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**Minireview**

# Stress for Stress Tolerance? A Fundamentally New Approach in Mammalian Embryology[1]

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## ABSTRACT

**In vitro culture, storage, and manipulation of gametes and embryos require meticulously adjusted conditions to avoid or minimize the harmful effects of uncontrolled stress. However, recent work indicates that a well-defined and properly applied stress may induce general adaptation and increase tolerance to various in vitro procedures. The aim of this review is to summarize reports on the effects of stress on gametes and embryos of several species. Treatment with sublethal doses of high hydrostatic pressure (HHP), or osmotic, heat, or oxidative stress resulted in increased morphological survival, fertilizing ability, or developmental potential after various in vitro or in vivo procedures. HHP treatment of spermatozoa, oocytes, embryos, and embryonic stem cells increased fertilizing ability, developmental competence, and differentiation and improved results after cryopreservation, parthenogenetic activation, intracytoplasmic sperm injection, and somatic cell nuclear transfer. Osmotic stress of oocytes resulted in higher developmental rates after cryopreservation, parthenogenetic activation, and somatic cell nuclear transfer. Heat shock was reported to increase developmental competence of parthenogenetically activated oocytes. Although cellular and subcellular mechanisms supposedly contributing to these processes require further research, the new principle, i.e., to improve the stress tolerance by a defined sublethal stress, may outline a completely new strategy in mammalian embryology, as well as cryopreservation of other cells and tissues with remarkable theoretical and practical consequences.**

*embryo, hydrostatic pressure, ovum, sperm, stress*

## INTRODUCTION

The dominant approach in the fifty years' history of applied embryology in mammals has been a rather defensive one. The laboratory phase of the work is considered to be potentially dangerous for gametes and embryos [1, 2], and efforts were mostly focused on eliminating these dangers. However, current understanding of the in vivo processes is limited; several factors that seem to be important in vivo may arrest development in vitro, and our preparedness to apply even the known requirements is restricted by technical, financial, ethical, and legal limits [3, 4]. Accordingly, in spite of considerable worldwide efforts, embryos generated in vitro mostly have compromised physiology, gene expression, and development. The idea that a laboratory procedure may actually improve gamete performance and embryo quality, i.e., the establishment of an in vitro method that increases the general resistance, fertilizing ability, and/or developmental competence of gametes and embryos, if ever considered, is generally regarded as pure fantasy by most embryologists.

In the forthcoming chapters the establishment of an in vitro technique that increases the general resistance and performance of gametes, embryos, and stem cells is reported. The principle of this approach is to apply a sublethal stress for the cells in order to increase the subsequent stress tolerance, morphological intactness, fertilizing ability, and/or developmental competence. High hydrostatic pressure (HHP), used purposefully as a sublethal stressor first, is presented, followed by the discussion of studies using osmotic or oxidative stresses. Factors potentially contributing to this phenomenon will be discussed, and the future application possibilities will also be outlined.

## HIGH HYDROSTATIC PRESSURE

Applying sublethal stress treatment to gametes and embryos in order to improve their viability comes, interestingly, from the unexpected lessons of former food microbiology studies.

The use of high-pressure technology to preserve the quality of food products dates back as far as the early 1900s with the observations of Hite [5] on the extended shelf life and quality of milk and the sterilization of fruits. Pressures in the range of

