of the oocyte have been considered the most significant parameters affecting the success of *in vitro* fertilization (IVF) (Krisler, 2004). The efficiency of oocyte maturation in human IVF procedures is still suboptimal; during assisted reproductive cycles, only approximately 5% of fresh oocytes produce a baby (Patrizio and Sakkas, 2009). In fact, cytoplasmic competence in oocytes, that is, the ability to produce embryos with high developmental potential, is poorly defined biochemically.

The transition from oocyte to fertilized egg (zygote) involves many changes, including protein synthesis, protein and RNA degradation, and organelle remodeling. These changes occur concurrently with the meiotic divisions that produce the haploid maternal genome. Accumulating evidence indicates that the cell-cycle regulators that control the meiotic divisions also regulate the numerous changes that accompany the oocyte-to-zygote transition. In studies described below, a PTAT treatment promoted competence of the maturing oocyte to produce a better quality embryo.

Pig

Germinal vesicle stage oocytes surrounded by cumulus cells (cumulus-oocyte complexes /COCs/) were aspirated into 0.5 ml straws in TCMH medium. Straws were then treated with 20 MPa for 60 minutes, at room temperature (24°C; PTAT-treated group). Control groups included GV oocytes kept at 24°C for 60 min too (Control group I), and unaffected oocytes kept in the incubator in IVM (*in vitro* maturation) medium for the corresponding time (Control group II). Following that, COCs were released from straws and incubated in the original IVM medium until the start of the parthenogenetic activation. Results showed that PTAT treated oocytes developed into blastocysts with a higher chance than controls (54% vs. 39% and 47% in PTAT-treated oocytes vs. Control I and Control II oocytes, respectively); with cell numbers being increased as well (60 vs. 47 and 48 in PTAT-treated oocytes vs. Control I and Control II oocytes, respectively) (Pribenszky et al., 2008).

The study was repeated independently by Kurome et al. in 2011 (unpublished results of Mayuko Kurome, Barbara Kessler and Eckhard Wolf; Molecular Animal Breeding and Biotechnology, LMU Munich, Germany) with a slight modification: PTAT treatment of the COCs was performed at 38°C. In total, 608 COCs were used in seven replicates; they were distributed into treatment and control groups. Similarly to previous result, PTAT treatment increased blastocyst rate (24.3% vs. 20.0% in PTAT vs. Control oocytes), and significantly increased blastocyst cell numbers (80.9 vs. 58.7 in PTAT vs. Control oocytes).

In the presented studies, oocytes treated with a single controlled hydrostatic pressure stress at the beginning of the maturation process developed to the blastocyst stage with a higher chance than controls. Whereas maturation and cleavage rates were not affected at either of the studies, treatment significantly increased the cell number of the blastocyst with $p < 0.01$ in both cases. PTAT treatment of immature oocytes increased embryo quality during *in vitro* embryo production.

3.3.3.3. PTAT treatment to improve cryotolerance of oocytes

Oocyte cryopreservation has become one of the most challenging approaches to restore fertility of chemo- and radiation therapy treated women with compromised ovarian function (Falcone and Bedaiwy, 2005) and improve reproductive flexibility of *in vitro* assisted reproductive technologies in humans (Porcu and Venturoli, 2006; Vajta and Kuwayama, 2006; Vajta and Nagy, 2006). In animals, endangered species and premium genetics from specific breeds could be rescued, whereas the expense and disease transmission during storage and transportation could be minimized (Vajta, 2000). Although vitrification of human oocytes can result in pregnancy rates comparable to that of the fresh ones as the result of newly defined techniques (Cobo et al., 2008; Rienzi et al., 2010), improvements are still needed, especially in animals, including pigs (Somfai et al., 2012).

Pig

A total of 1668 porcine *in vitro*-matured oocytes were used by Du et al. (2008a,b,c) in control and treatment groups, treated in TCMH as described above. Significantly higher blastocyst rates ($p < 0.01$) were obtained in the groups treated with 20 MPa pressure levels, with either 70 (11.4 ± 2.4%) or 130 (13.1 ± 3.2%) minutes long equilibration times between treatment and the start of the vitrification, when compared with the control group without PTAT treatment where no blastocysts were obtained. The influence of temperature at PTAT treatment on further embryo development was also investigated. Treatments of 20 MPa with 70 min post-PTAT equilibration were performed at 37 or 25°C. Oocytes pressurized at 37°C had a significantly higher blastocyst (14.1 ± 1.4%) rate than those treated at 25°C (5.3 ± 1.1%; $p <$

0.01) or untreated control (1.3 ± 1.3%). Overall, PTAT considerably improved developmental competence of vitrified pig *in vitro*-matured oocytes.

Human

Discarded *in vitro*-matured or not fertilized human oocytes were used for this pilot study. Oocytes (110) were randomly distributed to treatment or control groups. Treatment groups were treated as described above. Morphological survival and parthenogenetic activation were the tools to assess survival after cryopreservation. MediCult's Oocyte Freezing kit was used for cryopreservation as described by the manufacturer (MediCult, Denmark, today Origio, Denmark). The parthenogenetic activation protocol included 10 µM calcimicin treatment for 5 minutes, followed by incubation with 5 mM DMAP for 3 hours. None of the activated oocytes developed to the blastocyst stage, possibly due to their inappropriate cytoplasmic maturation; however, PTAT treatment increased rates of both survival and activation rate (67% vs. 62%, and 40 vs. 30% for PTAT vs. Control oocytes, respectively) (Matyas et al., 2010; Pribenszky et al., 2010a).

3.3.3.4. PTAT treatment to improve the efficacy of somatic cell nuclear transfer (SCNT)

Du et al. (2008a,b) aimed to investigate the *in vitro* and *in vivo* developmental competence and cryotolerance of embryos produced by hand-made cloning (HMC) after PTAT treatment of the recipient oocytes. *In vitro* matured porcine oocytes were treated with 20 MPa as described above, then equilibrated for 2 hours before enucleation. Two cell lines (from Day 40 fetuses of Yucatan and Danish Landrace breeds /LW1 – 2/) were used as donor cells for nuclear transfer. After 7 days of *in vitro* culture, blastocyst rates and mean cell numbers were determined. Randomly selected blastocysts were vitrified with the Cryotop method. The blastocyst rate was significantly higher in the PTAT-treated groups compared with the control groups at both cell lines (Yucatan: 57% vs. 28.9%; LW1-2: 68.2% vs. 46.4% in PTAT vs. Control). Cell number of the blastocysts was similar; however, subsequent blastocyst vitrification resulted in significantly higher survival rate in the PTAT group compared with the control group after thawing (62% vs. 30% in PTAT vs. Control).

Representative embryo transfer of reconstructed embryos from the PTAT group resulted in the birth of two healthy piglets by natural delivery.

The authors concluded that PTAT treatment of porcine oocytes before HMC improved *in vitro* developmental competence and cryotolerance and supported embryonic and foetal development, as well as pregnancy establishment and maintenance during pregnancy, up to the birth of healthy piglets (Du et al., 2008a,b).

3.3.4. *In vivo* results

PTAT-treated and transferred bovine embryos resulted in the birth of healthy offspring (Siqueira et al., unpublished data).

As a result of the insemination of PTAT-treated fresh or cryopreserved spermatozoa, 491 and 531 healthy piglets were born, respectively, not being different from the control in sex ratio, weight, stillbirth and malformations (Horváth et al., 2016; Kuo et al., 2007; Pribenszky et al., 2009).

Following artificial inseminations of sows with PTAT-treated and cryopreserved boar semen, rates of non-return of oestrus and pregnancy (86.2% vs. 64.7%, and 82.3% vs. 60.8% in PTAT vs. Control, respectively), and total numbers of piglets and live piglets were significantly higher (10.8 vs. 8.0, and 94. vs. 7.3 in PTAT vs. Control, respectively) in the PTAT-treated group. There was no significant difference in birth weight, weaning weight, and preweaning losses among piglets in the PTAT and Control groups (Horváth et al, 2016).

Embryos developing from PTAT-treated porcine oocytes utilized as nucleus recipients in somatic cell nuclear transfer were transferred, which resulted in the birth of two healthy piglets by natural delivery (Du et al., 2008b).

3.3.5. Gene expression

A fundamental understanding of the PTAT technique`s mechanism of action at the molecular level is still lacking. However, different groups have shown that the PTAT-increased cryotolerance of embryos or spermatozoa is related to the altered expression of genes connected to growth arrest, oxidative stress, cell pluripotency, apoptosis and lipid metabolism.

3.3.5.1. Preimplantation stage embryos

PTAT-induced transcriptional changes of mouse blastocysts included the upregulation of Azin1, Gas5, Gadd45g and Sod2 immediately after the treatment, and Gadd45g after 120 min culture. The study demonstrated that hydrostatic pressure activates short- and long-term growth arrest and oxidative stress-related genes (Bock et al., 2010).

Similar phenomena, that is, upregulation of antioxidant defense-related genes (Sod2 and GPX4) together with lipid synthesis (Erg25) and stress tolerance-related (Hsp70) genes were also observed in PTAT-treated bovine blastocysts. The relative abundance of the related mRNAs was highest one hour after the end of the stress treatment. The cryotolerance of the PTAT-treated blastocysts was, similarly, highest if vitrification followed one hour after the stress treatment (Siqueira et al., 2009 and 2011).

Jiang et al. (2016) treated bovine blastocysts with three different PTAT treatments (40, 60 and 80 MPa) in combination with three recovery periods (0, 1 h, 2 h post HHP) before vitrification. Re-expansion rates were significantly higher at 40 and 60 but lower at 80 MPa after vitrification-warming in the treated groups than controls. Microarray analysis and gene ontology analysis indicated that PTAT at 40 and 60 MPa promoted embryo competence through down-regulation of genes in cell death and apoptosis, and up-regulation of genes in RNA processing, cellular growth and proliferation. In contrast, 80 MPa up-regulated genes in apoptosis, and down-regulated protein folding and cell cycle-related genes. Moreover, gene expression was also influenced by the length of the recovery time after PTAT. The significantly over-represented categories were apoptosis and cell death in the 1 h group, and protein folding, response to unfolded protein and cell cycle in the 2 h group compared to 0 h. These results suggest that PTAT promotes competence of vitrified bovine blastocysts through modest transcriptional changes (Jiang et al., 2016).

PTAT-treated ovine blastocysts were processed for gene expression analysis 1,5 hours after the end of PTAT exposure. Significantly lower abundance for BAX and OCT4 transcripts were observed in PTAT-treted embryos than in the control group (Bogliolo et al., 2011).

3.3.5.2. Semen

In porcine spermatozoa PTAT treatment induced a significant increase in the production of several proteins that are supposed to play a crucial role in the process of fertilisation. Ubiquinolcytochrome C reductase complex core protein 1, perilipin and carbohydrate-binding protein AWN precursor were identified as pressure-stress response proteins being significantly higher in PTAT-treated samples measured after treatment, following 5 hours of equilibration and also post-thawing (Huang et al., 2009a). As spermatozoa are regarded as being transcriptionally inactive cells, the above proteins may be the result of posttranscriptional stabilization of the mRNA by the sublethal stress, as was described earlier in chondrocytes (Kaarniranta et al., 1998).

3.3.5.3. Oocytes

Global gene expression profiling of PTAT-treated porcine oocytes and the parthenogenetically activated or cloned embryos developed from these oocytes identified several PTAT-responsive genes. 44 transcripts were altered by PTAT treatment, with most exhibiting lower expression in PTAT-treated oocytes. Genes involved in embryonic development were prominent among the transcripts affected by PTAT (Lin et al., 2014).

3.4. Other sources of stress to improve oocyte viability

Several other sources of stress also may improve cell competence if applied in a predetermined, controlled manner.

3.4.1. Osmotic stress treatment of oocytes

3.4.1.1. Stress tolerance and protocol setup

Temporary increase of NaCl concentration on cryotolerance and developmental competence of porcine oocytes was tested by Lin et al. (2009a,b). Survival rates were compared after 1 h exposure to seven elevated NaCl concentrations (from 0.25 to 4%, equivalent to 361 to 1306 mOsmol). Survival rate after treatment was only reduced at 1.5 and 2% NaCl concentrations (710 and 850 mOsmol).

In subsequent experiments, oocytes were exposed to 593 mOsmol NaCl, equilibrated for 1 or 2 h, vitrified, then subjected to parthenogenetic activation or used as recipients for somatic cell nuclear transfer. Blastocyst rates increased in both cases after NaCl treatment compared with untreated controls. (64% and 65% vs. 45% in NaCl-treated 1h and NaCl-treated 2h vs. Control, respectively; Lin et al., 2009a). In a forthcoming experiment, further chemicals were tested to provide increased osmotic pressure of the treatment medium, including NaCl, sucrose or trehalose applied at the same osmotic level (588 mOsmol). Subsequently, COCs were incubated in IVM medium for 1 h at 38.5°C in 5% CO_2 with maximum humidity. After this recovery period, cumulus cells were removed and the oocytes were subjected to further treatments, as described below (Lin et al., 2009b).

3.4.1.2. Hyperosmotic treatment to improve cryotolerance of oocytes

Porcine *in-vitro*-matured cumulus–oocyte complexes were exposed to 588 mOsmol NaCl, sucrose or trehalose solutions for 1 h, allowed to recover for a further 1 h, vitrified (using the Cryotop method), warmed and subjected to parthenogenetic activation (as described above). Both Day 2 (Day 0 = day of activation) cleavage (46%, 44%, 45% and vs. 26% for NaCl-, sucrose-, and trehalose-treated vs. Control, respectively) and Day 7 blastocyst rates (6%, 6%, 7% vs. 1%, for NaCl-, sucrose-, and trehalose-treated vs. Control, respectively) were increased after osmotic treatments compared with untreated controls (Lin et al., 2009a,b).

3.4.1.3. Hyperosmotic treatment to improve the efficacy of somatic cell nuclear transfer (SCNT)

The COCs were treated with 588 mOsmol NaCl, sucrose or trehalose, then used as recipients for SCNT (Day 0). Cleavage rates on Day 1 did not differ between the NaCl-, sucrose-, trehalose-treated and the untreated control groups, but blastocyst rates on Day 6 were higher

in all treated groups (64%, 69%, 65% vs. 47%, for NaCl-, sucrose-, and trehalose-treated vs. Control, respectively) compared with control. Cell numbers were significantly lower in the sucrose- and trehalose-treated groups, whereas the NaCl-treated group did not affect cell number compared with the control group. In conclusion, treatment of porcine oocytes with osmotic stress improved blastocyst rate, but not the cell number after enucleation and SCNT (Lin et al., 2009a,b).

3.4.2. Oxidative stress treatment of oocytes

3.4.2.1. Stress tolerance and protocol setup

The effect of controlled oxidative stress to *in vitro*-matured bovine oocytes was investigated based on previous studies about PTAT treatments. For all experiments, immediately before fertilization, cumulus-oocyte complexes (COCs) were incubated in various concentrations (low, 0.01 or 0.1 µM; medium, 1 or 10 µM; high, 100 µM; or very high, 1 mM) of H_2O_2 for 1 h before fertilization. Because pyruvate can neutralize the effect of peroxide (Morales et al., 1999), the different concentrations were diluted in maturation medium without pyruvate. Meanwhile, two control groups were kept in maturation medium without pyruvate and normal maturation medium, respectively. After incubation, COCs were washed in HEPES-TALP. After H_2O_2 exposure, mature oocytes were fertilized. At 24 h post-insemination, presumed zygotes were denuded and cultured in modified synthetic oviduct fluid (SOF) medium. Embryos were evaluated for blastocyst development, total cell number and apoptotic cell ratio. Fertilization and penetration rates were comparable between the groups; only very high concentrations of H_2O_2 (1 mM) resulted in significantly lower fertilization (22.1%) and penetration rates (34.4%) in comparison with all other groups (Vandaele et al., 2010).

3.4.2.2. Oxidative stress treatment to improve *in vitro* maturation and *in vitro* embryo production

Exposure of oocytes to high H_2O_2 concentration (50–100 mM) produced more blastocysts in comparison with the control group (47.3 and 31.8%, respectively). The mean number of blastomeres at the blastocyst stage (7 days post-insemination) varied between 100 and 120 and was not different between groups. Interestingly, the mean apoptotic cell ratio of the high H_2O_2 group did not differ from the control, but was significantly lower in comparison with low or medium H_2O_2.

Short-term exposure of mature COCs to high concentrations of H_2O_2 induced stress tolerance during further development, manifested as enhanced embryo development, but it did not affect apoptosis in blastocysts. In contrast, low to medium H_2O_2 concentrations significantly increased apoptosis in Day 7 blastocysts. Neither the higher glutathione levels in mature

oocytes nor the enhanced penetration or fertilization rate was the cause of the positive effect of H_2O_2 on oocyte competence. The beneficial effect of H_2O_2 on embryo development should therefore be related to other (uncharacterized) effects of H_2O_2, presumably on the oocyte.

The positive effect of H_2O_2 at the end of the maturation period is not mediated by increased glutathione content or improved fertilization, but is an effect on the long term up to the morula or blastocyst stage more than 4 days later (Vandaele et al., 2010).

3.4.3. Mechanic stress treatment of oocytes

3.4.3.1. Stress tolerance and protocol setup

Mizobe et al. (2010) tried to mimic the ciliary beating of oviductal epithelial cells and contraction of oviductal smooth muscle by applying mechanic stimuli to oocytes.

Authors hypothesized that mechanical vibration of dishes containing oocytes and embryos during *in vitro* maturation and *in vitro* culture might improve production efficiency of blastocysts. Mechanical vibrations at a frequency of 20 Hz and accelerations of ±0.33 G and ±0.11 G in the x-axis and y-axis directions, respectively, were applied. The COCs were cultured with mechanical vibration for 5, 10 or 60 s at intervals of 10, 30, 60 or 90 min. Control COCs were cultured without mechanical vibration. After culture, some oocytes were examined for *in vitro* maturation. The control group was activated and cultured without mechanical vibration. The effects of durations and intervals of mechanical vibration during *in vitro* maturation on the nuclear maturation and parthenogenetic development of oocytes were examined. In a forthcoming experiment, oocytes matured without mechanical vibration were activated and cultured with mechanical vibration for 5 s at intervals of 10, 30, 60, 180, 360 or 720 min. Control oocytes were cultured without mechanical vibration. The effects of mechanical vibration during *in vitro* maturation of recipient oocytes and ⁄ or *in vitro* culture after reconstruction on the development of SCNT embryos were also examined. Oocytes matured with or without mechanical vibration for 5 s at intervals of 60 min were enucleated and fused with donor cells. Fused embryos were cultured with (for 5 s at intervals of 60 min) or without mechanical vibration. Mechanical vibration during *in vitro* culture after activation did not affect the blastocyst formation of oocytes (Mizobe et al., 2010).

A different group has investigated a further mechanic stimulus, sheer stress, by applying a non-static, namely tilting culture system. To set tilting parameters, uniform radial velocity, the maximum tilt angle and the holding time at the maximum tilt angle were defined. First, the plate is tilted to the positive maximum tilt angle. Second, the tilting plate is held static, next, the plate is tilted to the negative maximum tilt angle. Last, the tilting plate is held with no motion.

The maximum tilt angle that caused mineral oil to spill out was defined and set the limit of the maximum tilt angle to approximately 20°. Furthermore, excess uniform radial velocity also induces spill out of the mineral oil. The minimum radial velocity at which the mineral oil spilled from the 35-mm dish was 240°／s when the maximum tilt angle was 20°. The oil did not spill out when the tilt angle was 10°. It was necessary to increase the maximum tilt angle and radial velocity to move embryos in the microdrop. However, this study set the maximum tilt angle and radial velocity so that they did not result in the spilling out of the mineral oil, but still allowed the observation of embryo motion in the video rate recording. Thus, the maximum tilt angle was 10–20° and the radial velocity was approximately 1°／s. Frozen 2-cell stage embryos were cultured at a maximum tilt angle of 20° with a holding time of 1 min. The plate was rotated at 1°／s to reach a total tilt of 20°. Frozen-thawed human embryos were also used to test the system. As the consequence, the culture system used did not affect blastocyst rate, but increased cell number of the embryos by 16–26% (Matsuura et al., 2010).