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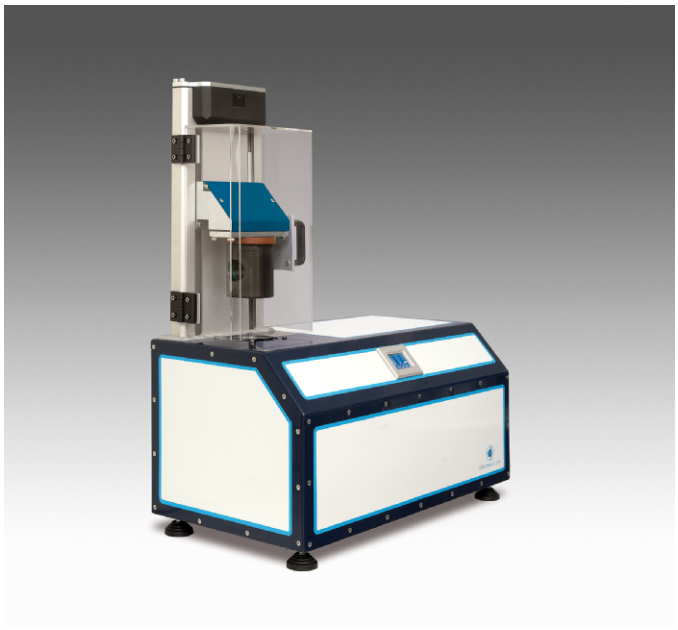

FIG. 1. A programmable hydrostatic pressure-generating device, HHP Machine 1400. The capacity of the chamber is 1400 cc, the range of use is between 1 MPa and 40 MPa. This device was used in the experiments with large sample volumes, e.g., with bull semen, boar semen, or fetal chord blood. The closing system is automatic, driven by electric engines. Photo courtesy of Cryo-Innovation Technologies.

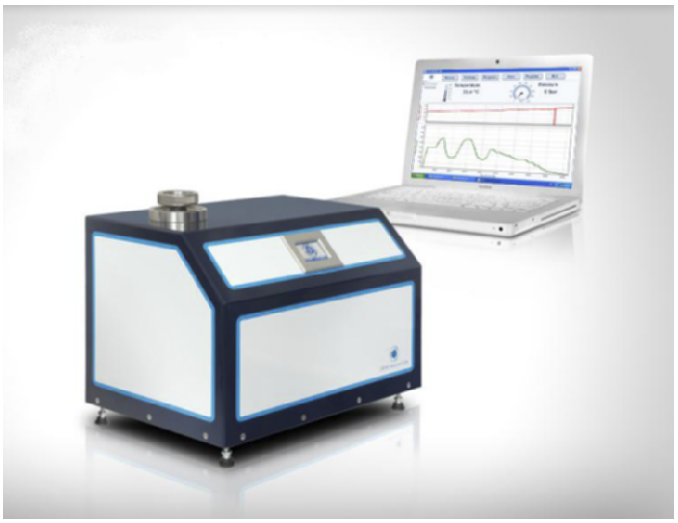

FIG. 2. A programmable hydrostatic pressure-generating device, HHP Machine 100. The capacity of the chamber is 100 cc, the range of use is between 2 MPa and 90 MPa. This device was used in the experiments with small sample volumes, e.g., with embryos, oocytes, or embryoid bodies. The closing system is manual. The custom-made software shows, records, and saves all treatment data and gives the option of designing special pressure profiles (e.g., oscillation). Photo courtesy of Cryo-Innovation Technologies.

400–650 MPa reduced the microbial load in foodstuffs, with minimal adverse effects on the product compared to other preservation 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. [6] have reported that the proliferation of *Listeria monocytogenes* was not decreased but significantly increased as a consequence of sequential treatment with cold shock and HHP. It appears that biological effects of the first sublethal treatment have preconditioned the bacteria, protecting them from the detrimental effects of the second sublethal treatment.

This observation eventually motivated research to apply sublethal stress in assisted reproduction to improve success rates of in vitro procedures such as cryopreservation, in vitro culture, somatic cell nuclear transfer, extended in vitro storage, or even artificial insemination.

Initially for a sublethal stressor, HHP treatment was chosen due to its unique and outstanding features: 1) acts instantly and uniformly at every point of the sample, 2) HHP features zero penetration problems or gradient effects, 3) HHP can be applied with the highest precision, consistency, reliability, and safety.