3.4.3.2. Mechanic stress treatment to improve *in vitro* maturation and *in vitro* embryo production

Mechanical vibration during *in vitro* culture (IVC) after activation did not affect blastocyst formation (11.6 ± 5.2–16.5 ± 3.0% vs. 12.1 ± 1.0 in treated groups vs. Control) of *in vitro* activated porcine oocytes. However, blastocyst formation rates after activation of oocytes matured with mechanical vibration for 5 s at intervals of 30–60 min or for 10 s at intervals of 60 min were higher than those of oocytes matured without mechanical vibration (25.7 ± 2.0–28.1 ± 2.7% vs. 12.3 ± 1.4% and 25.8 ± 1.8% vs. 15.7 ± 1.9%, respectively). Therefore, mechanical vibration enhanced the cytoplasmic maturation of *in vitro*-matured pig oocytes, resulting in improvement of their parthenogenetic development (Mizobe et al., 2010).

In another study, the effects of continuous tilting of the culture dish during IVM of porcine COCs and／or IVC following IVF of oocytes on early development to the blastocyst stage and the cell number of blastocysts were investigated. The tilting culture during IVM has improved the diameter of COCs after IVM, whereas maturation itself was unaffected. Although the results and their interpretation is conflicting (blastocyst rate and average cell number in the control group: 28.3% and 30.4; group tilted during IVM: 29.1% and 30.1, group tilted during IVC: 24.9% and 34.1), the authors concluded that tilting culture was beneficial for embryo development (Koike et al., 2010).

3.4.3.3. Mechanic stress treatment to improve the efficacy of somatic cell nuclear transfer (SCNT)

Mechanical vibration during IVM and／or IVC with different intervals and frequencies, as described before, was used. Mechanical vibration for 5 s at intervals of 60 min during *in vitro*

maturation of oocytes did not affect fusion with miniature pig somatic cells after enucleation. However, the blastocyst formation rate of SCNT embryos was improved ($p < 0.05$) by mechanically vibrating recipient oocytes for 5 s at intervals of 60 min during *in vitro* maturation, regardless of the presence or absence of the same treatment during *in vitro* culture. The treatment did not affect cell number of the blastocysts (Mizobe et al., 2010).

4. MATERIALS AND METHODS

4.1. Oocyte and embryo retrieval, *in vitro* culture

4.1.1. Animals used for oocyte and embryo production

4.1.1.1. Mouse

For oocyte and embryo production four-twelve weeks old B6D2F1 (female C57BL/6 x male DBA/2) female and CD1 male mice were used. Animals were housed under standard conditions (22 ± 2 °C; 12 h dark/12 h light; water and food ad libitum).

All procedures were approved by the Animal Care and Use Committee of Szent István University, Faculty of Veterinary Science.

4.1.1.2. Zebrafish

Wild-type zebrafish (*Danio rerio*) embryos were obtained from the breeding unit of Department of Aquaculture, Szent István University, Gödöllő, Hungary. Parents (egg/sperm donors) were housed according to standard procedures at 25 ± 2°C, pH 7.0 ± 0.2 and a conductivity of 525 ± 50 mS (system water) with a 14:10 hour light-dark photoperiod in a ZebTec (Tecniplast, Buguggiate, Italy) recirculating zebrafish housing system. The system water is RO water amended with artificial sea salts (30g/ L Coral Ocean Plus salt from ATI GmBH, Germany and 30g/L NaHCO3 from Sigma-Aldrich, MO, US). The housing system from Tecniplast supplied the water and automatically adjusted the pH and conductivity. The fish were fed twice a day with SDS–Small Granular (Akronom, Budapest, Hungary) or Zebrafeed by Sparos (Olhão, Portugal).

Studies were conducted in the approved laboratory animal unit of Department of Aquaculture of Szent István University, Hungary (permission number: PEI/001/1719-2/2015, issued by Government Office for Pest County, Hungary). Experiments including the breeding procedure were approved by the Animal Welfare Committee of Szent István University, Hungary and by Government Office for Pest County, Hungary (permission number: XIV-1-001/2301-4/2012, issued to Szent István University, Hungary).

4.1.2. Oocyte and embryo production

4.1.2.1. Mouse

For embryo production female mice were superovulated by intraperitoneal injection of 10 IU of pregnant mare serum gonadotropin (PMSG; Werfaser, Alvetra & Werfft, Austria) in the afternoon at 14.00 p.m., followed by 5 IU of human chorionic gonadotropin (hCG, Choragon

5000 IU, Ferring, Deutschland) 48 hours later. After hCG administration, females were mated with fertile males in monogamous pairs. Females with a vaginal plug – as a proof of copulation – were used for embryo harvesting.

For oocyte production female mice were superovulated by the previously described hormonal treatment, but the injections were administered at 20.00 pm (10 IU of PMSG, followed by 5 IU of hCG 48 hours later). After the hCG injection females were kept in their cage without a male mouse. On the next morning females were used for oocyte harvesting.

4.1.2.2. Zebrafish

Parents were spawned in pairs each week in double breeding tanks (Tecniplast, Buguggiate, Italy) that allow eggs to fall through the perforated bottom of the inner tank to avoid cannibalism by the parents. Ninety pairs provided embryos for these experiments. Parents were placed into the 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.

4.1.3. Oocyte and embryo harvesting

4.1.3.1. Mouse

Mouse oocytes and embryos were harvested by flushing the oviduct or the uterus with Flushing Medium (Medicult, Denmark).

One-cell stage embryos were harvested from the oviducts of copulated females on the day when the vaginal plug was observed (Day 0.5 after hCG injection). Zygotes were freed from cumulus cells by exposure to 0.1% hyaluronidase in G-MOPS Plus (Vitrolife, Sweden) for a few minutes 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.

Oocytes were harvested by flushing the oviduct 13 hours after hCG injection, in the morning. The oocytes were freed from cumulus cells by exposure to 0.1% hyaluronidase in G-MOPS for a few minutes.

4.1.3.2. Zebrafish

Following spawning, the parents were removed from the breeding tanks by removing the inner tank with the perforated bottom. The embryos were collected from the outer tank by filtering

the water of the tank. All eggs fertilized in the breeding tanks were pooled, and then groups of 100 or 200 embryos were formed and incubated in 15-cm Petri-dishes at 26°C until treatment.

4.1.4. Embryo culture

4.1.4.1. Culture of mouse embryos

Mouse embryos of each developmental stages were cultured in the incubator in 30-µl microdrops of potassium simplex optimized medium, supplemented with amino acids (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.

4.1.4.2. Culture of zebrafish embryos

Zebrafish embryos were cultured in 15-cm Petri dishes filled with system water, at 26°C. The water around the embryos was completely changed once a day. At the stage of 5 days post-fertilization (dpf), each group of larvae was individually placed into 15-cm Petri-dishes and fed once a day with a mixture of banana worms (*Panagrellus nepenthicola*) and SDS 100 feed dissolved in system water. Banana worms were derived from the Banana Worm Starter Culture of the University of Veterinary Medicine, Budapest. The water around the larvae was completely changed once a day, 90 minutes after feeding. Larval development was monitored daily under a Leica M205FA stereomicroscope (Leica, Wetzlar, Germany) following the water change. Mortalities and possible developmental defects (tail malformations, pericardial edema, trunk curvature) were recorded. Health status was monitored daily, including normal feeding behavior and appetite, intact skin and normal social behavior. Groups of larvae were placed into 3.5-L culture tanks in the recirculating housing system on 15 dpf. The tanks were cleaned daily, and mortalities were recorded.

4.2. PTAT treatment

4.2.1. Loading the oocytes and embryos into sample container for PTAT treatment

Before the PTAT treatment oocytes and embryos of all treatment groups were evaluated morphologically. Only oocytes and embryos that were spherical and symmetrical with no extruded material in the perivitelline space and with an intact zona pellucida, and embryos that had blastomeres in stage-specific number with uniformity in size, color and density, were included in the experiment.

Before the PTAT treatment the biological material is placed into an appropriate cell container, e.g. luer lock syringe, 0.25-ml or 0.5-ml ministraws, 5-ml maxistraws, insemination or transfusion bags or custom-made plastic boxes, in appropriate handling media. The container must be filled up without air bubbles, and then properly sealed with heat, plastic caps or iron balls.

Zebrafish embryos, mouse embryos or oocytes of each treatment day were randomly distributed into control and treatment groups. Treatment groups were loaded into the appropriate sample containers, as in the following paragraphs. Control group represented the group of oocytes or embryos left at room temperature in the equivalent sample container as the treatment group, for the corresponding time, while a group of embryos or oocytes was left in the incubator unaffected.

4.2.1.1. Mouse embryos and oocytes

Embryos or oocytes of each treatment day were distributed into control and treatment groups according to the daily experimental plan. Each treatment group were then aspirated into separate 0.25-ml artificial straws (IMV, L'Aigle, France) in G-MOPS Plus, without air bubbles. All operations were performed on 37°C heated stage. Straws were sealed by plastic plug (**Figure 3**), then were PTAT treated.

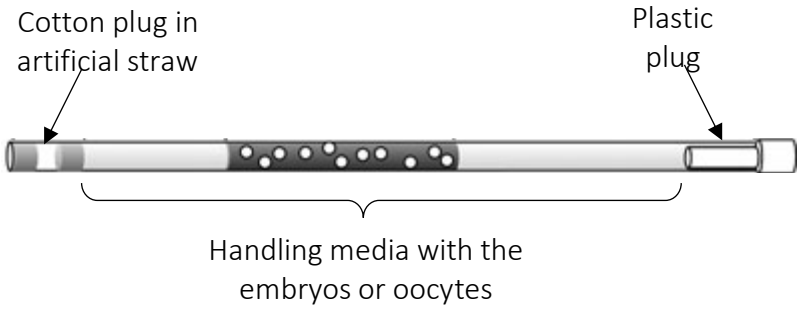

Figure 3. Oocytes or embryos loaded into artificial straws ready for PTAT treatment. Straws were closed by plastic plugs.

4.2.1.2. Zebrafish embryos

Embryos of each treatment day were distributed into control and treatment groups according to the daily experimental plan. Embryos of each treatment group were then aspirated into separate 2-ml luer lock syringes (B.Braun Melsungen AB, Melsungen, Germany) in system water without air bubbles and locked by plastic luer lock caps (**Figure 4**). All operations were performed on room temperature (22-25°C).

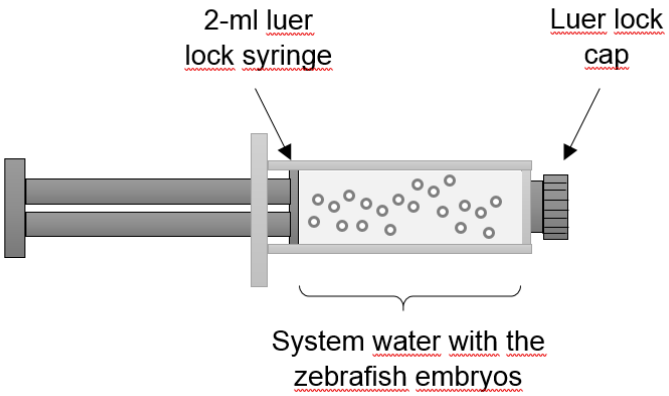

Figure 4. Luer-lock syringe and cap utilized as sample container for PTAT treatment of zebrafish embryos.

4.2.2. Description of the PTAT treatment device

The treatments were performed by a computer controlled hydrostatic pressure device GBOX 2010 (Applied Cell Technology Ltd., Budapest, Hungary; **Image 1**).

Image 1. GBOX 2010 for the PTAT treatment of biological samples.

After sealing the container loaded with the biological sample, containers are placed into the pressure chamber of the GBOX 2010 that was previously filled with distilled water (**Figure 5**) and heated up to the required temperature (e.g., body or room temperature). The pressure chamber is closed, and the machine executes the pressure program according to the set parameters.

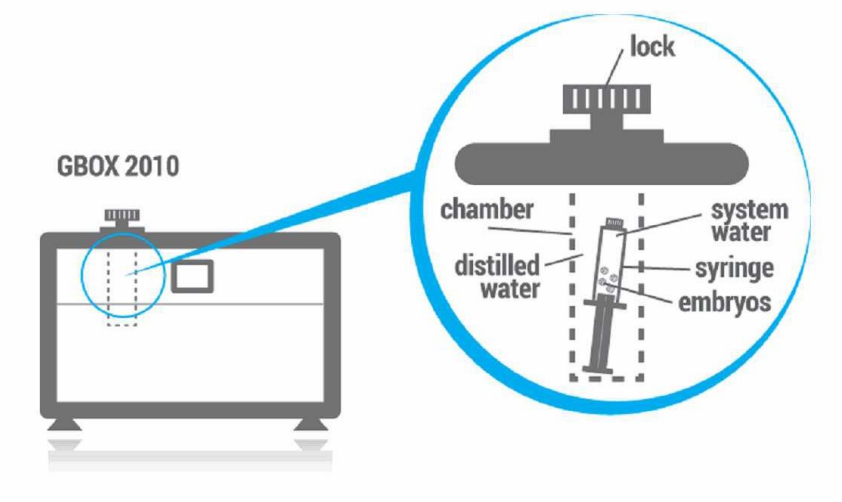

Figure 5. Construction of GBOX 2010, showing the pressure chamber containing a syringe with embryos.

The capacity of the pressure chamber is 100 cm^3, the range of use is between 2 MPa and 90 MPa (one pascal (Pa), equal to one newton per square metre [N/m^2 or $kg \cdot m^{-1} \cdot s^{-2}$] or $145{,}04 \cdot 10^{-6}$ psi). The custom-made software runs the adjusted preconditioning treatment by controlling hydrostatic pressure build up, holding and coming back to atmospheric pressure, and the temperature in the pressure chamber. In the following studies rate of pressurization and decompression to atmospheric pressure was the default setting of the device, 10 MPa/min.

4.2.3. PTAT treatment of embryos and oocytes

The amount, duration, and temperature of PTAT treatment is different according to the treated biological material, and has been determined by the stress tolerance tests (Study 1). In Study 1 the stress treatment was followed by a morphological evaluation with Zeiss AxioVision 40 software, on Zeiss Axio Observer A1 (Carl Zeiss MicroImaging Inc., Thornwood, NY, USA), on 37°C heated stage (Tokai Hit, Japan).

4.2.3.1. Fish embryos

Based on the results of the stress tolerance tests, zebrafish embryos were PTAT-treated with 5 MPa pressure for 90 minutes on 25°C, 24 hours post-fertilization, at 26-somites or Prim-5 stage in the zebrafish embryo chilled storage study (Study 3).

4.2.3.2. Mouse oocytes

Based on the results of the stress tolerance tests, for the developmental competence study (Study 4), and the gene expression study (Study 5), mouse oocytes were treated with 20 MPa pressure for 60 minutes, on 36°C.

4.3. Chilled storage of zebrafish embryos

Chilling was performed according to the modified protocol of Desai et al. (2015b). Briefly, embryos were placed into 50-ml screw-cap centrifuge tubes (Axygen, Union City, CA, USA) 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. The temperature was monitored (**Figure 6**) and kept constant at 0.0–0.3°C during the entire chilling period. Cooling profile was measured using a K-type thermocouple connected to a Digi-Sense DualLogR thermometer (Eutech Instruments, Singapore). 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 in Chapter 4.1.4.2. (Culture of zebrafish embryos).

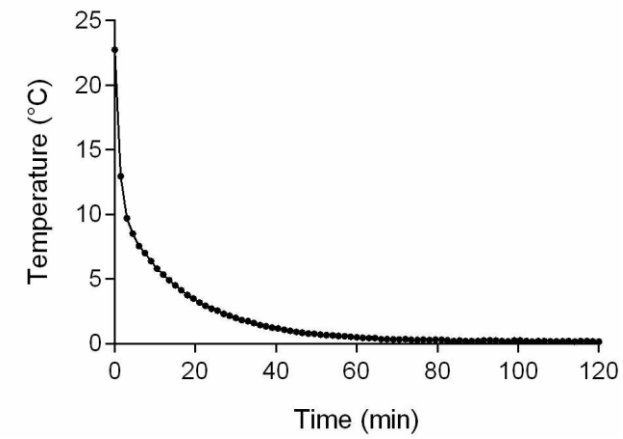

Figure 6. Chilling curve applied for zebrafish embryos. Groups of 26-somites or Prim-5 stage embryos were placed on ice in zebrafish system water containing cryoprotectants. Cooling occurred according to the following cooling rates: 3.16°C/min for 4.5 minutes, 0.28°C/min for 22.5 minutes, 0.06°C/min for 30 minutes, and 0.01°C/min for 33 minutes. Embryos were kept at 0.0–0.3°C for additional 22.5 hours (24 hours in total).

4.4. 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 at a sex ratio of 3:1 (three Leopard danio to one experimental fish) to keep track of the zebrafish during experiments. Spawned Leopard danio fish were derived from the same source, from the breeding unit of Department of Aquaculture, Szent István University, Gödöllő, Hungary. Following spawning, fish were removed from the spawning tanks, and embryos were incubated in 10-cm Petri-dishes until 10 dpf, as described in 4.2.2. (Culture of zebrafish embryos).

4.5. 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).

4.5.1. Vitrification

For the vitrification Equilibration Solution (ES) and Vitrification Solution (VS) were prepared from Washing Solution (WS). G-MOPS Plus (Vitrolife, Sweden) was used for WS, which was supplemented with 7.5% dimethyl sulfoxide (DMSO) and 7.5% ethylene glycol (EG) for ES, and 15% DMSO, 15% EG and 15% sucrose for VS.

In a 50-mm Petri dish one 20-µl WS droplet and two 20-µl ES droplets (ES1 and ES2) were placed forming a triangular figure, with 1 mm distance from each other. A third ES droplet (ES3) was also placed into the Petri dish, a little further from the triangle (**Figure 7A**).

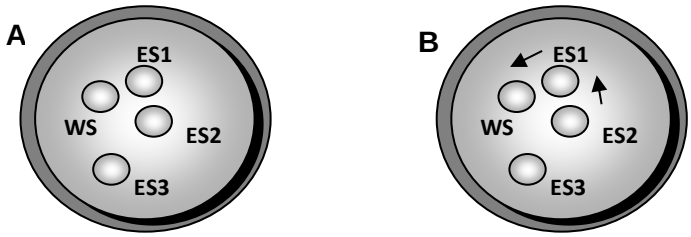

Figure 7. (A) Preparation of ES droplets and WS droplet for vitrification, (B) equilibration by merging WS with ES1 droplet, and then with WS+ES1 with ES2 droplet.

Another Petri dish was also prepared, with 100 µl VS in it. The dishes were prepared when the oocytes were ready for the vitrification procedure, and were kept at room temperature.

The denuded oocytes were placed into the WS droplet in the first dish. Then, the WS droplet was mixed with the ES1 droplet with the pipette tip, and the oocytes were let to equilibrate for 3 minutes. Then the mixed WS+ES1 droplet was mixed with the ES2 droplet, and the oocytes were let to equilibrate for another 3 minutes (**Figure 7B**).

After the 3-minutes long equilibration in the WS+ES1+ES2 droplet, the oocytes were placed into the ES3 droplet, where they were kept for 9–15 minutes, until they recovered their normal size and morphology.

Then oocytes (maximum 3 at the same time) were aspirated with minimum volume of ES solution using a capillary. The oocytes were placed on the surface of VS droplet in the second dish, and after expelling any remaining ES solution out of the capillary, they were aspirated and expelled in the VS droplet several times. Then oocytes were placed beside the black mark on the Cryotop sheet with minimum amount of VS solution (**Figure 8**), and within 60 seconds after being placed into the VS droplet, the Cryotop was put into the liquid nitrogen directly.

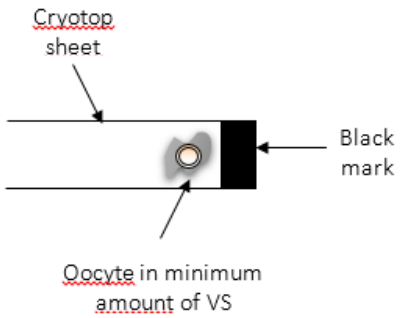

Figure 8. Tip of Cryotop sheet with an oocyte in a minimum amount of VS on it.