Experiments followed a common scheme. First, samples (spermatozoa, oocytes, embryos, or embryonic stem cells) are exposed to different levels (5–80 MPa) of hydrostatic pressure applied for various times in the range of 30–120 min (using programmable hydrostatic pressure devices; Cryo-Innovation Ltd., Budapest, Hungary) to determine the sublethal zone.

Generally, for HHP treatment, oocytes, embryos, spermatozoa, embryoid bodies, or other cells or tissues are loaded into suitable containers (e.g., 0.25- or 0.5-ml ministraw, 5-ml maxistraw, 100- to 500-ml transfusion bag) in conventional culture or extender media (e.g., TCM-199, M2, or G-MOPS for oocytes and embryos) and sealed hermetically, without air bubbles. Subsequently, containers are placed into the pressure chamber (filled with distilled water) of the pressurizing device that was previously 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 (magnitude and duration of the pressure, treatment temperature, pressure profile). Figures 1 and 2 show programmable HHP machines used for the stress treatment of biological materials.

At the second phase of experiments, samples are exposed to this sublethal dose, then incubated for 5–120 min under normal culture conditions for recovery. Subsequently, the required intervention (cryopreservation, insemination, parthenogenetic activation, in vitro fertilization, in vitro maturation or culture, or enucleation followed by somatic cell nuclear transfer) is performed. Results are assessed by investigating morphology as well as functional parameters, including motility, membrane integrity, fertilizing ability, and developmental competence. In each experiment, treatment groups are compared with a single control group where HHP treatment was omitted.

Surprisingly, the pressure tolerance limit of mammalian gametes and embryos was found in the 20–80 MPa zone, where cells survived 30- to 120-min treatments without any loss of their viability, although the highest hydrostatic pressure that these cells normally encounter is less than 0.2 MPa. The optimal and sublethal pressure ranges for gametes and embryos of different mammalian species are shown in Table 1.

The following paragraphs summarize HHP effects according to cell types in various ART procedures.

### *Blastocysts*

Mouse in vivo-derived blastocysts were tolerant of HHP, allowing a pressure treatment of 60 MPa for 30 min. This treatment has been shown to dramatically improve survival rates after rapid freezing [7]. Bovine embryos produced in vitro

TABLE 1. Optimal and sublethal pressure ranges for gametes and embryos.

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

could be treated with even higher pressure (80 MPa, 45 min), again with the result that their cryotolerance during traditional freezing was increased considerably [8]. Later, similar results were also achieved in the same model with modified pressure parameters (60 MPa, 1 h) and by replacing traditional freezing with vitrification [9]. The latter method improved results also in control embryos; however, HHP treatment further enhanced the outcome, resulting in higher survival, reexpansion, and hatching rates. The strongest effect was observed when vitrification was preceded by 1-h equilibration after the HHP treatment [9]. Bogliolo et al. [10] have tested the sublethal hydrostatic pressure stress on the quality of fresh and vitrified ovine blastocysts and found that 40 MPa and 70 min of treatment at 38°C improved the quality of the embryos by increasing their cell number and reducing the proportion of picnosis. The HHP treatment exerted a positive effect in vitrified blastocysts and decreased the number of picnotic nuclei.

To explain the molecular background of the phenomenon, HHP-induced transcriptional changes of mouse blastocysts were assessed by selecting nine genes from various stress-related pathways. The mostly upregulated genes were antizyme inhibitor 1 (*Azin1*), growth arrest specific 5 (*Gas5*), growth arrest and DNA-damage-inducible 45 gamma (*Gadd45g*), and superoxide dismutase 2 (*Sod2*) immediately after hydrostatic pressure treatment, and *Gadd45g* after 120 min culture. This study demonstrated that HHP activates short and long-term growth arrest and oxidative stress-related genes [11]. Similar phenomena, i.e., upregulation of antioxidant defense-related genes (*Sod2* and *Gpx4*) together with lipid synthesis (*Sc4mol*, also known as Erg25) and stress tolerance-related (*Hspa*, also known as Hsp70) genes, were observed in HHP-treated bovine blastocysts, too. The relative abundance of the related mRNAs was the highest 1 h after the end of the stress treatment, which corresponded with the observation that the cryotolerance of the HHP-treated blastocyst was the highest if vitrification followed 1 h after the stress treatment [12].