4.5.2. Warming

For the warming of the vitrified oocytes Thawing Solution (TS) and Dilution Solution (DS) were prepared in advance. TS contained 3.42 g sucrose dissolved in 10 ml G-MOPS Plus, while DS contained the half amount of sucrose (1.71 g) in 10 ml G-MOPS Plus.

Four 30-mm Petri dishes filled with 2.5 ml solutions were required for the thawing procedure; the first contained TS, the second DS, while the last two contained WS (as WS G-MOPS Plus was used, as described in the vitrification protocol). The TS dish was warmed up to 37°C, the other three dishes were kept on room temperature.

As the first step of thawing, from the liquid nitrogen the Cryotop sheet was quickly plunged into the TS dish. After the oocytes came off of the sheet, they were placed on the center of the dish. One minute after being put into the TS solution, the oocytes were placed on the bottom of the DS with small amount of TS. The oocytes swelled temporarily and began to shrink in DS in 3 minutes. Then oocytes were transferred onto the bottom of WS1 dish with small amount of DS. After being kept in WS1 for five minutes, oocytes were placed onto the surface of WS2 dish with minimum amount of WS1, then were kept here for 5 minutes before being put into the incubator in appropriate culture media (**Figure 9**).

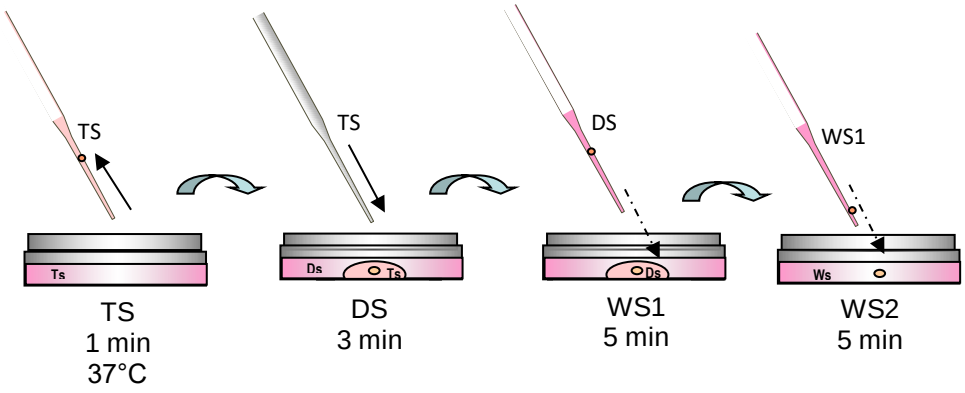

Figure 9. Placing the oocyte to from TS to DS, WS1, and WS 2 for warming after vitrification.

4.6. *In vitro* fertilization of mouse oocytes

To evaluate their developmental competence, mouse oocytes were fertilized by the intracytoplasmic sperm injection (ICSI) method, or activated parthenogenetically.

4.6.1. Preparation of mouse sperm for ICSI

Spermatozoa of CD1 male mice were used for the fertilization of the mouse oocytes. 12 hours after the hCG injection of the female mice, sperm was harvested form the epididymis of the male mouse. The caudae epididymides were punched with a 26 gauge needle and the sperm cells were let to swim out in a 30 mm Petri dish containing 1 ml of G-MOPS Plus. After incubation at 37°C under 5% CO_2 in air for 1 hour, the upper portion of the medium was collected and examined. Over 80% of spermatozoa displayed high motility.

4.6.2. Immobilization of sperm and ICSI

After the 1-hour long incubation of the sperm suspension, when the oocytes had been harvested, 6 µl of epididymal sperm suspension was suspended in 5 µl of 12% polyvinylpyrrolidone (PVP) solution. A single spermatozoon was drawn, tail first, into the injection pipette just to the head–midpiece junction. The injection was made in G-MOPS Plus. Application of a few piezo-pulses separated the head from the tail (Kuretake et al., 1996). Isolated sperm heads were injected immediately into mouse oocytes.

4.6.3. Examination of oocytes and embryos following ICSI procedure

After the injection with mouse sperm heads, mouse oocytes were incubated in KSOM+AA for 6–8 hours and examined with an Olympus IX- microscope with Hoffman differential interference optics for evidence of activation. Embryos showing two pronuclei and a second polar body were cultured further in KSOM+AA medium.

4.7. 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, followed by careful flushing in G-MOPS Plus. Then the oocytes were incubated in 5 mM DMAP (6-dimethylaminopurine) solution (solvent: KSOM+AA, Embryomax, Millipore, Billerica, USA) under mineral oil (Ovoil, Vitrolife, Sweden) for 3 hours. After the treatment oocytes were cultured *in vitro* in 30-µl microdrops of KSOM+AA under mineral oil at 37°C with 6% CO_2 and 90% humidity in air.

4.8. Determination of blastocyst cell number

4.8.1. Immunocytochemistry of pre-implantation embryos

Blastocysts' total cell number and inner cell mass (ICM) cell number have been determined with whole-mount immunostaining. After ICSI procedure, embryos were cultured for 96 hours until they reached the blastocyst stage. At blastocyst stage zona pellucida was removed by a short incubation in acidic Tyrode's solution (Sigma-Aldrich, USA) and the embryos were fixed in 4% paraformaldehyde (Sigma-Aldrich, USA) for 15 minutes. Then embryos were permeabilized with ionic detergent 0.1% Triton X-100 (Sigma-Aldrich, USA) and blocked with phosphate buffered saline solution supplemented with 1% bovine serum albumin.

Anti-OCT4 antibodies (Polyclonal Rabbit 19081, Santa Cruz Biotechnology) were used as primary antibodies, in 1:100 dilution, during overnight incubation at 4˚C, and Alexa Fluor 594 anti-rabbit secondary antibodies (Molecular Probes, USA) in 1:2000 dilution for 1 hour at room temperature. After immunostaining for OCT4, blastocysts were mounted on the slides and covered with Vectashield (Vector Laboratories, USA) containing 4'-6-Diamidino-2-phenylindole (DAPI, Sigma-Aldrich, USA).

4.8.2. Cell counting

The assessment for cell number counting was made using digital images on different focal planes, obtained with a Zeiss optical sectioning apotome microscope equipped with an HBO lamp. The cells presented in different focuses were counted and summarized. The total cells were identified by their blue (DAPI staining) fluorescence; ICM cells were identified by their red (Alexa fluor 594) fluorescence.

4.9. Mouse embryo transfer

4.9.1. Preparation of psudopregnant recipients

The pseudopregnant recipient females used for embryo transfer were obtained by natural mating with vasectomized males as the seminal secretions produced by a sterile male are required for the uterus to become receptive to the transferred embryos. To obtain a recipient, a maximum of 2 females of 8 weeks to 6 months of age were placed with a vasectomized male in the afternoon. The following morning, females were checked for the presence of a vaginal copulation plug. As mating usually occurs during midnight, the day of vaginal plug detection is considered to be 0.5 days post mating (Bermejo-Alvarez et al., 2014).

4.9.2. Embryo transfer into recipients' uterus

Females with a vaginal plug were anesthetized with intraperitoneal injection of Ketamine (0.1 mg/g) and Xylazine (0.01 mg/g). After immobilization and loss of reflexes the ovary, oviduct, and a small portion of the upper uterus were carefully pulled out of the abdominal cavity by grabbing the adipose tissue around them through a small incision on the flank region between the last rib and the hips. The oviduct was punctured with a 27 gauge needle, and a glass pipette with 130-150 µm external diameter containing the media with the embryos in it between two tiny air bubbles was inserted through the orifice into the uterus. The embryos and the two air bubbles before and after them were gently blown into the uterus. After removing the pipette from the uterus, the oviduct and ovary were placed back to the abdominal cavity by grabbing the adipose pad. The recipients were identified by ear punching and were observed until recovery from anesthesia (Bermejo-Alvarez et al., 2014).

4.10. Gene expression investigations

4.10.1. RNA purification

Total RNA was isolated from three biological replicates of 30–44 treated and 24–36 control embryos using the RNeasy Plus Micro Kit (Qiagen, Hilden, Germany). The RNA integrity number (RIN) was determined with an Agilent 2100 Bioanalyzer instrument using an RNA 6000 Pico Chip Kit (Agilent Technologies, Palo Alto, CA, USA).

4.10.2. RNA amplification and microarray analysis

The TrueLabelling-PicoAMP kit (SABiosciences, Frederick, MD, USA), which uses a two-round RNA amplification procedure, was utilized to amplify an appropriate quantity of complementary © RNA using 240 pg total RNA from each sample. 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). All microarray data were deposited in the NCBI Gene Expression Omnibus (GEO) database (GEO Accession no. GSE28411).

4.10.3. Complementary DNA synthesis and reverse transcription qPCR (RT-qPCR)

Isolated RNA was reverse transcribed using the SuperScript VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Primers for representative genes were designed using Primer3 software (Rozen and Skaletsky 2000). mFOLD software was used to avoid interfering secondary DNA structures (Zuker 2003), and

the specificity of the primers was predicted by BLASTn software (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The H2A histone family, member Z (*H2afz*), Hypoxanthine guanine phosphoribosyl transferase (*Hprt1*) and Peptidylprolyl isomerase A (*Ppia*) genes served as endogenous controls because they have been shown to be reliable reference genes during *in vivo* and *in vitro* preimplantation development (Mamo et al., 2007).

Primers were optimised using four-point twofold serial dilution standard curves. 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 (PCR) verification. Each real-time PCR reaction mixture contained 0.25 embryo equivalent cDNA template, 300nM each primer and 50% SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich) in a total volume of 15 µl. The RT-qPCR reactions were set up using the QIAgility liquid handling robot and were performed on a Rotor-Gene Q cycler (Qiagen). The cycling parameters were as follows: 94°C for 3 min initial denaturation, followed by 40 cycles of 95°C for 5 s, 60°C for 15 s and 72°C for 30 s. Melting curve analysis and agarose gel electrophoresis confirmed the specificity of the primers and the absence of genomic (g) DNA contamination. The RT-qPCR experiments were performed according to Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009).

4.11. Statistical analysis

4.11.1. Stress tolerance tests

In case of the stress tolerance studies data were analyzed by a generalized linear model with logit link and binomial error distribution (logistic regression). The only independent factor was the treatment type that was tested by a likelihood ratio test in each experiment. All treatment groups were compared to Control group by Dunnett type multiple comparison procedure (Dunnett, 1955). For the stress tolerance studies statistical calculations were done in R 2.7.2. (R Development Core Team, 2007). For model fitting the “MASS” package (Venables and Ripley, 2002), and for multiple comparisons, the “multcomp” package (Holthorn et al., 2007) were used. The P-value < 0.05 was considered significant.

4.11.2. Zebrafish embryo chilled storage study

All data were analyzed using GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA). The survival data were analyzed by Kaplan-Meier nonparametric tests, nonparametric logrank tests and Cox-regression tests (using Stata/SE 14 software, StataCorp LP, College Station, TX, USA). To analyze morphology and fertility data, the appropriate

ANOVA followed by Tukey's or Sidak's post-hoc test as required or Student's paired t-test (when comparing two variables) were performed. A P-value < 0.05 was considered significant. Values are presented as the mean ± SEM.

4.11.4. Statistical and functional analysis of mouse embryo gene expression studies

The raw data of 41 534 probe sets of the Agilent 4 X 44K whole mouse genome chip were subjected to LOWESS 50ormalization, processed by GeneSpring GX 11 (Agilent Technologies) and analysed by asymptotic t-tests. Two-tailed P<0.05 and fold changes ≥1.5 were considered significant. Because the P-values were not corrected by a multiple test, the microarray analysis was further subjected to an extensive RT-qPCR validation. Pathway and annotated clustering analysis were performed using the functional annotation tools of DAVID Bioinformatics Resources (Dennis et al., 2003; Huang et al., 2009b) to determine the significantly over-represented Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. P-values retrieved from the functional analysis were corrected using the Benjamini Hochberg procedure (Benjamini and Hochberg 1995). To validate the gene expression profiling results, data from three biological replicates were analysed by RT-qPCR for each gene using the Relative Expression Software Tool 2008 V2.0.7 (Pfaffl et al., 2002).

5. STUDY RESULTS

Our experiments followed the common scheme of our research team's other PTAT-related studies' (**Figure 10**). First, in Study 1, target objects (oocytes, or embryos in different developmental stages) were exposed to different levels (5–80 MPa) of hydrostatic pressure applied for various times (15–120 min), to determine the sublethal zone. Following the pressure treatment morphological alterations and developmental competence were evaluated. In addition, in this early stage of the investigations, in Study 2 we also assessed the *in vivo* long-term effects of the stress treatment.

At the second phase of the experiments, in Study 3 and Study 4, samples were exposed to the predefined treatment, then the required procedures (cryopreservation or chilled storage, *in vitro* fertilization and culture, embryo transfer) were performed. Results were assessed by evaluating morphology as well as functional parameters, including fertilization and developmental competence, blastocyst cell number, and investigation of the individuals developing from the treated gametes or embryos. In each experiment, treatment groups were compared with a control group where PTAT treatment was omitted.

In Study 5, in the third phase of experiments our goal was to reveal the background of PTAT's beneficial effects on oocyte and embryo cryosurvival. We therefore assessed and compared the gene expression alterations due to PTAT treatment in treated and control oocytes.

General scheme of the experiments – how are the procedures and results interconnected?

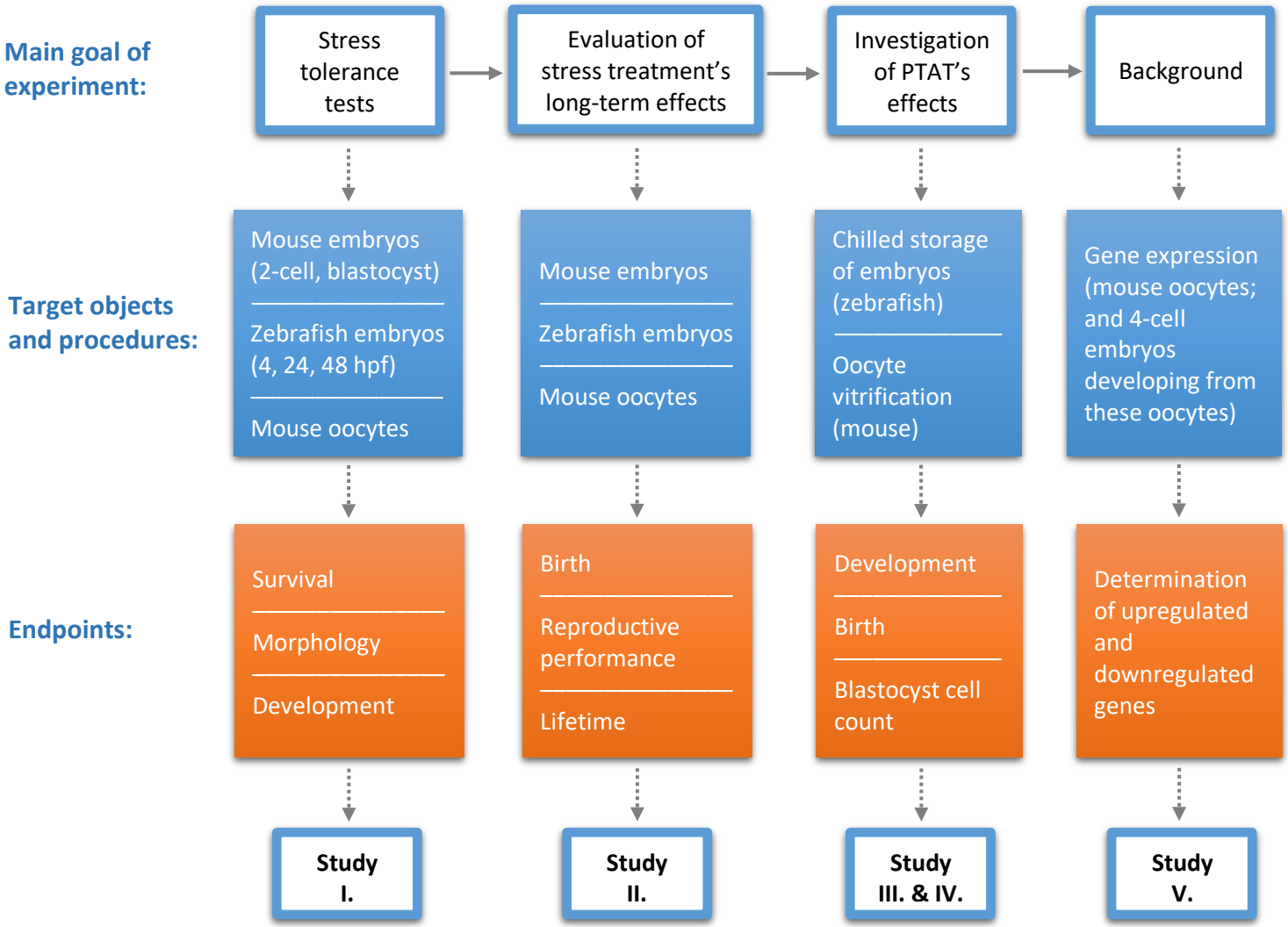

Figure 10. Overview of the experiments.

5.1. Study 1 – Stress tolerance tests

5.1.1. Experimental design

In this experiment 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 (embryos and oocytes) were exposed to various magnitudes of hydrostatic pressure for various durations, and their morphology and developmental potential were measured. Control group represented the group of oocytes or embryos left at room temperature (Control RT) in the equivalent sample container as the treatment groups, for the corresponding time, while a group of embryos or oocytes was left in the incubator on body temperature (Control BT), unaffected. 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, etc.).

Three experiments were performed to analyze the stress tolerance of different embryonic stages followed by an *in vivo* evaluation (**Figure 11**).

5.1.1.1. Experiment I: Mouse embryo stress tolerance tests

In order to correctly evaluate the effects of hydrostatic pressure on mouse embryos, three different types of embryos were used: two-cell stage embryos, *in vitro* produced blastocysts, and *in vivo* produced blastocysts.

Twenty embryos from each group were exposed to different hydrostatic pressures from 20 to 80 MPa (by 20 MPa increments) for various times (60 and 120 min), at room temperature (22 to 26°C).

In each experiment the pressure treatment was followed by a morphological evaluation and measurement of size alterations, and then embryos were cultured for 3 to 6 days, while morphological and developmental changes were recorded in every 24 h, to evaluate the possible harmful effects of the treatment.

5.1.1.2. Experiment II: Zebrafish embryo stress tolerance tests

Zebrafish embryos in three different developmental stages were used in the tests: 4 hours, 24 hours, and 48 hours post-fertilization.

Groups of 100 embryos in 3 replicates were exposed to various levels of pressure and duration between 5 and 40 MPa for 15 and 90 minutes in each of the three developmental stages. All treatments were performed at 25°C. The effect of the stress treatment was evaluated based on the survival rates and continued *in vitro* development of the treated embryos, which has been registered daily.

5.1.1.3. Experiment III: Mouse oocyte stress tolerance tests

Groups of oocytes were exposed to different hydrostatic pressures (10, 20 and 40 MPa) for 60 minutes, at room temperature (22°C). Morphology and survival rates were recorded following the treatments to evaluate the direct effects of stress. Longer (90 or 120 minutes) treatments were not tested, as their introduction to mouse oocyte vitrification and *in vitro* fertilization protocol would not have been possible due to their length.

In each experiment the stress treatment was followed by a 60 minutes long equilibration in the incubator under standard culture conditions. After the incubation oocytes were pathenogenetically activated in order to check their ability for normal cleavage and embryo development.

The reason of using parthenogenetic activation in this experiment was that we planned to use the results of the experiment in the stress tolerance tests of human oocytes. However, due to ethical reasons it is not permitted to create a human embryo for experimental purposes. Parthenogenetic activation and development following this technique was a good alternative to verify the functionality of human oocytes instead of *in vitro* fertilization.