### *Spermatozoa*

HHP treatment has improved the performance of bovine and porcine spermatozoa. Porcine ejaculated semen was treated with 30 MPa pressure for 90 min, then used for artificial insemination according to the farm routine 4 or 24 h after semen collection. The treatment did not increase pregnancy or farrowing rates, but a significant increase of litter size was observed. A positive effect was also demonstrated by in vitro investigations, where the lifespan of routinely stored boar semen increased after HHP treatment [13]. Further experiments have demonstrated that HHP treatment considerably improved the cryotolerance of porcine semen [14, 15]. As inefficiency of artificial insemination with cryopreserved semen hampers the widespread application of this technology worldwide, these results may offer a breakthrough in pig breeding [16]. Increased postthaw motility, membrane integrity, and fertility were also achieved by the HHP treatment of bull spermatozoa [17].

HHP treatment of spermatozoa also increased the production of several proteins that are considered to play a crucial role in the process of fertilization. Ubiquinol-cytochrome C reductase complex core protein 1, perilipin, and carbohydrate-binding protein AWN precursor were identified as HHP response proteins, being significantly higher in HHP-treated samples measured immediately after treatment, following 5 h of equilibration, and also postthawing [15].

### *Oocytes*

Porcine oocytes were found to be relatively sensitive to HHP; accordingly, a 20-MPa pressure for 60 min proved to be the optimal treatment to increase stress tolerance [18]. The treatment has induced more than tenfold increase in blastocyst rates after vitrification and parthenogenetic activation [18, 19]. In another experiment, pressure-treated oocytes were enucleated and used as recipients for somatic cell nuclear transfer. Both blastocyst rates and the survival of these blastocysts after vitrification have increased significantly. The strongest effect was observed when 1 to 2 h recovery time was applied between the end of the HHP treatment and the initiation of vitrification or enucleation. Transfer of cloned embryos derived from HHP-treated oocytes has resulted in two healthy piglets [20]. Similarly, sublethal HHP stress treatment of immature, GV-stage porcine oocytes resulted in an increased blastocyst rate and higher blastocyst cell numbers following in vitro maturation, parthenogenetic activation, and in vitro culture [21]. In all of these experiments, T2 medium (HEPES-buffered TCM-199 containing 2% cattle serum) was used as holding medium during the HHP treatment of the oocytes.

A recent study has examined the effect of the $Ca^{+}$ content of the treatment media used at HHP treatments of porcine oocytes. The efficiency of activation was tested at different pressure levels and media, including T2 and mannitol-PVA fusion medium with (MPVA+$Ca^{2+}$) or without $Ca^{2+}$ and $Mg^{2+}$ (MPVA). The results showed that HHP did not induce parthenogenetic activation in T2 but only in MPVA+$Ca^{2+}$ with low $Ca^{2+}$ concentration and MPVA without $Ca^{2+}$. The highest activation efficiency was achieved with 10 min HHP treatment using 10 or 20 MPa for oocytes in MPVA+$Ca^{2+}$ or MPVA, respectively. In light of these results, the possible source of $Ca^{2+}$ during activation was investigated. It was found that even after a total of 30 min wash with TL-HEPES-PVA buffer without $Ca^{2+}$ before HHP treatment in MPVA, the oocytes could still be activated, indicating the possibility of an intracellular $Ca^{2+}$ source. It was concluded that parthenogenetic activation could be induced by HHP in certain holding media with low or zero $Ca^{2+}$ content [22].

The stress tolerance of mouse oocytes was comparable to that of porcine ones. Treatment of MII phase mouse eggs with 20 MPa pressure for 60 min at 37°C before vitrification with the Cryotop technique has resulted in increased survival after

warming and intracytoplasmic sperm injection and increased pregnancy rate and litter size [23].