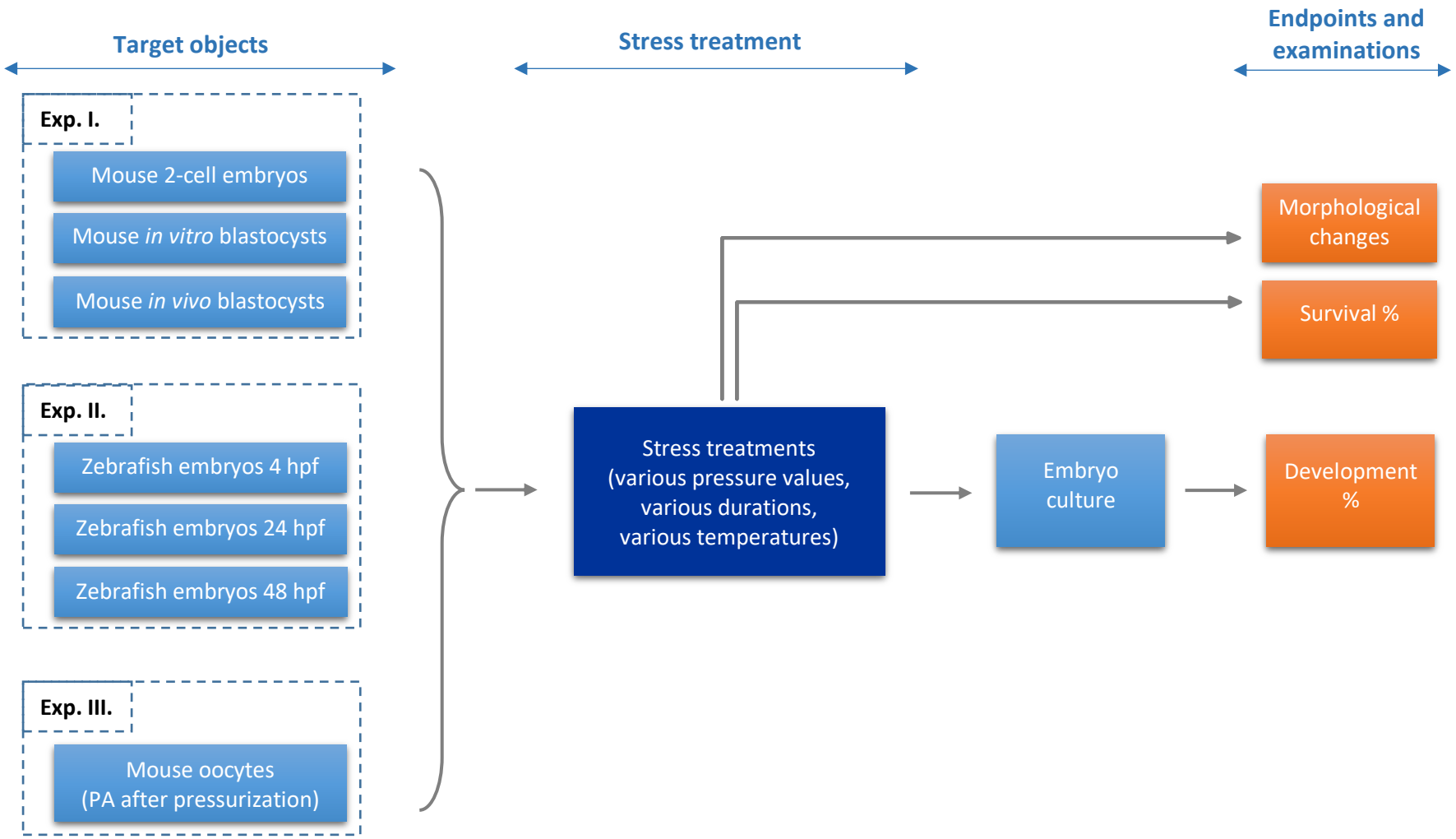

Figure 11. Experimental design of pressure tolerance tests (Study 1).

5.1.2. Results

5.1.2.1. Experiment I: Mouse embryo stress tolerance tests

5.1.2.1.1. Stress tolerance of two-cell stage mouse embryos

Altogether 459 two-cell stage embryos were used in this study. Number of embryos per group was between 9 and 20. All treatments were replicated three times. Embryos responded to the stress treatment with no, moderate or intense reversible or irreversible morphological change, depending on the magnitude and duration of the treatment. While the 20 and 40 MPa treatments caused no macroscopic damage in the two-cell stage embryos (**Figure 12**), the 60 and 80 MPa treatments caused disruptions in the blastomeres, membrane damage and cell death.

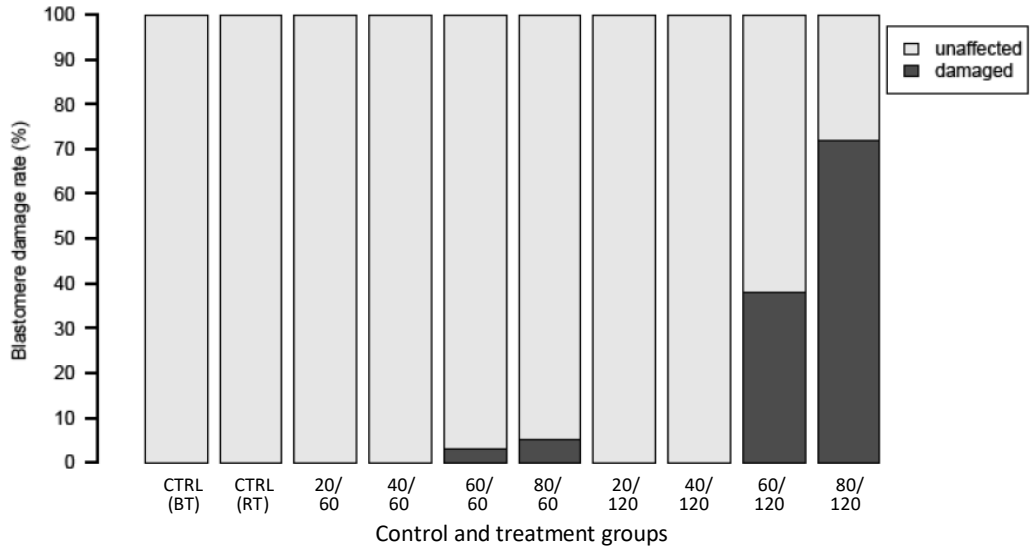

Figure 12. Blastomere damage (%) seen in the different treatment groups after various stress treatments of two-cell stage embryos. Treatments are indicated in MPa/min, at room temperature. BT: embryos kept in the incubator; RT: embryos left at room temperature in sample container.

A reversible reduction of the blastomere size was visible in case of two-cell stage embryos treated with 60 MPa for 120 minutes (**Image 2A**), while embryos treated with the highest stress treatment level (80 MPa for 120 minutes) resulted in irreversible membrane damage (**Image 2B**).

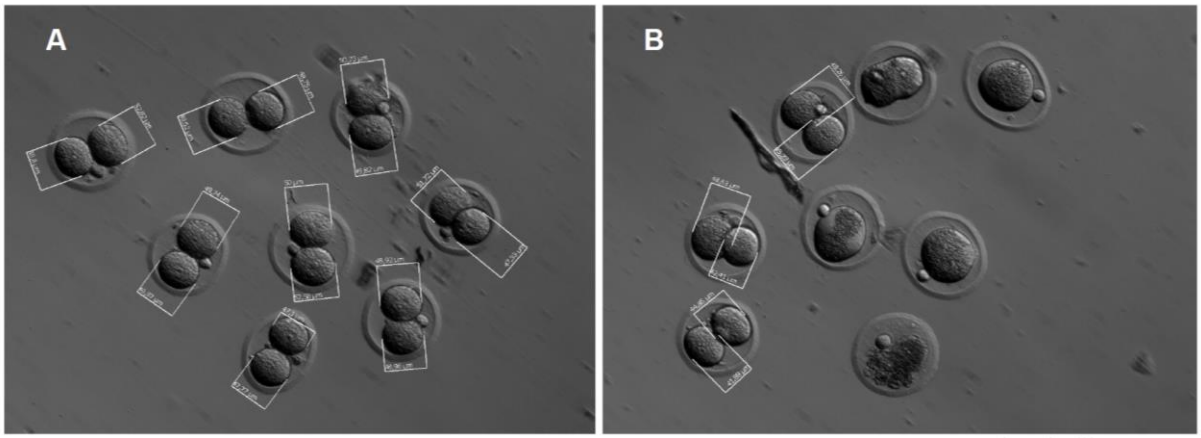

Image 2. Morphological alterations in two-cell stage embryos treated with 60 MPa for 120 min (**A**), and with 80 MPa for 120 min (**B**). The white calipers are computer generated size measurements.

The average size of the blastomeres has reduced gradually when pressure was increased (**Figure 13.** Average size of the blastomeres of the embryos treated at the two-cell stage, after the treatment (Mean ± SEM). Treatments are indicated in MPa/min. Values without a common superscript differed (P < 0.05).).

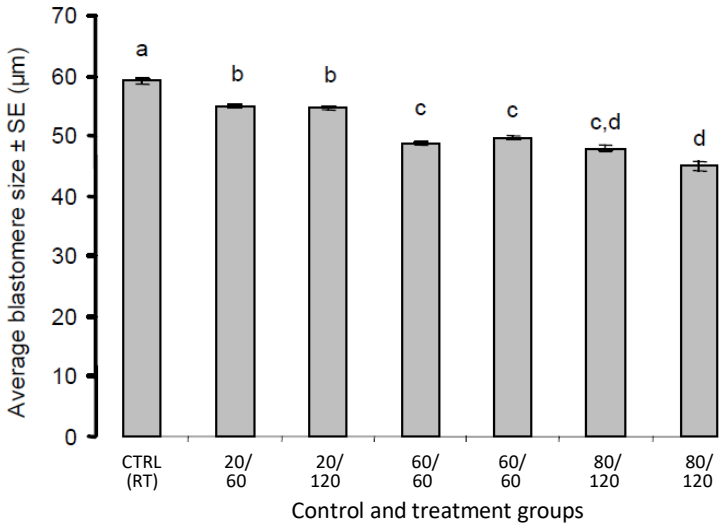

Figure 13. Average size of the blastomeres of the embryos treated at the two-cell stage, after the treatment (Mean ± SEM). Treatments are indicated in MPa/min. Values without a common superscript differed (P < 0.05).

After *in vitro* culture, two-cell stage embryos regained normal blastomere size, developed to the blastocyst stage and then hatched. *In vitro* developmental rates of the treated two-cell stage embryos are shown in **Figure 14**. Both 120 min and 80 MPa treatments resulted in lower blastocyst formation rates compared to the control (**Figure 14A**). These treatment groups had lower hatching rate as well (**Figure 14B**).

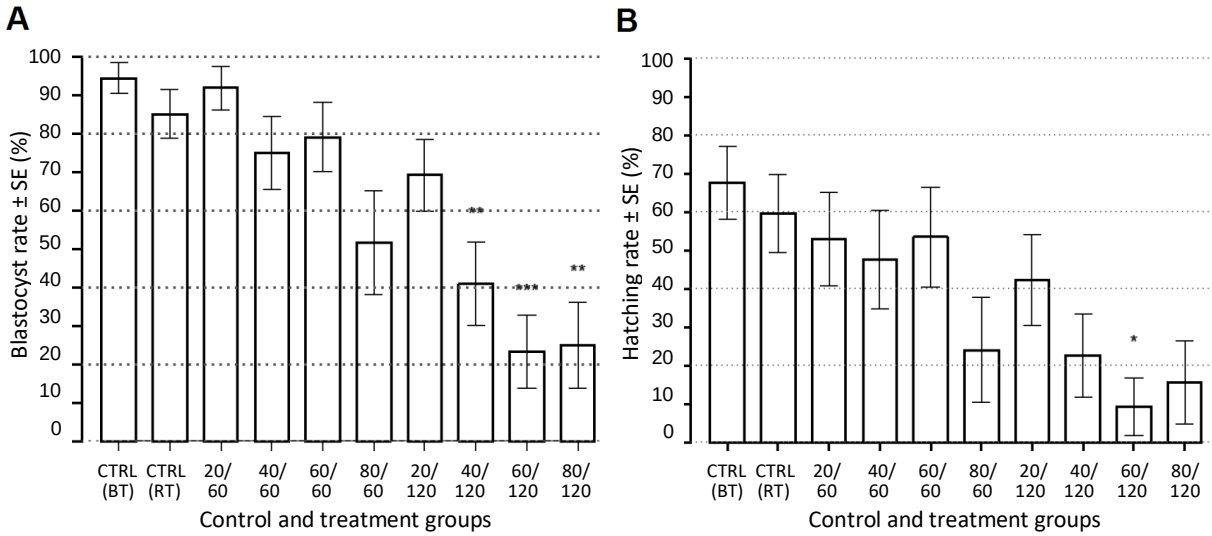

Figure 14. Blastocyst developmental rates (**A**) and hatching rates (**B**) after different stress treatments of two-cell stage mouse embryos. CTRL BT: embryos left in the incubator unaffected; CTRL RT: embryos kept at room temperature without pressurization; Stress treatment groups: MPa/min; *: P < 0.05; **: P < 0.01; ***: P < 0.001 groups different compared to CTRL RT.

5.1.2.1.2. Stress tolerance of *in vitro* cultured or *in vivo* derived expanded mouse blastocysts

Aggregately 402 *in vitro* produced and 464 *in vivo* derived blastocysts were used in this study. Number of embryos per treatment or control groups varied between 7 and 20. All treatments were replicated three times. Embryos responded with no, moderate or intense reversible morphological change, depending on the magnitude and duration of the stress treatment (**Image 3**).

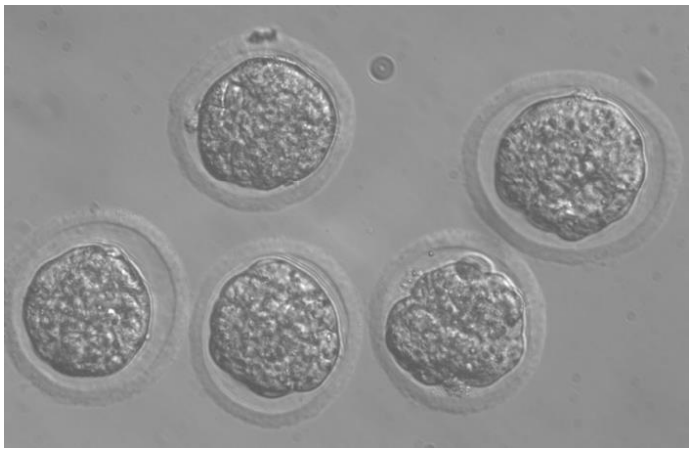

Image 3. Expanded blastocysts compacted after 60 MPa 60 min treatment. 2-4 h post treatment blastocoel re-expanded and embryos hatched as controls. After transfer normal pups were born.

While 20 MPa treatments caused no change in the morphology of the expanded blastocysts, 40 MPa or larger impacts caused reversible collapse of the blastocoel. **Figure 15** shows the treatment related reversible changes in the morphology of the blastocysts.

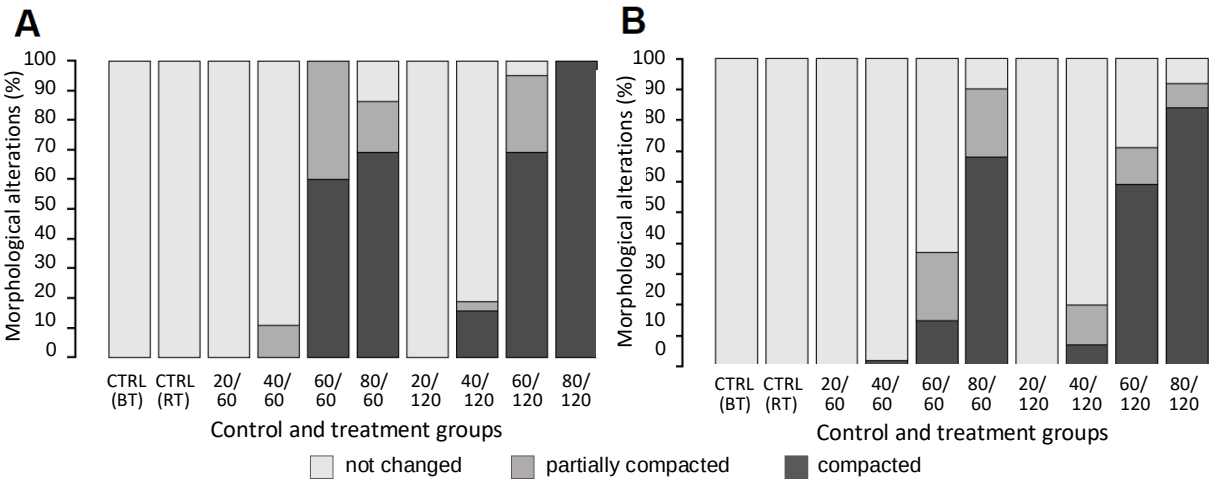

Figure 15. Morphological alterations seen in the different treatment groups after various stress treatments of *in vitro* cultured blastocysts (**A**), and *in vivo* derived blastocysts (**B**). Compacted means full collapse of the blastocoel, while "partially compacted" refers to visible collapse of the blastocoel. Treatments are indicated in MPa/min, at room temperature. CTRL BT: embryos kept in the incubator; CTRL RT: embryos left at room temperature.

There were no significant changes in the hatching rates of the treatment groups compared to the control (**Figure 16**).

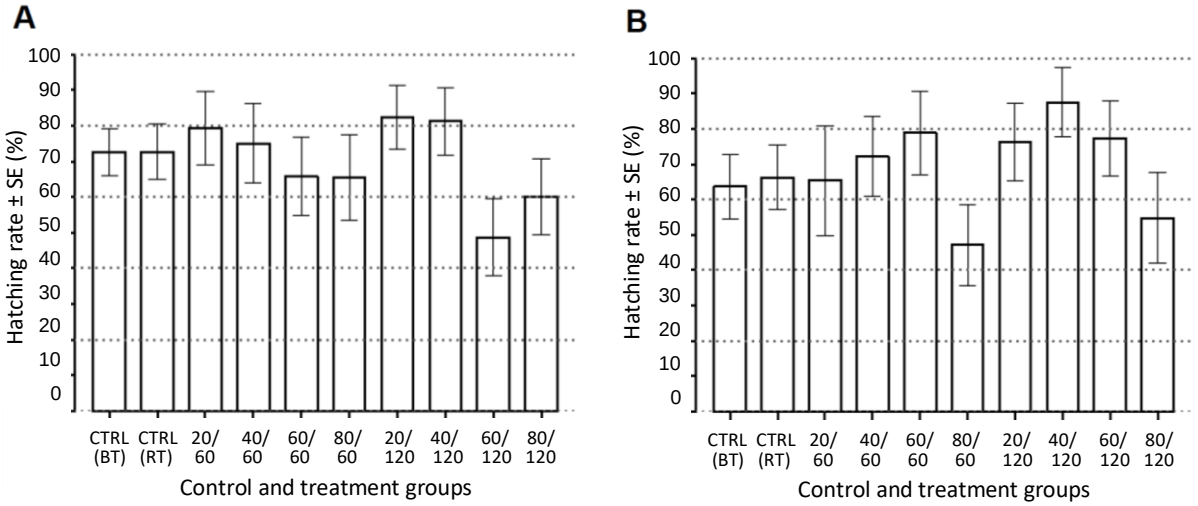

Figure 16. Hatching rates after different stress treatments of *in vitro* cultured mouse blastocysts (**A**), and *in vivo* derived mouse blastocysts (**B**). CTRL BT: embryos left in the incubator unaffected; CTRL RT: embryos kept at treatment temperature without pressurization; Stress treatment groups: MPa/min.

5.1.2.2. Experiment II: Zebrafish embryo stress tolerance tests

Groups of 100 embryos in 3 replicates for each treatment groups were used for the experiment. PTAT tolerance test results showed that the 40 MPa treatment had a distinct harmful effect on the embryos, while this detrimental impact was milder in the 20 MPa groups. Contrarily, several 5 and 10 MPa groups had similar or slightly better survival rates compared to the controls (**Table 2**).

Table 2. Survival rates (%) of zebrafish embryos following various pressure treatments in three different developmental stages

Developmental stage	Pressure duration	Pressure magnitude			
		5 MPa	10 MPa	20 MPa	40 MPa
4 hours post-fertilization	0 min (CTRL)	85.0	89.0	77.6	80.0
	15 min	84.3	88.3	46.0	4.2
	45 min	84.6	82.7	14.0	0
	90 min	88.9	80.6	19.6	0
24 hours post-fertilization	0 min (CTRL)	95.5	97.1	97.0	93.0
	15 min	94.3	92.9	90.4	43.7
	45 min	77.0	94.3	94.2	0
	90 min	96.2	96.7	91.0	0
48 hours post-fertilization	0 min (CTRL)	95.9	97.5	97.5	93.0
	15 min	87.4	94.8	97.6	84.3
	45 min	98.7	94.8	94.5	2.4
	90 min	96.6	93.7	93.8	0

The aim of the stress tolerance tests was to find the right amount and duration of pressure for the planned experiments focusing on chilled storage of the embryos. Regarding that former results published by Liu et al. (1998) and Lahnsteiner et al. (2009) showed the highest chilling tolerance around 24 hour-post-fertilization stage, the evaluation of the treatment's long-term effects and the later chilling experiments were performed on the embryos at this stage, using the stress treatment (5 MPa, 90 minutes) that was found to be the most beneficial for embryo development in this stage.

5.1.2.3. Experiment III: Mouse oocyte stress tolerance tests

Altogether 175 oocytes were used in the experiment. The results of the stress tolerance tests were evaluated based on continued *in vitro* development following parthenogenetic activation. As shown in **Table 3**, the blastocyst rate at all treatment levels were comparable to control.

Table 3. Blastocyst rates following various pressure treatments, and number of oocytes utilized for the experiment

Treatment group MPa/min	Blastocyst rate	No. of replicates	No. of oocytes
CTRL	18%	2	22
10/60	19%	2	48
20/60	20%	2	48
40/60	19%	2	47

5.2. Study 2 – Evaluation of stress treatment's long-term effects

For the evaluation of *in vivo* long-term effects of the treatment, 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. For the PTAT treatment, the parameters that were found to have the optimal effect in the stress tolerance studies (Study 1) have been used. Besides these investigations, further long-term effects evaluations have also been performed in Study 3 (on zebrafish embryos) and 4 (on mouse oocytes). In these experiments PTAT and chilled storage (in Study 3), or PTAT and vitrification (in Study 4) have been tested to evaluate the long-term effects of the treatment (**Figure 17**).

5.2.1. Experimental design

5.2.1.1. Experiment I: Long-term effects of PTAT treatment on zebrafish embryos

For this purpose, a group of 200 zebrafish embryos 24 hours post-fertilization treated with 5 MPa pressure for 90 minutes on 25°C, and a non-treated group of another 200 embryos were cultured for 30 days. Hatching and/or embryo survival was compared on 6, 10 and 30 days post-fertilization. The offspring morphology was compared on 10 and 30 days post-fertilization. Experiments were replicated four times.

5.2.1.2. Experiment II: Long-term effects of PTAT treatment on mouse embryos

For the same purpose, twelve expanded mouse blastocysts were treated with 60 MPa for 60 minutes. These PTAT-treated blastocysts, and seven controls were transferred into two, 3.5 Day pseudopregnant recipients. The longevity and the reproductive performance of the offspring were investigated until the next generation.

5.2.1.3. Further investigations to evaluate PTAT' long-term effects in Study 3 and Study 4

Further investigations were also performed in the latter phases of the experiments. In these, PTAT's and certain additional procedures' (e.g. chilled storage, vitrification, intracytoplasmic sperm injection) long-term effects were evaluated simultaneously.

5.2.1.3.1. PTAT-treated and chilled-stored zebrafish embryos

The long-term effects of PTAT treatment were tested in zebrafish embryos which underwent chilled storage too, besides the stress treatment. For details and results see Study 3.

5.2.1.3.2. PTAT treated and vitrified mouse oocytes

Similarly, PTAT-treated and then vitrified oocytes were also investigated for the long-term effects of the treatment. These experiments will be discussed in Study 4.

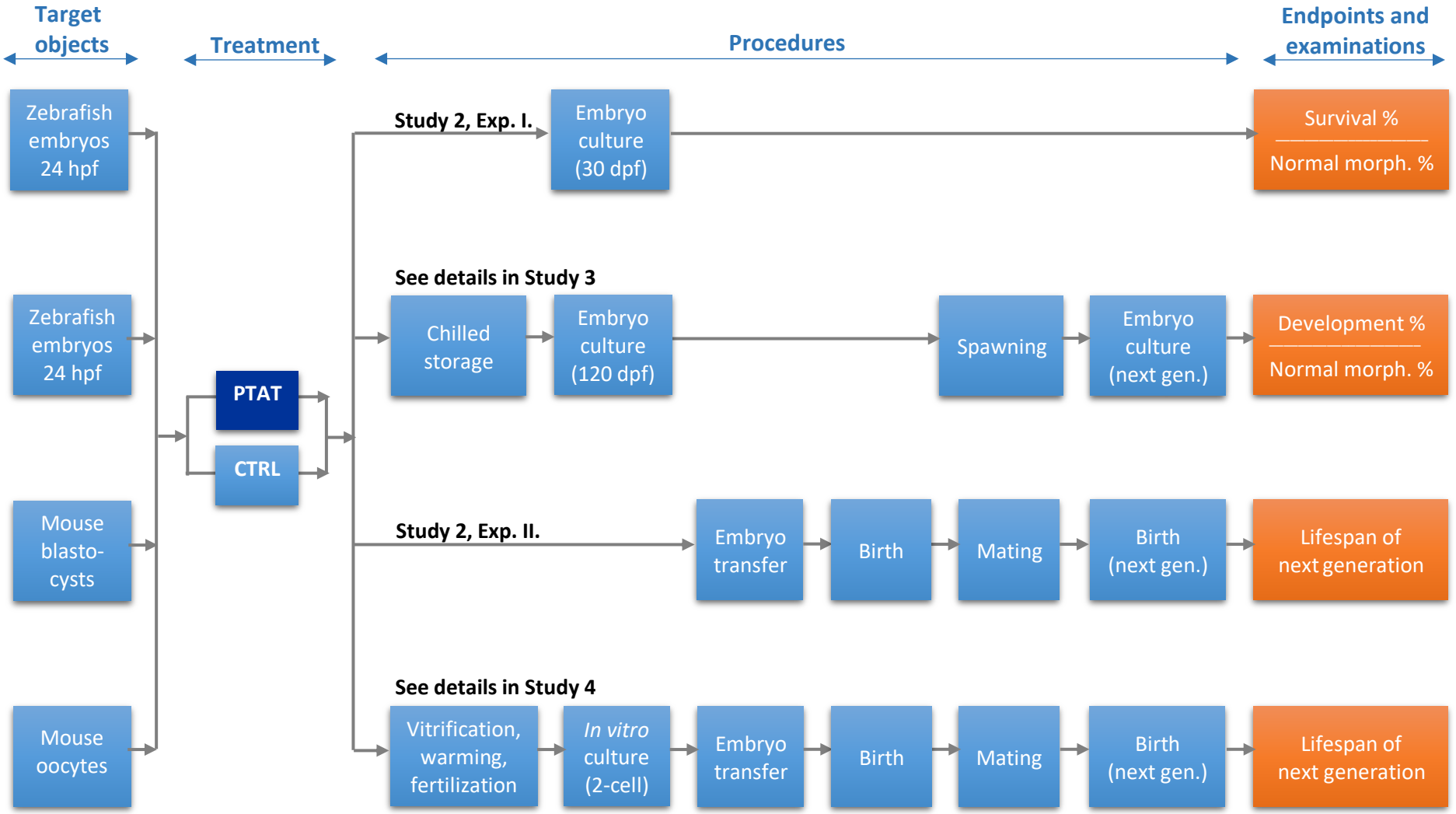

Figure 17. Overview of experiments for the evaluation of PTAT's long-term effects. These effects were investigated in Study 2 on zebrafish and mouse embryos, but further tests were also performed in Study 3 (on zebrafish embryos) and 4 (on mouse oocytes). In these experiments PTAT and chilled storage (in Study 3), or PTAT and vitrification (in Study 4) were tested from to evaluate the long-term effects of the treatment (see details in relevant chapters).

5.2.2. Results

5.2.2.1. Experiment I: Long-term effects of PTAT treatment on zebrafish embryos

The impact of PTAT treatment on zebrafish embryo survival, development and morphology was studied by examining two matching groups of animals (control and 5 MPa / 90 min PTAT treated, 24 hours post fertilization) cultured according to standard housing conditions. Embryo survival and further development was not adversely affected by PTAT. Control and PTAT-treated groups showed similar development without any differences in the hatching rate by 6 days post-fertilization (98.7 ± 0.2% vs. 99.3 ± 0.3% for PTAT vs. Control). Similarly, survival was the same on 10 days post-fertilization (95.4 ± 2.4% vs. 98.1 ± 0.6% for PTAT vs. Control) and 30 days post-fertilization (69.1 ± 4.8% vs. 69.8 ± 0.8% for PTAT vs. Control) after fertilization. The morphological characteristics (prevalence of normal morphology, occurrence of tail abnormalities and pericardial edema) of the two groups were identical, both groups showed normal morphology.

5.2.2.2. Experiment II: Long-term effects of PTAT treatment on mouse blastocysts

Out of the 12 mouse blastocysts (treated with 60 MPa pressure for 60 min) 12 healthy pups (six males and six females) were born. After weaning three males died. The remaining six females and three males were bred, five females became pregnant, and 41 healthy pups were born plus one litter that was cannibalized. Out of the 41 pups, two has died at approximately two months of age, with reduced body weight, and further 14 were victims of cannibalism. At 15 months after the birth of the transferred embryos five females and three males plus nine female granddaughters were alive.

In the Control group seven healthy pups were born (3 males and 4 females). After weaning one male died. After breeding, four females became pregnant and 32 healthy pups were born, 10 were victims of cannibalism. At 15 months after the birth of the transferred embryos three females and two males plus eight female granddaughters were alive.

5.3. Study 3 – Effect of PTAT on developmental potential of chilled-stored zebrafish embryos

5.3.1. Experimental design

As a continuation of Study 1 and Study 2, showing that zebrafish embryos can survive and develop normally following the optimal PTAT treatment, two consecutive experiments were planned in order to test the beneficial effects of PTAT on survival after chilled storage of the embryos (**Figure 18**). The sample size used in each experiment was calculated based on the results of our pilot study which aimed to develop the most effective treatment protocol (two sample mean and standard deviation).

5.3.1.1. Experiment I: Embryo survival and continued development after PTAT treatment and chilled storage.

We hypothesized that PTAT treatment of embryos before chilled storage will increase their survival rate. Experimental groups (n=200 per group) were PTAT preconditioned or incubated at 25°C, then placed at 0.0–0.3°C for 24 hours (PTAT chilled and Control chilled groups, respectively) and then cultured for 30 days. A third, untreated un-chilled group was used to monitor the breeding system.

Hatching rates on 6 days post-fertilization (dpf), survival rates every day, and morphology on 10 dpf and 30 dpf were evaluated. Experiments were replicated four times.

5.3.1.2. Experiment II: Embryo development to maturity and ability to produce healthy offspring after PTAT treatment and chilled storage.

We hypothesized that PTAT treatment of embryos before chilled storage will actually enable them to develop to maturity and be able to produce healthy offspring. Four experimental groups were created. Chilled groups (n=100 per experimental groups) were treated with PTAT or incubated at atmospheric pressure and then kept at 0.0–0.3°C for 24 hours (PTAT chilled group and Control chilled group, respectively). Unchilled groups (n=100 per group) were treated with PTAT or left at atmospheric pressure and then incubated at 25°C in parallel with the chilled storage of the chilled groups (PTAT group and Control group, respectively). Subsequently, all groups were cultured separately until the proposed sexual maturity (~90–120 days), when the fish were spawned.

Ten fish from the unchilled groups and all fish from the PTAT chilled group that reached maturity (n=5) were propagated with six mating attempts for six consecutive weeks. After fertilization, development of the offspring was evaluated on 5 dpf and 10 dpf. The morphological characteristics of Day 10 larvae were recorded and quantified. Fertilization experiments were replicated six times.

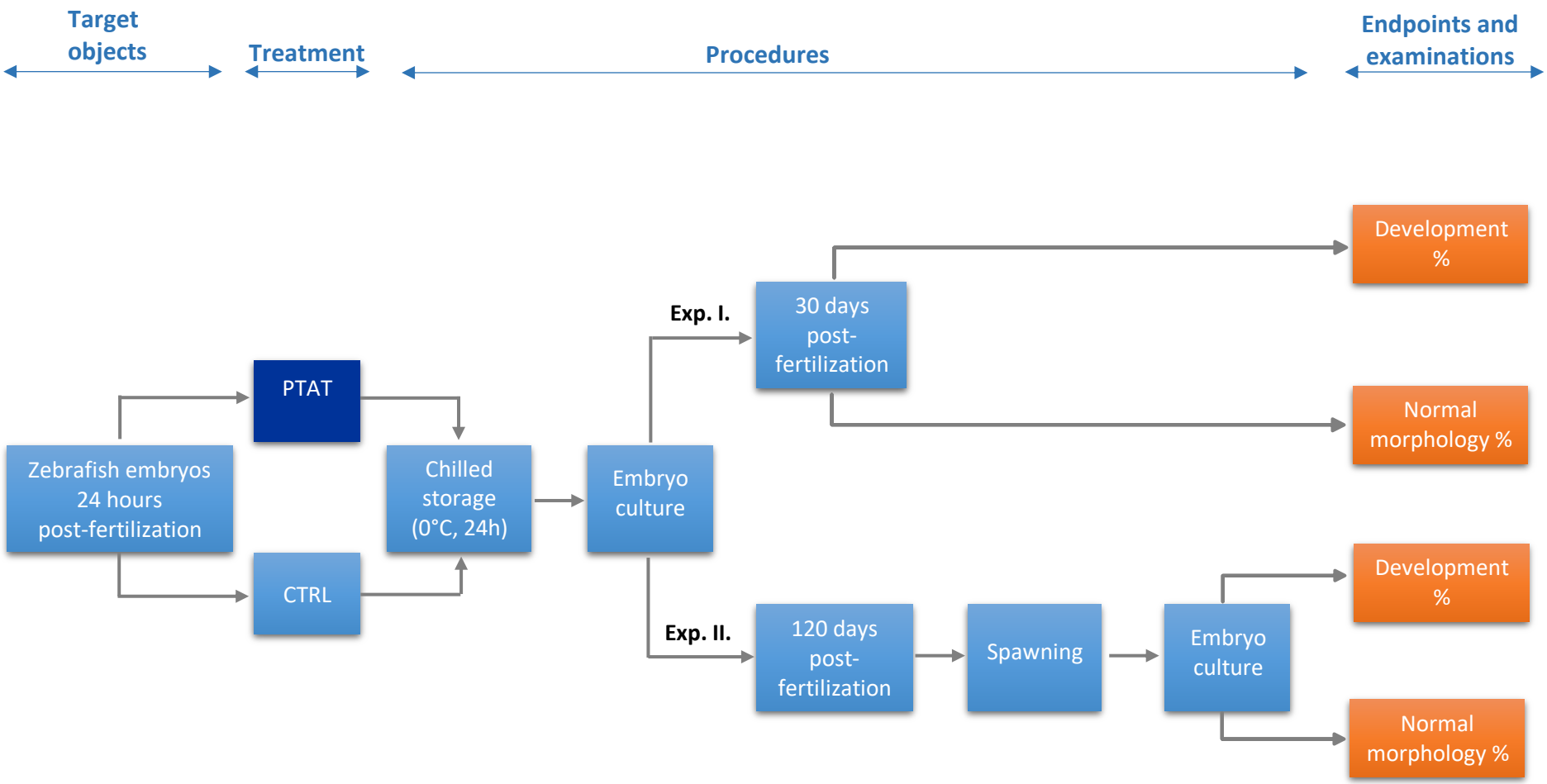

Figure 18. Experimental design of Study 3.

5.3.2. Results

5.3.2.1. Experiment I.: Embryo survival and development after PTAT treatment and chilled storage

The chilling sensitivity of 26-somites or Prim-5 stages zebrafish embryos with or without PTAT preconditioning was examined in this part of the study. The effects of PTAT and chilling on development, hatching, survival and morphology were evaluated compared to Control group, in which embryos were chilled without PTAT preconditioning.

Chilling for 24 hours has arrested the development of embryos and in both groups embryos remained in the 26-somites or Prim-5 embryo stages by the end of the chilling process (**Image 4**).

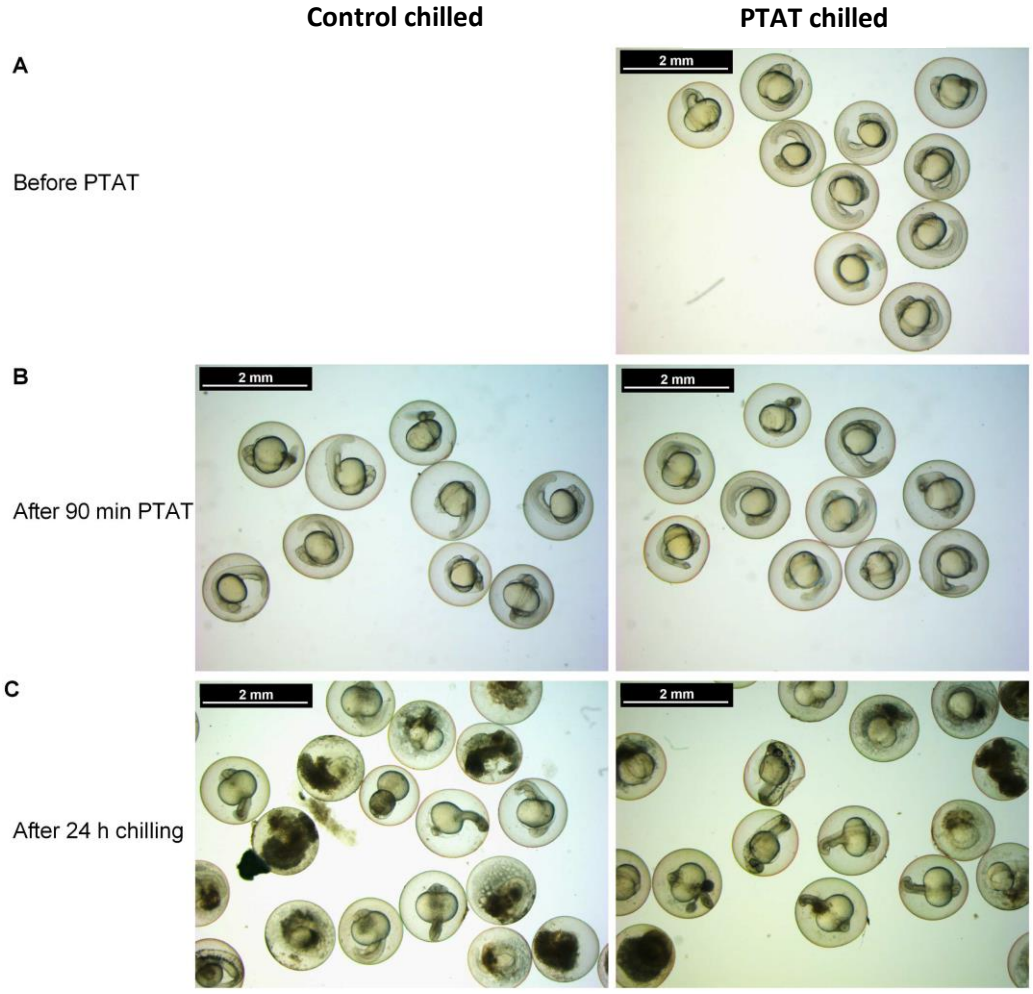

Image 4. Appearance of zebrafish embryos before and after PTAT treatment and after chilling. Zebrafish embryos at 26-somites and Prim-5 stages were submitted to PTAT treatment (**A**). The preconditioning caused no morphological changes on embryos (**B**). The 24-hour-long storage on ice arrested the development, after chilling (**C**) the embryos showed the same developmental stages as before. The beneficial effect of PTAT treatment was remarkable right away after the chilled storage.