### *Embryonic Stem Cells*

First results achieved with HHP treatment and subsequent cryopreservation of mouse embryonic stem cells was reported by Dinnyes et al. [24]. Mouse embryonic stem cells were used to prepare embryoid bodies (EBs). At Day 4 after EB formation, EBs were treated with 60 MPa for 30 min at 24°C (the same parameters as those used to treat mouse blastocysts [7]) and cryopreserved using the solid surface vitrification technique. After warming, the capability of differentiation into cardiomyocytes was defined by microscopic observation of the beating EBs and cardiomyocyte-specific IHC staining.

Studies showed that the pressure treatment did not affect the survival and the differentiation rate in the nonvitrified groups. However, the applied HHP treatment highly improved the efficiency of in vitro differentiation toward cardiac lineages (74.6% vs. 36.9%, respectively) [24].

Data are presented in Table 2.

## OSMOTIC AND OXIDATIVE STRESS

Results listed above have stimulated research to investigate other agents to induce sublethal injury and increase subsequent stress tolerance. In the first series of experiments, porcine oocytes were treated with sublethal concentrations of NaCl (593 and 1306 mOsm). After a subsequent recovery period followed by vitrification and parthenogenetic activation or somatic cell nuclear transfer, blastocyst rates were significantly increased in the hyperosmotic NaCl-treated groups compared to controls [25]. In a further study, the effect of different osmotic agents was compared. Oocytes were exposed to 588 mOsm NaCl, sucrose, or trehalose solutions for 1 h, recovered for 1 h, then vitrified and parthenogenetically activated. All three treatments resulted in elevated blastocyst rates compared to the controls. When parthenogenetic activation was replaced with somatic cell nuclear transfer, all sublethal hyperosmotic treatments increased blastocyst rates, but treatment with trehalose and sucrose resulted in significantly reduced cell number within the blastocysts [26].

Controversially, osmotic stress has been found to be detrimental to oocytes in different studies, using similar osmotic pressure values but different treatment times [27, 28]. Nevertheless, these studies did not aim to optimize parameters for sublethal stress treatment.

Vandaele et al. [29] has reported the effects of short-term hydrogen peroxide ($H_2O_2$) exposure to bovine in vitro matured cumulus-oocyte complexes (COCs) on subsequent preimplantation embryo development and apoptosis. Mature COCs were incubated in $H_2O_2$ at concentrations ranging from 0.01 to 100 μmol/L, then fertilized and cultured in vitro. Oocyte incubation with 50–100 μmol/L of $H_2O_2$ resulted in a significantly higher blastocyst yield (47.3%) than the one achieved without $H_2O_2$ (31.8%), whereas apoptotic cell ratio was inversely related with $H_2O_2$ concentration. It was also shown that the stress tolerance after $H_2O_2$ exposure was mediated neither by increased glutathione content in treated oocytes nor by enhanced fertilization or penetration [29] (Table 2).

## HEAT STRESS

Several experiments conducted to simulate the effect of environmental heat in vivo have shown that even a slight increase (3.5°–4.5°C) in the temperature of the embryo-holding media for a limited period of time (60–120 min) has reduced blastocyst and hatching rates after in vitro fertilization and culture both in cattle and pig [30, 31]. Milder elevated temperature (2.5°C) for a longer period of time (6 h) caused mitochondrial damage and developmental arrest in bovine embryos [32].

Considering that in these models the authors were not aware of the need to calibrate the sublethal dose and apply it systematically, it was unexpected that Isom et al. [33] found increased developmental competence of parthenogenetically activated porcine oocytes when a 42°C heat stress was applied for 9 h immediately after activation. As no such effect was observed on in vitro fertilized or cloned embryos, the interpretation of these results still requires further analysis and additional research information.