As it is shown in **Figure 19**, chilling survival was significantly improved by PTAT preconditioning. By 6 days post-fertilization (dpf), 37.6 ± 3.4% of embryos had hatched in the PTAT chilled group, compared to the 23.0 ± 3.8% for the Control chilled group. By 10 dpf, heartbeat was detected in 17.1 ± 3.5% of PTAT chilled larvae compared to 4.3 ± 1.7% of Control chilled ones. On one hand, all chilled controls died by 19 dpf. On the other hand, the average survival rate in the PTAT chilled group on 30 dpf was 2.3 ± 1.0%. Kaplan-Meier analysis and the logrank test based on daily individual data until 30 dpf (**Figure 19**) revealed that the PTAT preconditioning was statistically significant for fish survival (P<0.0001). A semi-parametric Cox-regression provided similar results (P<0.001).

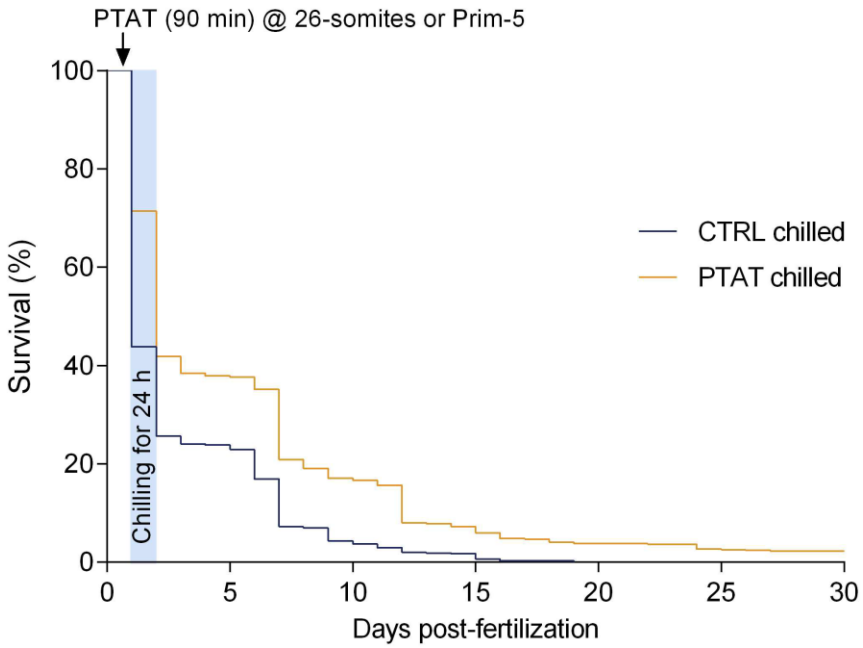

Figure 19. PTAT preconditioning decreases chilling sensitivity, and enhances embryo survival after chilling. Kaplan-Meier estimates of zebrafish embryo survival in the first 30 day-post-fertilization. 200–200 embryos were randomized to PTAT and Control groups, experiments were replicated four times. Embryos at 26-somites and Prim-5 stages were exposed to PTAT for 90 minutes or incubated at atmospheric pressure, then were chilled on ice for 24h (PTAT chilled and CTRL chilled, respectively). No viable control embryo in the CTRL chilled group was found after 19 dpf in any of our experiments. PTAT preconditioning was statistically significant for fish survival (P<0.0001).

Embryo morphology was evaluated 10 days after fertilization by registering normal morphology, the occurrence of tail abnormalities and pericardial edema. More morphologically normal embryos were detected in the PTAT chilled group than in the Control chilled group (42.5 ± 23.7% vs. 22.1 ± 14.3% normal morphology; P = 0.1919; **Figure 20**). By 30 dpf, 66.7 ± 19.2% of PTAT chilled individuals showed normal morphology.

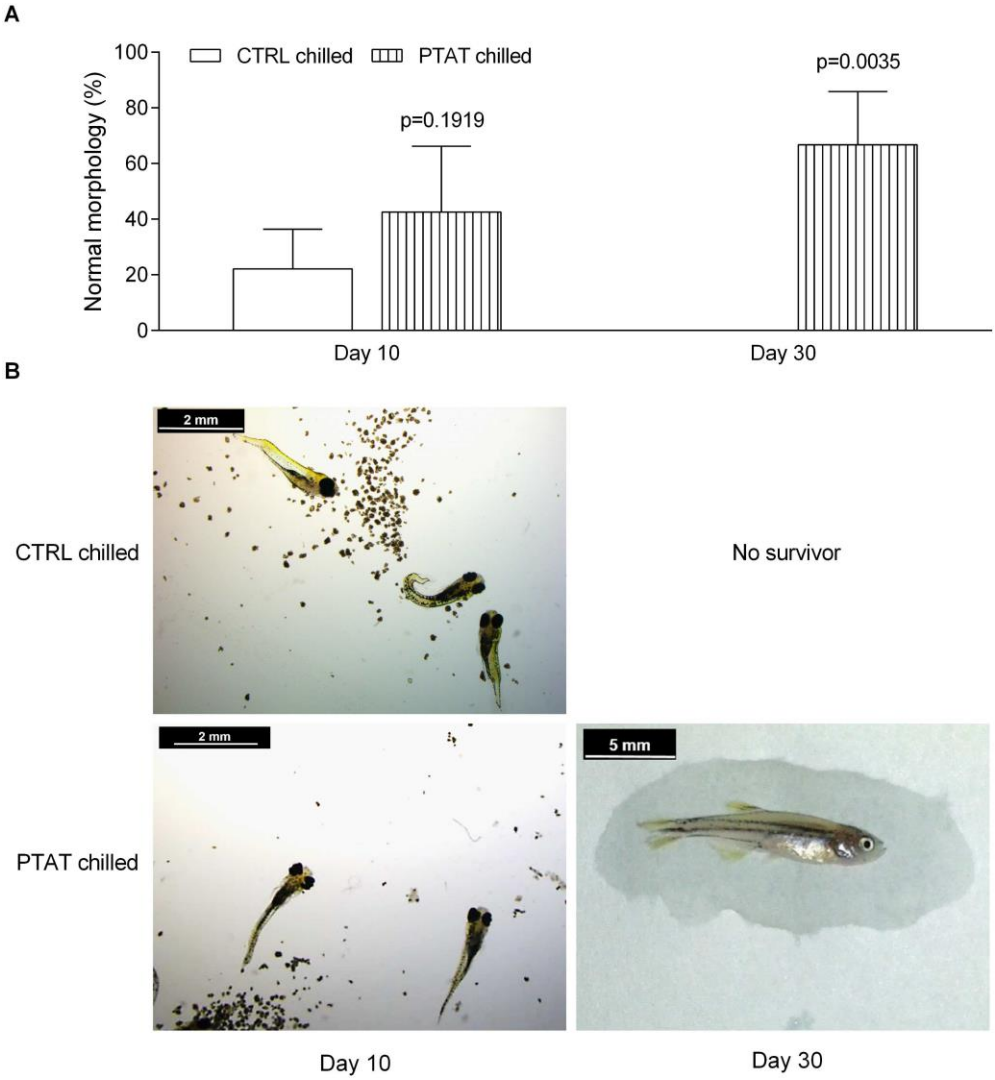

Figure 20. PTAT treatment improves the ratio of fish with normal morphology after 24 h chilled storage. Larval/fish morphology was evaluated on 10 dpf and 30 dpf. (**A**) Mean values (± SEM) of ratio of morphologically normal larvae/fish per experiments (four repetitions) are presented. Remarkably higher percentage of morphologically normal larvae/fish was detected in the PTAT-treated chilled group, compared to the untreated chilled controls. No viable fish was found in the Control chilled groups on 30 dpf. (**B**) Illustration of morphological abnormalities detected on 10 and 30 dpf.

5.3.2.2. Experiment II.: Embryo development to maturity and ability to produce healthy offspring after PTAT treatment and chilled storage

Knowing that PTAT preconditioning was successful, we next hypothesized that this treatment would enable fish to reach maturity and produce healthy offspring.

In the second experimental phase, all fish in the Control chilled group died by 30 dpf (as in Exp. I), whereas the PTAT chilled group grew to maturity. Thus, fertility results, viability and morphology of the offspring were compared to the Unchilled control and PTAT groups. The two unchilled groups and the PTAT chilled group didn't differ in terms of fertilization rates (77.8 ± 2.9% for Control, 73.5 ± 4.0% for PTAT and 67.2 ± 7.3% for PTAT chilled group; **Figure 21A**), and the viability of offspring on 10 dpf (88.1 ± 2.2% for Control, 87.3 ± 2.8% for PTAT,

and 78.1 ± 4.3% for PTAT chilled group; **Figure 21B**). Larvae with normal morphology on 10 dpf were similar in all groups (data not shown).

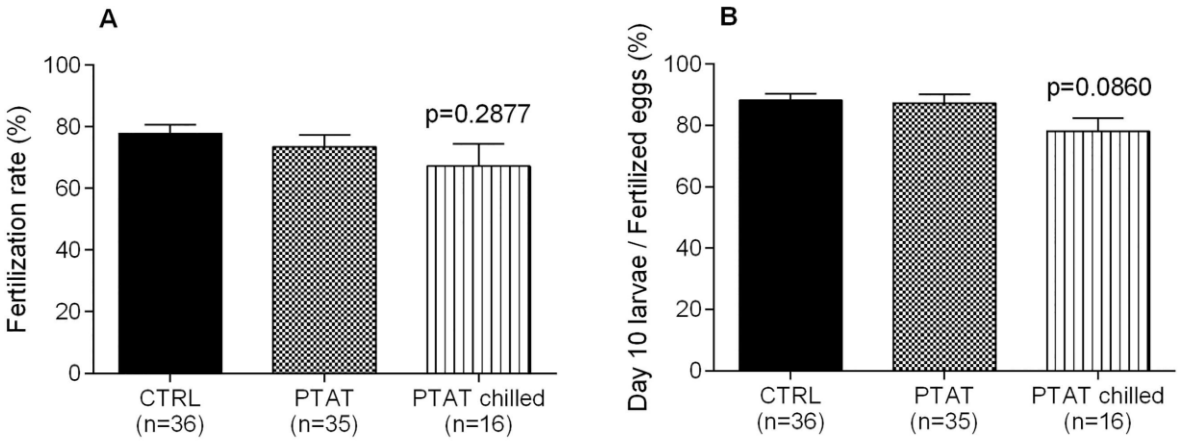

Figure 21. PTAT helps to preserve fertility potential of chilled embryos. (**A**) Average fertilizing capacity of adults developed from embryos chilled after PTAT (PTAT chilled), compared to the Unchilled control (CTRL) and unchilled PTAT (PTAT) groups. The fertilization rates are comparable. (**B**) Percentage of viable offspring on 10 dpf. The offspring of fish derived from PTAT treated chilled embryos develop in the normal pace and have normal morphology. Mean values (± SEM) of four replicates are presented.

5.4. Study 4 – Effect of PTAT on developmental potential of vitrified mouse oocytes

5.4.1. Experimental design

As described in Study 1 and Study 2, the optimal PTAT treatment parameters have been successfully determined for mouse oocytes, and it have been proved that they can survive and develop normally following vitrification and parthenogenetic activation. In the present experiments mouse oocytes were vitrified, warmed, *in vitro* fertilized and cultured till the blastocyst stage following which they were transferred or used to define cell number. (**Figure 22**).

5.4.1.1. Experiment I.: Developmental competence

In this experiment PTAT-treated and Control mouse oocytes were vitrified, warmed, fertilized by intracytoplasmic sperm injection (ICSI), and *in vitro* cultured until blastocyst stage. Post-vitrification and post-fertilization survival rates, ratio of embryos reaching 2-cell and blastocyst stages were used as endpoints for the investigation.

5.4.1.2. Experiment II.: Blastocyst cell number

In this phase of the experiment PTAT-treated and Control mouse oocytes were vitrified, ICSI-fertilized and *in vitro* cultured until reaching the blastocyst stage. Then, the total cell number and inner cell mass (ICM) cell number of the embryos were determined.

5.4.1.3. Experiment III.: Embryo transfer and evaluation of PTAT's long term-effects in the next generation

In this experiment PTAT's long-term effects were tested, together with the procedures described in this Study: oocyte vitrification, *in vitro* fertilization by ICSI, and *in vitro* culture. The aim of this experiment was to check whether the offspring originating from the PTAT-treated, vitrified, and fertilized oocytes are able to develop normally until birth, after reaching maturity to reproduce normally, and after producing offspring to have normal lifespan in both the parent and the offspring generation.

For this purpose, PTAT-treated, vitrified oocytes were ICSI-fertilized, then cultured *in vitro* until two-cell stage. Embryos reaching the two-cell stage were transferred to pseudopregnant recipients. The first endpoint of the experiment was birth rate following transfer. Then, mouse pups were reared until reaching maturity, and mated with each other. The second endpoint was ability to produce healthy offspring, and as third endpoint lifespan of the original individuals was investigated.

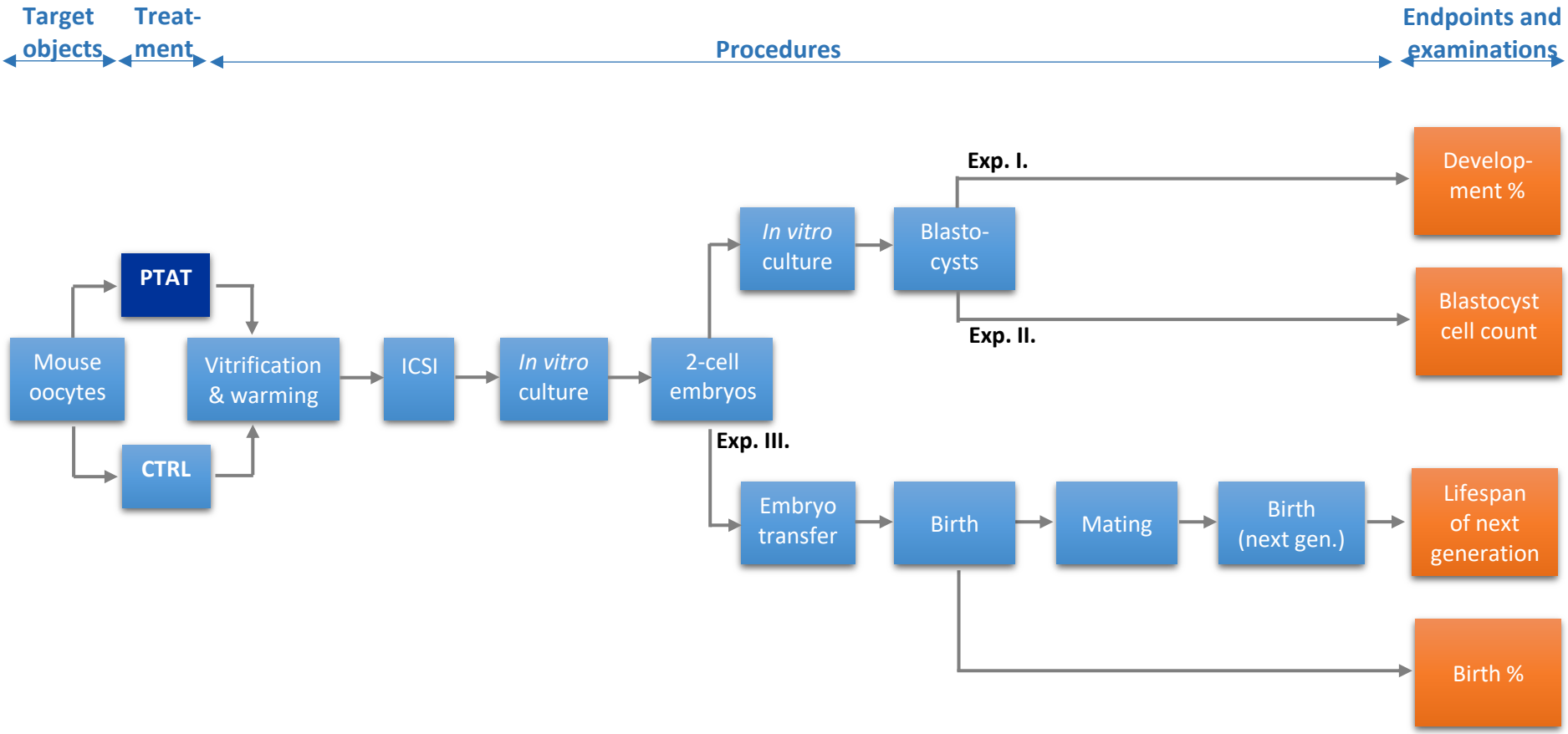

Figure 22. Experimental design of Study 4.

5.4.2. Results

5.4.2.1. Experiment I.: Developmental competence

In this experiment PTAT-treated and Control oocytes were vitrified, warmed, fertilized by intracytoplasmic sperm injection (ICSI), and *in vitro* cultured until blastocyst stage. Post-warming survival rates of PTAT-treated and non-treated mouse oocytes were similar between the groups (83% vs. 79% in PTAT vs. Control), but cleavage and blastocyst rates were significantly higher (70% vs. 62% in PTAT vs. Control, respectively) in the oocytes that were PTAT treated before vitrification (**Table 4**).

Table 4. *In vitro* continued development of oocytes with or without PTAT treatment after vitrification/ warming, ICSI and IVC

	No. of oocytes	No. of oocytes survived vitrification	No. of oocytes survived ICSI	No. of 2-cell stage embryos	No. of blastocysts per fertilized oocyte
Control	272	208 (79%)	135 (65%)	84 (62%)	68 (50%)
PTAT treated	292	235 (83%)	167 (71%)	117 (70%)	103 (62%)

5.4.2.2. Experiment II.: Blastocyst cell number

In the second experiment blastocysts' total cell number and inner cell mass (ICM) cell number was determined with whole-mount immunostaining. ICM cells were identified by their OCT4 expression, which was detected by their red (Alexa fluor 594) fluorescence. Total cells were identified by their blue fluorescence caused by DAPI, which binds to the DNA of the cells in the blastocyst (**Image 5**).

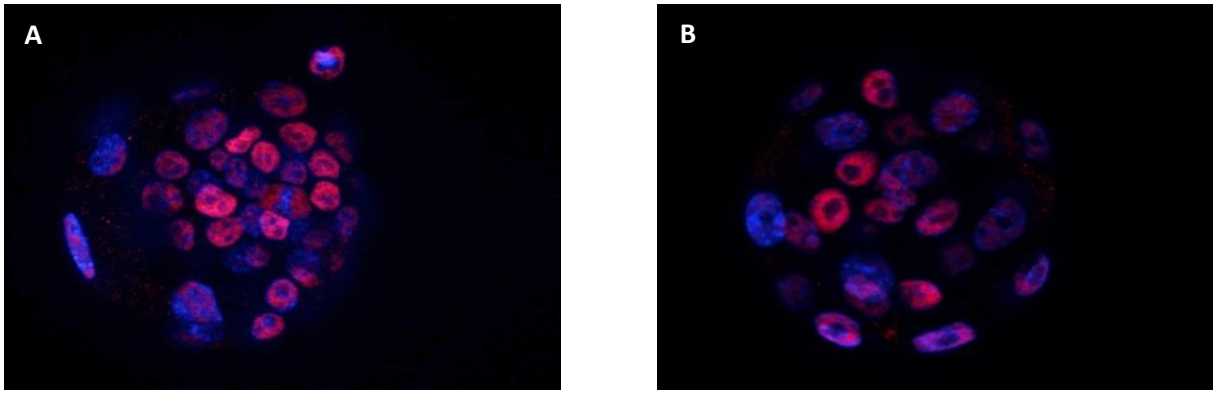

Image 5. Images of blastocysts showing OCT4 (red fluorescence) and DAPI positive (blue fluorescence) blastomeres. (**A**) PTAT treated blastocyst; (**B**) Control blastocyst

The assessment for cell number counting was made using digital images on different focal planes. The cells presented in different focuses were counted and summarized. Blastocyst ICM cell number was significantly higher in the blastocysts developing from oocytes that were PTAT treated before vitrification (50.9 vs. 45.9 in PTAT vs. Control; **Table 5**).

Table 5. Cell number of blastocyst from PTAT treated / not treated ICSI fertilized mouse oocytes

	No. of blastocysts used for cell count	No. of OCT4 positive cells /ICM cells/ (SD)	No. of DAPI cells /total cells/ (SD)
Control	23	17.2 (3.8)	45.9 (12.9)
PTAT treated	23	21 (4.9)	50.9 (12.5)

5.4.2.3. Experiment III.: Embryo transfer and evaluation of PTAT's long term-effects in the next generation

In the third phase of this study PTAT-treated and Control oocytes were vitrified, warmed, fertilized by intracytoplasmic sperm injection (ICSI), and *in vitro* cultured until two-cell stage. Then embryos were transferred into pseudopregnant recipients' uterus.

Post-warming survival rate of PTAT-treated and Control mouse oocytes was similar between the groups (86% vs. 83% in PTAT vs. Control), but cleavage rates, and, most importantly, birth rates were significantly higher (73% vs. 68%, and 27% vs. 15% in PTAT vs. Control, respectively) in the oocytes that were PTAT treated before vitrification (**Table 6**).

Table 6. Continued *in vitro* development and fertility data of embryos coming from treated / not treated vitrified-warmed ICSI fertilized mouse oocytes

	No. of oocytes	No. of oocytes survived vitrification	No of oocytes survived ICSI	No. of 2-cell stage embyos	Transferred	No. of pups born
Control	207	172 (83%)	117 (68%)	67 (57%)	67	10 (15%)
PTAT treated	220	190 (86%)	139 (73%)	102 (73%)	101	27 (27%)

Following the birth of mouse pups originating from the PTAT-treated and Control oocytes, pups were reared until reaching maturity, and mated with each other. Lifespan of the original individuals developing from the treated oocytes, and their offspring's fitness was investigated (**Image 6**). Lifetime, fitness and reproductive performance of the two groups were similar and comparable to the physiological data.

Image 6. Examples of offspring from the different groups. Mouse pups originate from (**A**) PTAT treated, (**B**) Control oocytes. Oocytes were vitrified/warmed, ICSI-fertilized, *in vitro* cultured until 2-cell stage, then transferred to pseudopregnant recipients.

5.5. Study 5 – Investigation of PTAT's effects on gene expression

The aim of the present study was to analyze the impact of PTAT treatment during mouse early preimplantation development using gene expression microarrays. We studied murine oocytes and 4-cell embryos derived from treated oocytes to evaluate the PTAT-induced transcriptional response after embryonic genome activation (EGA).

5.5.1. Experimental design

To achieve a comprehensive, genome-scale analysis of the PTAT's effect, we performed transcriptome profiling experiments on PTAT-treated mouse oocytes. The differences between the gene-expression profiles were examined on untreated and PTAT-treated mouse oocytes at two different stages (**Figure 23**) in Experiment I and II.

5.5.1.1. Experiment I.: Gene expression profile of PTAT-treated oocytes, at oocyte stage

After the PTAT program was completed, oocytes were frozen at -80°C to be fixated in this developmental stage for the gene expression investigation.

5.5.1.2. Experiment II.: Gene expression profile of PTAT-treated oocytes, followed by *in vitro* fertilization and culture until 4-cell stage

After the PTAT program oocytes were fertilized by ICSI and cultured *in vitro* for two days, until reaching the 4-cell stage. Fertilization and *in vitro* culture were performed as described in the Materials and Methods. After reaching the 4-cell stage, these embryos were also fixated at -80°C.

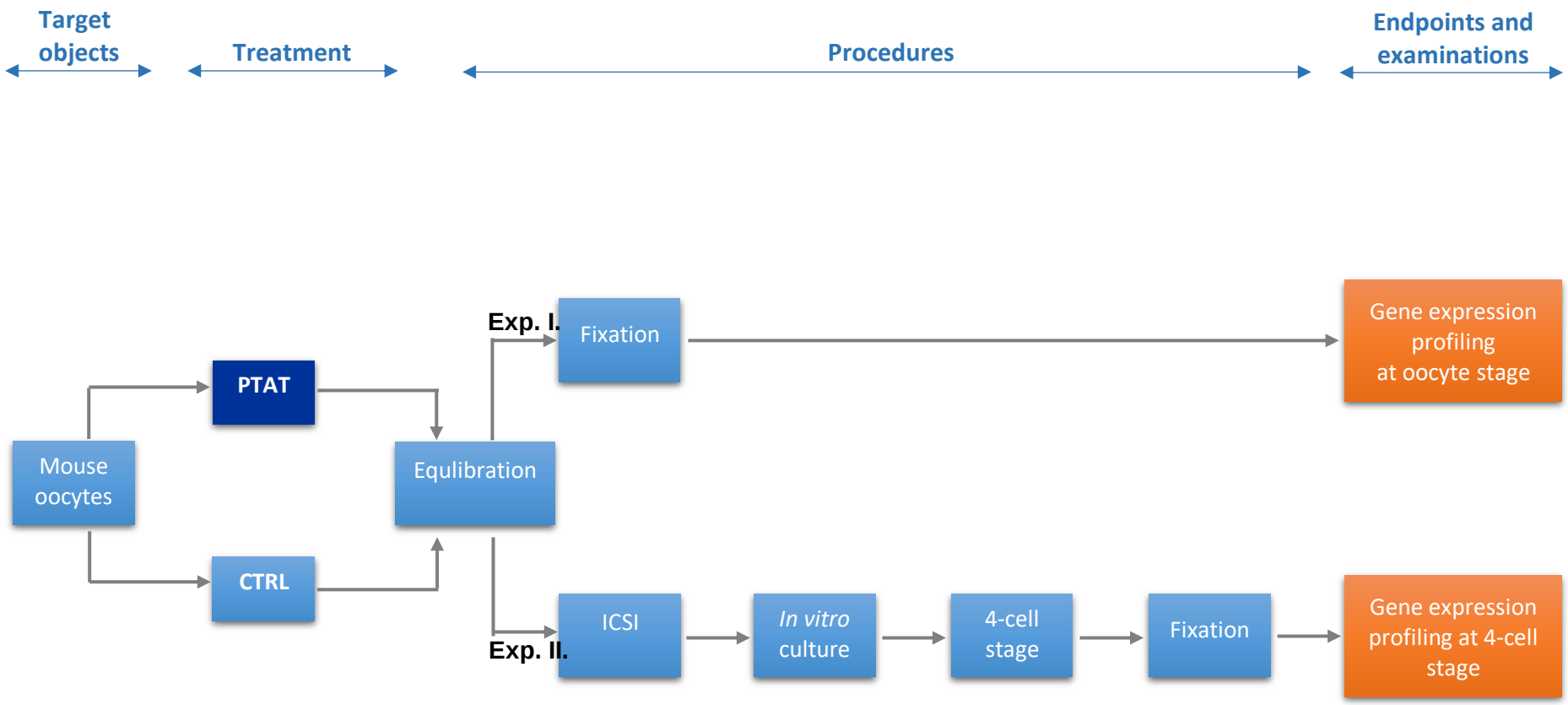

Figure 23. Experimental design of Study 5.

5.5.2. Results

To achieve a comprehensive, transcriptome-scale analysis of the effect of PTAT, we performed global gene expression profiling experiments on untreated or PTAT-treated mouse oocytes, and 4-cell embryos that were developed from these oocytes following ICSI-fertilisation and culture.

Gene expression array was performed in oocyte stage in four biological replicates of 50 PTAT-treated and 50 control oocytes. For the investigation of the 4-cell stage embryos' gene expression 153 control and 170 PTAT-treated oocytes were used in four replicates, out of which 82 control and 100 PTAT-treated 4-cell stage embryos developed (53% and 58%, respectively).

To verify the results obtained from the microarray analysis, RT-qPCR was performed on 20 selected genes (twelve genes for the oocyte investigations, and eight genes for the 4-cell embryos).

5.5.2.1. Experiment I: Gene expression changes in PTAT-treated oocytes

Based on the microarray results validated by RT-qPCR, no significant changes were observed at the oocyte level following PTAT treatment. These results along with the high RNA Integrity Numbers (8.7-9.2) of the RNA isolated from PTAT-treated oocytes, indicated that the optimally adjusted PTAT treatment did not induce global RNA degradation.

5.5.2.2. Experiment II: Gene expression changes in 4-cell embryos developing from the PTAT-treated oocytes

In contrast, at the four-cell stage, 250 upregulated and 255 downregulated genes ($P < 0.05$; fold change ≥ 1.5) were identified by asymptotic t-tests, without multiple correction. The most significant 25 upregulated and downregulated genes are listed in **Table 7** and **Table 8**.

Table 7. The top 25 upregulated genes detected in 4-cell embryos in response to PTAT. Abbreviations: SPRY, SP1A kinase of Dictyostelium and rabbit Ryanodine receptor; SMEK, suppressor of mek1; RUN, RPIP8/UNC-14/NESCA; ATM, ataxia telangiectasia mutated; DEAH, Asp-Glu-Ala-His; MEK1, mitogen-activated protein kinase 1; IQ, Ile-Gln; DCN1, defective in cullin neddylation.

Gene symbol	Entrez GeneID	P-value	Fold change	Description
1110004E09Rik	68001	0.00007	2.25	RIKEN cDNA 1110004E09 gene
Klk1b24	16617	0.00016	2.00	Kallikrein 1-related peptidase b24
Pgd	110208	0.00020	2.24	Phosphogluconate dehydrogenase
Eva1b	230752	0.00041	2.62	Eva-1 homologue B
Gadd45a	13197	0.00095	1.98	Growth arrest and DNA-damage-inducible 45 a
Cdca5	67849	0.00097	1.73	Cell division cycle associated 5
Dcun1d1	114893	0.00125	6.23	DCN1, defective in cullin neddylation 1, domain containing 1
Phlda2	22113	0.00188	2.02	Pleckstrin homology-like domain, family A, member 2
Iqce	74239	0.00213	1.79	IQ motif containing E
Gmfb	63985	0.00236	3.15	Glia maturation factor, b
Mpeg1	17476	0.00288	1.82	Macrophage expressed gene 1
Epc2	227867	0.00308	1.51	Enhancer of polycomb homologue 2
Ccdc85b	240514	0.00347	2.69	Coiled-coil domain containing 85B
Dhx33	216877	0.00350	2.04	DEAH (Asp-Glu-Ala-His) box polypeptide 33
Trim3	55992	0.00358	1.63	Tripartite motif-containing 3
Rad17	19356	0.00390	1.66	RAD17 homologue
Atmin	234776	0.00393	2.07	ATM interactor
Fbxo30	71865	0.00474	1.50	F-Box protein 30
Stat3	20848	0.00578	1.79	Signal transducer and activator of transcription 3
Plekhm1	353047	0.00635	1.52	Pleckstrin homology domain containing, family M (with RUN domain) member 1
Kif5c	16574	0.00644	2.59	Kinesin family member 5C
Smek1	68734	0.00650	1.69	SMEK homologue 1, suppressor of mek1 (Dictyostelium)
Spata5	57815	0.00661	1.70	Spermatogenesis associated 5
Bspry	192120	0.00665	1.78	B-Box and SPRY domain containing
Metap1	75624	0.00679	1.67	Methionyl aminopeptidase 1

Table 8. The top 25 downregulated genes detected in 4-cell embryos in response to PTAT. Abbreviations: FBR-MuSV, Finkel–Biskis–Reilly murine sarcoma virus; ER, endoplasmic reticulum; PHD, plant homeodomain.

Gene symbol	Entrez GeneID	P-value	Fold change	Description
Uqcr11	66594	0.00005	1.68	Ubiquinol-cytochrome c reductase, complex III subunit XI
Fam159b	77803	0.00046	4.16	Family with sequence similarity 159, member B
Fam65c	69553	0.00059	2.06	Family with sequence similarity 65, member C
Pcnt	18541	0.00064	2.36	Pericentrin
Mastl	67121	0.00100	2.62	Microtubule associated serine/threonine kinase-like
Fbxo6	50762	0.00121	1.58	F-Box protein 6
Acot13	66834	0.00124	3.97	Acyl-CoA thioesterase 13
Bptf	207165	0.00127	1.89	Bromodomain PHD finger transcription factor
Rpl34	68436	0.00178	1.79	Ribosomal protein L34
Emc10	69683	0.00191	6.47	ER membrane protein complex subunit 10
Gm6905	628696	0.00199	1.95	PREDICTED: similar to ribosomal protein L11
Fau	14109	0.00200	1.76	FBR-MuSV ubiquitously expressed (fox derived)
Rnaseh2b	67153	0.00223	2.44	Ribonuclease H2, subunit B
Mrps18c	68735	0.00225	2.03	Mitochondrial ribosomal protein S18C
Rps16	20055	0.00234	1.81	Ribosomal protein S16
Coa6	67892	0.00285	1.94	Cytochrome c oxidase assembly factor 6
Cebpz	12607	0.00290	2.47	CCAAT/enhancer binding protein zeta
Rps25	75617	0.00348	1.66	Ribosomal protein S25
Gon4 L	76022	0.00373	2.87	Gon-4-like
Sdhd	66925	0.00374	4.77	Succinate dehydrogenase complex, subunit D, integral membrane protein
Lztr1	66863	0.00400	2.54	Leucine-zipper-like transcriptional regulator, 1
Anapc13	69010	0.00407	2.24	Anaphase promoting complex subunit 13
Hypk	67693	0.00416	1.77	Huntingtin interacting protein K
Tmem145	330485	0.00433	1.83	Transmembrane protein 145
Lrrc58	320184	0.00443	1.92	Leucine rich repeat containing 58

RT-qPCR is the gold standard for accurate and sensitive gene expression analysis (Hellemans and Vandesompele 2011) and it is frequently used as a validation tool to confirm microarray gene expression results (Morey et al., 2006). For each of the eight genes selected for RT-qPCR in the 4-cell stage, the direction of change detected by RT-qPCR agreed with the microarray analysis ($P \leq 0.015$; **Figure 24**), confirming the validity of the microarray data (Pearson correlation coefficient $r = 0.93$).

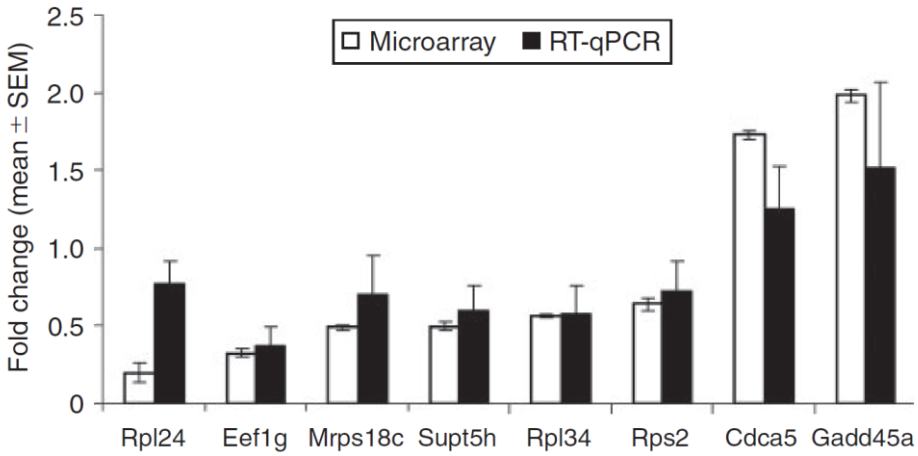

Figure 24. RT-qPCR verification of microarray data of 4-cell embryos developed from oocytes after intracytoplasmic sperm injection. Relative expression of selected genes in PTAT-treated samples compared with untreated controls is given as the mean ± s.e.m. of three biological replicates. The direction of change detected by RT-qPCR was consistent with the results of microarray analysis ($P \leq 0.015$).

We then analysed the function of the differentially expressed genes by identifying the affected pathways using the functional annotation tools of DAVID Bioinformatics Resources. We found 21 Gene Ontology (GO) categories to be significantly over-represented, mostly in the Cellular Component group (**Table 9**).

Table 9. Functional annotation chart showing the over-represented Gene Ontology (GO) categories of the significantly changed genes at the 4-cell stage identified by functional annotation tools of DAVID Bioinformatics Resources. The multiple test corrected P-values are shown in the Benjamini column. The percentage of downregulated genes represents the number of downregulated genes compared with all the regulated genes for a particular term. Ratios higher than 90% are bolded.

Category	GO term	Benjamini	No. up-regulated genes	No. down-regulated genes	% Down-regulated genes
Biological process					
	Translation	1.00×10^{-11}	2	35	**95**
Molecular function					
	Structural constituent of ribosome	2.20×10^{-19}	1	32	**97**
	Structural molecule activity	1.10×10^{-9}	5	34	87
	RNA binding	6.00×10^{-3}	7	27	79
	Nucleotide binding	8.30×10^{-3}	47	31	40
Cellular component					
	Ribosome	1.60×10^{-21}	1	37	**97**
	Ribonucleoprotein complex	2.60×10^{-18}	3	48	**94**
	Non-membrane-bounded organelle	5.30×10^{-8}	18	68	79
	Intracellular non-membrane-bounded organelle	5.30×10^{-8}	18	68	79
	Ribosomal subunit	2.10×10^{-5}	0	12	**100**
	Small ribosomal subunit	2.10×10^{-3}	0	7	**100**
	Cytosolic ribosome	2.50×10^{-3}	0	6	**100**
	Nucleolus	4.30×10^{-3}	6	14	70
	Cytosolic large ribosomal subunit	1.40×10^{-2}	0	4	**100**
	Mitochondrion	2.50×10^{-2}	17	32	65
	Membrane-enclosed lumen	2.70×10^{-2}	14	31	69
	Cytosol	2.70×10^{-2}	6	20	77
	Nuclear lumen	2.70×10^{-2}	11	25	69
	Organelle lumen	2.90×10^{-2}	14	29	67
	Intracellular organelle lumen	3.00×10^{-2}	14	29	67
	Large ribosomal subunit	3.70×10^{-2}	0	6	**100**

'Translation' was the only identified GO biological process, showing an extremely low multiple test corrected P-value. Consistent with this result, 'structural constituent of the ribosome' ($P \leq 2.20 \times 10^{-19}$) and 'ribosome' ($P \leq 1.60 \times 10^{-21}$) were the highest significantly enriched Molecular

Function and Cellular Component categories, respectively. Several additional translation-related GO categories were enriched in genes with altered expression, suggesting that protein synthesis was markedly affected by PTAT treatment. KEGG analysis further supported this notion because the 'ribosome' was the only significant pathway identified. Furthermore, functional characterization revealed that the over-represented categories contained mostly downregulated genes. The highest percentage (90%–100%) of downregulated genes was observed in the translation related categories (**Table 9**). Overall, the analysis strongly suggests that protein synthesis was inhibited in the 4 cell stage embryos developed from PTAT-treated oocytes.

6. DISCUSSION

As a general statement from the pioneering studies PTAT treatment positively affected efficiency of assisted reproductive technologies when applied throughout preimplantation development, from the gamete to the blastocyst (reviewed in Pribenszky et al., 2010b, Pribenszky and Vajta, 2011), suggesting that the treatment may regulate gene expression at an early stage. Indeed, recent studies confirmed the findings and showed that PTAT treatment improved the quality of the treated gametes or embryos, and altered the expression of several genes.