## IN VIVO CONSEQUENCES

Up till the date of the submission of this manuscript, 491 and 155 healthy piglets were born as a result of the insemination of HHP-treated fresh or cryopreserved spermatozoa, respectively [13, 16]. Piglets were not different from the control in sex ratio, weight, stillbirth, and malformations. Transfer of cloned embryos derived from HHP-treated oocytes has resulted in two healthy piglets [20].

HHP stress-treated blastocysts, after transfer, has resulted in 240 fit mouse pups, 12 of which were further bread to test lifetime and reproductive parameters not found to be different from the results of untreated controls in the two-generation study (Bock et al., unpublished results) [7].

## DISCUSSION

The phenomenon that a sublethal stress induces a response with temporary increase of a general, rather nonspecific resistance to various further stresses has been observed in almost all levels of life, from bacteria to multicellular organisms, including humans [34]. On the cellular level the reaction incorporates sensing, assessing, and then counteracting stress-induced damage, consequently increasing temporarily tolerance to such damage [35]. If the stress impact is over the limit of tolerance, programmed cell death (apoptosis) or necrosis occurs [36]. Stress-induced proteins may either reduce or facilitate the activation of the apoptotic cascade [37, 38].

Most proteins that are involved in the key functions of stress response belong to the chaperone family. These proteins are highly conserved and participate in various cellular functions, including protein, DNA, and chromatin stabilization and repair; cell cycle control; redox regulation; energy metabolism; fatty acid/lipid metabolism; and the elimination of damaged proteins [39, 40]. The response is regulated at transcriptional, translational, and/or posttranslational levels [41, 42].

This response was first detected after heat shock [43] and led to the discovery of the heat shock protein (HSP) family [44]. HSPs were later found to be involved in responses to various sudden changes in environment. Diverse stress signals may elicit very similar responses essentially by their common impact of deforming macromolecules, mainly membrane lipids, proteins, and/or DNA [35]. Hörmann et al. [45] compared the proteomic response of *Lactobacillus sanfranciscensis* to HHP, heat, cold, salt, acid, and starvation stress and concluded that different stresses produce overlapping subsets of stress-inducible proteins. The common set of proteins might be the basis for cross-protection.

HSPs play important roles in spermatogenesis, oogenesis, and embryo development [46]. Increased Hsp70 synthesis after various environmental stresses has been detected in mammalian preimplantation embryos before the genomewide activa-

TABLE 2. Results of stress treatment of gametes and embryos on the outcome of various assistance reproductive techniques.

| Type of cell treated | Aim | Results of HHP treatment (treated vs. control) | Results of hyper-osmotic or oxidative stress treatment (treated vs. control) |
|---|---|---|---|
| In vitro-produced bovine blastocysts | To improve cryotolerance | *77% vs. 61% of embryos hatched after vitrification | |
| In vitro-produced ovine blastocysts | To improve embryo quality | *161 vs. 124 cell number; *1.3 vs. 3.8 picnotic index | |
| | To improve cryotolerance | *2.3 vs. 4.8 picnotic index | |
| In vivo-produced murine blastocysts | To improve cryotolerance | *94% vs. 46% of embryos re-expanded after rapid freezing | |
| | To investigate effect on gene expression | *Upregulation of selected stress-related genes 0–120 min after treatment | |
| In vitro-matured porcine oocytes | To improve cryotolerance | *15% vs. 1% of oocytes developed to blastocysts after vitrification and parthenogenetic activation | *6%, *6%, *7% (NaCl, sucrose, trehalose, respectively) vs. 1% of oocytes developed to blastocysts after vitrification and parthenogenetic activation |
| | To improve somatic cell nuclear transfer | *57% vs. 28% of oocytes developed to blastocysts (Yucatan fibroblast donor cell line) | |
| | | *68% vs. 46% of oocytes developed to blastocysts (Large white donor cell line) | *64%, *69%, *65% (NaCl, sucrose, trehalose, respectively) vs. 47% of oocytes developed to blastocysts (Large white donor cell line) |
| | | 56 vs. 49 cells/blastocysts (Large white donor cell line) | 62, *48, and *47 (NaCl, sucrose, trehalose, respectively) vs. 62 cells/blastocysts (Large white donor cell line) |
| | To improve somatic cell nuclear transfer and cryotolerance | *62% vs. 30% of embryos survived vitrification | |
| In vitro-matured bovine oocytes | | | *47% vs. 32% blastocyst rate after treatment with $H_2O_2$ |
| Immature porcine oocytes | To improve in vitro maturation, parthenogenetic activation and embryo culture | *54% vs. 39% of oocytes developed to blastocysts<br>*60 vs. 47 cell number | |
| Ejaculated bull sperm | To improve cryotolerance | *54% vs. 45% of spermatozoa motile after slow freezing | |
| Ejaculated boar sperm | To improve cryotolerance | *59% vs. 37% of spermatozoa motile after slow freezing | |
| | To improve farrowing rates after insemination with cryopreserved sperm | *9.4–10 vs. 4.4–6.7 piglets/sow (pregnancy rate ~70% both groups) | |
| | To improve farrowing rates after insemination with fresh sperm | *12.1 vs. 11.1 piglets/sow (pregnancy rate ~80% both groups) | |
| | Proteomics (2D, mass-spec), protein profile before and after cryopreservation | *Ubiquinol-cytochrome c reductase complex core protein 1, perilipin, and carbohydrate-binding protein AWN precursor levels elevated after treatment, and remained elevated after thawing | |
| Mouse embryonic stem cells | To improve cryotolerance | *75% vs. 37% in vitro differentiation towards cardiac lineages | |

* Significantly different ($P \leq 0.05$); statistical method: generalized linear model.

tion of transcription [47–49]. Nevertheless, other factors, including elevated glutathione synthesis, may also play an important role in the stress response of embryos [50].

There is a growing amount of information about the molecular background of elevated stress tolerance induced by HHP or osmotic stress in gametes and embryos of different species.

Previous studies aimed to define the mechanisms early embryos use to survive in vitro culture conditions, including the response to the changing osmolarity. According to the findings of Bell et al. [51], a significant increase in *AQP3* and *9* mRNA was observed in the sucrose hyperosmotic treatment compared to standard medium and glycerol controls. Hyperosmotic sucrose treatment significantly increased embryonic apoptosis, which was negated in the presence of a MAPK8 blocker but not a MAPK14/11 blocker. MAPK14/11 activation was found to be a component of the rapid adaptive stress response mechanism that includes the effects of AQP mRNA expression and protein localization, whereas the MAPK8 pathway was a regulator of apoptosis [51].

Early preimplantation embryos are particularly sensitive to increased osmolarity. Baltz and Tartia [52] reported that embryos failed to develop from fertilized oocytes when osmolarity was increased. Early preimplantation mouse embryos require intracellular accumulation of glycine to provide osmotic support and thus control cell volume. They found that glycine-specific transporter, GLYT1, mediates osmoregulated glycine accumulation in mouse embryos, similar to human embryos [52].

Xie et al. [53] found that hyperosmolar sorbitol—as a single stressor—caused dose- and time-dependent apoptosis and decreases in embryo growth and cell number accumulation. Phosphorylated stress-activated protein kinase (SAPK) levels were induced proportionally to the amount of sorbitol added, suggesting strong correlation with the above observations. Embryos stressed by 400-1000 mM sorbitol died after 12 h. Apoptosis caused by sorbitol in embryos was found to increase in a dose-dependent manner: embryos cultured overnight in 200 mM sorbitol produced significant, or in 400 mM sorbital highly significant, increases in TUNEL intensity. The lowest hyperosmolar stresses, 10–25 mM sorbitol, produced insignificant increases in TUNEL [53].