6.1. Study 1 – Stress tolerance tests

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 (reviewed in Pribenszky et al., 2010b, Pribenszky and Vajta, 2011). 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. It has been examined if stage of development and the type of cell culture affect stress tolerance (~treatment parameters). The fine-tuning of protocols according to specific cell types and developmental stages is a very important step in order to define the most effective treatment of cells to be used before cryopreservation. The treatment of each of the referred cell types in the present study (except to mouse two-cell stage embryos, where cryopreservation experiments have not yet started) resulted in significantly increased cryosurvival (reviewed by Pribenszky et al., 2010b, 2012, and Pribenszky and Vajta, 2011).

Our own stress tolerance studies have shown, supporting previous experiences that the species and the stage of development affects the stress tolerance to hydrostatic pressure treatment. Zebrafish embryos' stress tolerance limit is around 40 MPa, however embryos 48 hours post fertilization were more resistant compared to 4 or 24 hours old embryos. Mouse oocytes survived and developed to blastocysts following parthenogenetic activation after 10, 20, and 40 MPa pressure treatments. Two cell stage mouse embryos treated with 40, 60 or 80 MPa for 120 min had a significantly decreased blastocyst formation rate compared to the Control group. There was a reversible morphological change following the treatments of the two-cell stage mouse embryos: increasing the magnitude of the pressure, the size of the blastomeres has gradually reduced by 8 to 25%. Impacts greater than 60 MPa may cause irreversible damage to the two-cell stage embryos including blastomere and membrane injuries. Mouse blastocysts, both *in vitro* cultured and *in vivo* derived, tolerated hydrostatic

pressure stress better than the two-cell stage embryos. 80 MPa/120 min treatment of the blastocysts proved to yield lower hatching rates than the rest of the groups, though this difference was still not significant. Depending on the magnitude of the hydrostatic pressure impact, expanded blastocysts responded with the collapse of the blastocoel, as it was described earlier (Pribenszky et al., 2005a). Both *in vivo* derived and *in vitro* cultured blastocysts collapsed depending on the treatment, though the reaction to pressure with the collapse of the blastocoel occurred at higher pressure in the *in vivo* group (at 60 MPa treatments all of the blastocysts exhibited a reversible collapse of the blastocoel in the *in vitro* cultured group, though only approximately half of them collapsed in the *in vivo* group). This might represent that *in vitro* culture conditions result in blastocysts being more sensitive to an environmental stress. The reversible collapse of the blastocoel to high hydrostatic pressure has been described in bovine blastocysts (Siqueira et al., 2011) and porcine blastocysts as well, and was affected by the treatment temperature (Pribenszky et al., unpublished results). According to previous results, the treatment temperature may affect the outcome of the treatment in pig. The treatment of porcine oocytes at body temperature resulted in higher blastocyst rate after cryopreservation and parthenogenetic activation compared to treatment at ambient temperature (Du et al., 2008b). Similarly, boar semen treated at body temperature yielded significantly higher cell survival rate compared to the outcome of the room temperature treatments (Pribenszky et al., 2006), though, at both experiments room temperature treatments also increased cell survival compared to untreated controls. The temperature of the PTAT treatment has to be adjusted to the state-of-the-art temperature management of the cryopreservation processes (e.g., if semen sample was cooled down to room temperature, then PTAT treatment shall not be done at 37°C). Furthermore, species specific differences might be important, as porcine zygotes and embryos are known for their extreme chilling sensitivity. Compared to other cells or tissues, the stress tolerance of semen or oocytes have been reported to be lower than that of the two-cell stage mouse embryos (Pribenszky et al., 2007, 2008, 2009).

6.2. Study 2 – Evaluation of the stress treatment's long-term effects

Following the stress tolerance studies the next step is to prove that the preconditioning procedure is free from long-term harmful effects.

In vivo derived mouse blastocysts treated with 60 MPa/60 min were transferred to recipients. The offspring were normal, as controls, no difference in sex ratio and malformations occurred. The original and the next generations originating from the control and treated embryos were similar in survival, sex ratio and the occurrence of cannibalism. Similarly, fish embryos treated with 50 MPa for 90 minutes 24 hours post fertilization developed into normal fish.

These findings support other studies, where the birth of approximately 400 piglets from PTAT treated fresh semen (Pribenszky et al., 2009) and 167 piglets from PTAT treated frozen/thawed semen (Kuo et al., 2007) did not differ in sex ratio, abortion and malformations from that of the controls. Horváth et al. (2016) also reported birth of healthy piglets with normal birth weight and weaning weight following artificial inseminations of sows with PTAT-treated and cryopreserved boar semen.

Healthy piglets were born from PTAT treated, enucleated somatic cell transferred oocytes as well (Du et al., 2008b). These studies confer that treating gametes and embryos with sublethal pressure stress may result in healthy offspring.

6.3. Study 3 – Zebrafish embryo chilled storage

The only promising way to preserve zebrafish embryos despite all attempts, is the cryopreservation of isolated blastomeres. However, these cells do not develop after thawing, therefore germ-line chimerism was a suggested method to preserve embryonic genetic material (Cardona-Costa and García-Ximénez, 2007).

As cryopreservation trials failed, most of the experiments focuses on developing cold- or chilled storage methods, however this area needs major developments as well. Lahnsteiner tested different temperature and exposure times at different stages of embryo development (Lahnsteiner et al., 2009). The maximal survival rate detected was from the Germ-ring stage to the Prim-25 stage after short (60–180 min) exposure at 4°C–8°C (above 70%). However, 5-somite stage embryos chilled at 1°C became nonviable within 6 hours.

A remarkable improvement was reported by Desai and his colleagues (2015a). They tested the impact of chilling on 50% epiboly stage embryos exposed to 0°C for different times using methanol as a cryoprotectant. Survival was evaluated in terms of the hatching rate. As was shown, 3–6 hours of chilling resulted in no significant differences in hatching rates (over 85%), while 18 and 24 hours of exposure, even with cryoprotectant, significantly decreased the hatching rates to less than 10% (Desai et al., 2015a).

Another approach is, instead of fine-tuning the various steps of the chilling or cryopreservation procedure, to apply a preconditioning treatment to the embryos before chilling.

In order to prove the beneficial effect of the PTAT preconditioning, we tested the technology on a single selected developmental stage. Regarding that former results published by Liu et al. (1998) and Lahnsteiner et al. (2009) showed the highest chilling tolerance around 24 hour-post-fertilization stage, our chilling experiments were performed on embryos at this stage of development.

In Study 1 the optimal pressure treatment has been defined, then in Study 2 PTAT it has been proved that the stress treatment did not have any long-term harmful effects in zebrafish embryos. Furthermore, in Study 3 the experiments showed the efficiency of PTAT preconditioning to support zebrafish embryo survival and continued development after chilled storage (Experiment I), and the long-term effects from pretreatment reaching maturity as well as having offspring after the chilled storage (Experiment II).

As shown in Experiment I, PTAT preconditioning significantly improved chilling tolerance of 26-somites or Prim-5 stage zebrafish embryos. Our results demonstrate that embryos chilled without PTAT pretreatment are able to hatch; however, none of the untreated controls survived beyond 19 day-post-fertilization, while several in the PTAT-preconditioned group grew past 30 dpf. Moreover, the beneficial effects of the PTAT treatment were noticeable in the increased ratio of larvae with normal morphology.

In comparison, previous studies reported very limited chilling survival of embryos from zebrafish or other fish species even though they used either hatching (Ahammad et al, 1998, 2003a, 2003b, Desai et al., 2015b) or stages close to hatching (Beirao et al., 2006) as endpoints of the experiments. To our knowledge, the present study is the first to report that fish embryos survive until 30 dpf after 24 hours of chilled storage. These results indicated that PTAT preconditioning significantly improves fish embryo chilling tolerance and thus encouraged us to extend the examination period until maturity and to test spawning.

In the second experimental round (Experiment II), we investigated if PTAT-treated and chilled zebrafish embryos can develop to sexual maturity and produce viable offspring. Our findings suggest that not only does PTAT treatment improve chilling tolerance and enhance survival, but fish developed from PTAT-pretreated and chilled embryos preserve their fertility potential and are able to produce healthy offspring. Because offspring of fish developed from chilled embryos developed at a normal pace with normal morphology, PTAT is also a safe procedure regarding reproduction.

Currently, no direct information is available on the exact molecular mechanism of how PTAT preconditioning enhances the chilling tolerance of zebrafish embryos. However, the possible mechanism might be outlined based on scientific publications. As reported recently (Long et al., 2013), zebrafish embryos possess the ability to improve their cold tolerance: mild cold stress preconditioning (16°C for 24 hours) of 96-hours-post-fertilization embryos could significantly increase the survival rate of forthcoming severe cold stress (exposure to 12°C). Gene ontology enrichment analysis revealed that RNA processing, ribosome biogenesis and protein catabolic processes were the most highly overrepresented pathways after cold-induction. Parallel to these results, PTAT treatment of bovine embryos increased the recovery

rate of forthcoming vitrification accompanied by analogous gene expression changes: genes involved in RNA processing, cellular growth and proliferation were up-regulated, while genes belonging to cell death and apoptosis were down-regulated (Jiang et al., 2016). Increased gene expression of RNA processing and ribosome biogenesis processes indicates that embryos respond to the preconditioning and prepare cells for the upcoming detrimental procedures. Similarly, down-regulation of cell death or apoptotic processes, while up-regulating catabolic processes of denatured or misfolded proteins, can improve cellular integrity.

Based on the results of Study 3, PTAT preconditioning has a significant beneficial effect to enhance general resistance and developmental competence of chilled zebrafish embryos. Regarding that the embryonic development is arrested during chilled storage this technology provides a solution to synchronize or to delay the development for experimental purposes. Moreover, treatment enabled to create viable offspring, which is a unique and remarkable step forward. Further improvement of PTAT-chilling/cryopreservation procedures have the potential for application in zebrafish shipment and trade between laboratories as well as gene preservation.

6.4. Study 4 – Mouse oocyte vitrification

A series of experiments have been performed in the present study using mouse eggs and procedures that are common in the human *in vitro* fertilization laboratory practice such as vitrification, fertilization by ICSI, *in vitro* culture until the blastocyst stage, and embryo transfer. We have expanded the scope by adding further endpoints to examine the safety and efficacy of the PTAT treatment of oocytes before vitrification. Vitrified-warmed eggs were fertilized by ICSI using a Piezo-driven system (Kuretake et al. 1996), since the piezo technique is required to the efficient microinjection of warmed mouse eggs. The use of piezo-ICSI is different from that of the human routine, where piezo is only used occasionally, although its use is spreading.

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 ICM 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, ICSI 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.

Formerly published findings showed that controlled stress treatment of oocytes helped to improve their survival and continued *in vitro* development during *in vitro* maturation (Pribenszky et al., 2008), cryopreservation (Du et al., 2008a,b,c; Pribenszky et al., 2008, 2010a; Mátyás et al., 2010), enucleation and somatic cell nuclear transfer (SCNT; Du et al., 2008a,b). The effect of the stress treatment was more tangible as the *in vitro* (and *in vivo*) development progressed, showing that it is the function of the cell that is preserved, maintained and enhanced primarily. Similarly, hydrostatic pressure stress treatment of mouse oocytes before vitrification resulted in similar post-warming survival, 20–25% increase in blastocyst rate and blastocyst cell number, and more than doubled birth rates, compared with untreated controls.

Further studies were conducted to test the preconditioning effect of other stressors. Four types of stressors were published to precondition oocytes with various success. Experiments with hydrostatic pressure, osmotic and oxidative stresses all followed the same principle: first, stress treatment protocols were defined by stress tolerance studies aiming to assess a stress level that does not cause irreversible damage to the cells, but improve their survival rates after different *in vitro* manipulations. Second, protocols were refined and were compared with untreated controls to define improvement levels and consistency. All of these studies were based on the premise that the sublethal stress treatment triggered cellular stress reaction, which actually improved the cells' resistance and at the posttranscriptional level.

Stress treatments of oocytes were performed in human (hydrostatic pressure), mouse (hydrostatic pressure) and porcine (hydrostatic pressure, oxidative, osmotic and mechanic stresses) species in processes like vitrification (hydrostatic pressure, osmotic stresses), *in vitro* maturation (hydrostatic pressure, oxidative and mechanic stresses) and SCNT (hydrostatic pressure, osmotic and mechanic stresses) (Matyas et al., 2010; Pribenszky et al., 2008, 2010a; Du et al., 2008a,b,c; Vandaele et al., 2010; Lin et al., 2009a,b, Mizobe et al., 2010; Matsuura et al., 2010). Hydrostatic pressure stress treatment (PTAT) proved to be the most effective according to the increasing blastocyst rate and cell number of the blastocysts originating from treated oocytes. Offspring was born from PTAT-treated eggs both following cryopreservation, ICSI and *in vitro* culture, and following enucleation, SCNT and *in vitro* culture (Du et al., 2008b). Present study described that PTAT did not affect gene expression level at the oocyte stage, whereas it was significantly affected after the activation of the embryonic genome (see in details in Study 5). Although several studies reported negative effects of environmental stress, authors of the cited experiments fine-tuned various stress effects to precondition (or pretreat)

cells. As a conclusion, controlled, sublethal stress treatment of oocytes improved oocyte performance in these studies, suggesting a rather general approach of the principle. However, stress types differ significantly if consistency and effect size is regarded.

6.5. Study 5 – Gene expression

Our aim was to investigate the effect of sublethal stress induced by PTAT on the whole transcriptome during mammalian preimplantation development. We performed gene expression microarray experiments initially on matured oocytes to study their response immediately after the PTAT. From fertilization until the onset of embryonic genome activation, the early embryo has to rely on its maternally inherited RNA and proteins (Bachvarova and De Leon, 1980; Cascio and Wassarman, 1982), since stored cytoplasmic components of the sperm do not play a major role during embryogenesis (Sutovsky and Schatten, 2000). Therefore, we studied whether stress induced by PTAT treatment of oocytes affected RNA abundance, e.g. by inducing selective degradation (Sirard, 2012). Since the embryonic genome activation occurs at the 2-cell stage in mouse embryos, initiating *de novo* RNA synthesis (Bolton et al., 1984; Li et al., 2010), 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 ICSI.

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, and the high RIN (RNA integrity number) values of the RNAs isolated from the PTAT-treated oocytes indicated that the treatment did not induce RNA degradation. However, the same stress did result in a marked effect on transcription at the 4-cell stage, i.e. after the embryonic genome activation.

The results of the microarray experiments 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 (Pribenszky et al., 2010a). Moreover, when PTAT treatment of matured oocytes was followed by further manipulations (cryopreservation, enucleation followed by SCNT) with a 2 hours equilibration between the PTAT and other procedures, a substantially increased developmental rate, higher blastocyst cell number and higher birth rate of healthy offspring were recorded (reviewed in Pribenszky et al., 2012). Our results were further supported by observations in porcine oocytes, where >99.99 % of the cell's transcriptome remained unaffected after exposure to the same PTAT treatment as in our study (Lin et al., 2013). However, the distinct expression pattern

detected at the 4-cell stage embryos can be explained by species differences. Moreover, the ICSI-technique applied in this study is more similar to the *in vivo* conditions than parthenogenetic activation or hand-made cloning.

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. In eukaryotes, the ribosomes are composed of four types of ribosomal RNA molecules and of 79 different ribosomal proteins (r-proteins) (Wool, 1979; Nakao et al., 2004). In our study, we identified 37 downregulated along with 1 upregulated genes, which are components of the ribosome, indicating that numerous r-protein genes were transcriptionally inhibited by the treatment (**Table 9**). Overall, the robust repression suggests that the PTAT stress inhibited ribosome assembly and thus transiently reduced the rate of the protein synthesis during preimplantation development.

Our results are consistent with the well-known phenomenon in microbes, where high pressure induces ribosomal dissociation (Schulz et al., 1976; Gross and Jaenicke, 1990; Gross et al., 1993; Niven et al., 1999; Alpas et al., 2003), and is suggested to be one of the principal causes of pressure-induced growth inhibition (Gross et al., 1993). Nevertheless, it should be noted that in *E. coli* the pressure-induced ribosome disassembly is completely reversible using pressure below 100 MPa; after the pressure is released, protein synthesis is resumed (Mackey and Mañas, 2008). Similarly, the inactivation of rat liver ribosomes after PTAT treatment *in vitro* was reported which was almost completely reversible when pressure under 120 MPa was used (Lu et al., 1997). The sublethal limit of PTAT treatment for different cell types (60-80 MPa) is comparable to these values (Pribenszky et al., 2010b; Pribenszky and Vajta, 2011).

In studies reporting improved oocyte and embryo survival and function following preconditioning by PTAT treatment, the best effect was observed when cells were allowed to recover for 1 to 2 hours in conventional culture conditions before the initiation of vitrification or enucleation (reviewed in Pribenszky et al., 2010b; Pribenszky and Vajta, 2011). Based on these observations, our profiling data suggest that the transiently reduced protein synthesis is not a prerequisite for developmental competence, at least over a short period of time. This finding supports the observation that the cleavage of fertilized rabbit oocytes can be accomplished in the absence of significant ribosomal RNA synthesis (Manes, 1971). It is possible that precisely adjusted PTAT stress may transiently suspend translation and precondition cells for the subsequent stress, after which ribosome reassembly initiates and translation is continued. Notably, in bovine *in vitro* fertilized blastocysts, vitrification-induced stress is concomitant primarily with the significant downregulation of the translation pathway, suggesting that the protein synthesis is particularly important during freezing-stress (Aksu et

al., 2012). Similarly, in PTAT-treated and vitrified bovine blastocysts downregulation of genes in cell death and apoptosis, and upregulation of genes in RNA processing, cellular growth and proliferation was observed, parallel with the beneficial effects of PTAT. In contrast, higher, harmful pressure level caused upregulation of genes in apoptosis, and downregulation in protein folding and cell cycle-related genes (Jiang et al., 2016). Ribosome biosynthesis is the most energy-consuming process in eukaryotic cells (Warner, 1999; Warner et al., 2001). Consequently, the transient arrest upon PTAT treatment might result in intracellular energy saving. For preimplantation embryos kept under physiological conditions molecular processes are performed at a relatively high rate to allow them to continuously cleave. Therefore, when they encounter a major stress e.g. freezing, they have to substantially increase the rate of catabolism to adapt to the altered environment. This hypothesis is further supported by the finding that frozen-thawed human embryos arrested prior to the blastocyst stage are metabolically more active in terms of amino acid turnover than their developing counterparts (Stokes et al., 2007). We hypothesize that the higher developmental competence of the stress-treated oocytes was supported by reduced metabolism rate and the accumulation of metabolites. This notion is in agreement with the Quiet Embryo Hypothesis, where preimplantation embryos with lower overall metabolism are more viable (Leese et al., 2008; Leese, 2012).

In our study we present the first comprehensive analysis describing the cellular response of mouse oocytes to the PTAT stress, as a model for possible future application in human medicine. In conclusion, although the HP treatment did not perturb the maternal RNA pool of mouse oocytes, an indirect but significant effect of the treatment became apparent after the embryonic genome activation at the four-cell stage, revealing a transcriptional footprint of PTAT-induced genes. 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.

7. NEW SCIENTIFIC RESULTS

In the initial phase of the experiments stress tolerance of oocytes and embryos in various developmental stages of several species have been investigated. The 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 evaluation of the treatment's long-term effects, 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.

In the second phase, long-term effects of the selected stress treatments were evaluated. 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 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 long-term effects have also been evaluated.

Then we demonstrated that stress-treated – or in other words PTAT (Pressure Triggered Activation of Tolerance) treated – zebrafish embryos had a significantly higher survival and better developmental rate following chilled storage, moreover the treated group had a higher ratio of normal morphology during continued development. 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. These results indicate that 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 our 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.

In the next phase we proved that PTAT increased the post-thawing and post-fertilization survival rates of treated mouse oocytes, 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. In addition, blastocysts developing from PTAT-treated oocytes had significantly higher total cell numbers and ICM cell numbers. 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. These findings prove that PTAT treated oocytes have significantly increased resistance to cryoinjuries, and thus an improved developmental competence compared to untreated controls. The results of the present study 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.

In the final phase we presented the first comprehensive analysis describing the cellular response of mouse oocytes to PTAT stress, as a model for possible future application in human medicine. 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.

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