Time- and dose-dependent apoptosis and cell cycle arrest responses in embryos were similar to those observed in somatic cells. The induction and role of SAPK in mediating these responses is also similar. However, the remarkable resistance of embryos to high concentrations of sorbitol suggests that part of its homeostatic response is different from that of somatic cells [53].

Lin et al. [25], after testing many concentration of NaCl, found, that using 588 mOsm media for 1 h was beneficial for improving cells' viability. Actually, this concentration was found to be harmful by Xie et al. [53], but the time of impact, at which increased cell death was found, was more than 5 h.

HHP treatment of bacteria has resulted in decreased overall protein production but has induced a relative elevation in synthesis of 55 proteins, most of them belonging to the stress protein family [54]. HHP also increased HSP level in chondrocytes predominantly at the translational level, i.e., by enhancing mRNA stability [42, 55].

The observed changes in the proteome of the HHP-treated porcine spermatozoa [15] are related to a response to the sublethal condition affecting lipid metabolism, redox regulation, and fertilizing ability, the protective effects of which could be detected by increased postthaw motility and increased litter size with normal offspring. On the other hand, no differences were found in the Hsp70 and 90 levels [15]. Although spermatozoa are transcriptionally inactive cells, elevated levels of proteins, including a spermadhesin, have been detected at different time points after the treatment. How these proteins take part in the defense mechanism of semen against stress that results in higher in vitro survival and higher fertilizing capacity requires further studies.

The gene expression study of HHP stress-treated mouse embryos provided information about stress-related cell cycle control [11]. The effect might be similar to that observed in *Escherichia coli*. Bacteria are increasingly sensitive to environmental disturbances in their fast-growing, proliferative phase, hence one of the protective reactions to sublethal stress is the temporary, reversible retardation or intermission of proliferation [54]. The elevated expression of oxidative stress-related genes in HHP-treated mouse and bovine embryos indicates a general stress response, as cellular redox potential has long been regarded as a key regulator of cellular stress response signaling.

The nature and intensity of the initial stress impact was reported to be important not only in the induction of stress protein expression but also in affecting cell viability. Highest expression of stress proteins may be detected when cell viability has already been reduced significantly [56]. For this reason, the fine-tuning of the stress treatment based on stress protein expression can be controversial. Additionally, empirical data with oocytes and embryos revealed that the highest protective effect of sublethal HHP or osmotic treatment was achieved if the second manipulation (cryopreservation, parthenogenetic activation, or enucleation) started 1 to 2 h after the end of the initial stress. These data support the finding that the highest expression of stress-related genes was found 1 h after the HHP treatment of bovine embryos. Moreover, formerly published expression kinetics of stress-related proteins revealed that the time of the peak expression varied between cells and stress impacts, but the first peak was found in the 1- to 3h period after impact [56].

## PERSPECTIVES

The unique possibility to improve the overall quality, i.e., stress tolerance, fertilizing ability, and developmental competence, of mammalian gametes and embryos opens new perspectives in laboratory embryology. Although the understanding of the exact molecular mechanism requires elucidation by further research, accumulating data unanimously demonstrate the beneficial effect of a well-defined sublethal stress including HHP or hyperosmotic treatment. The approach may result in considerable improvement from a wide range of procedures (in vitro fertilization, embryo culture, cryopreservation, etc.) in assisted reproductive techniques of mammals, including humans.

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## REFERENCES

1. Thompson JG, Mitchell M, Kind KL. Embryo culture and long-term consequences. Reprod Fertil Dev 2007; 19:43–52.
2. Gardner DK. Dissection of culture media for embryos: the most important

and less important components and characteristics. Reprod Fertil Dev 2009; 20:9–18.
3. Biggers JD. Thoughts on embryo culture condition. Reprod Biomed Online 2001; 4:30–38.
4. Leese HJ, Hugentobler SA, Gray SM, Morris DG, Sturmey RG, Whitear SL, Sreenan JM. Female reproductive tract fluids: composition, mechanism of formation and potential role in the developmental origins of health and disease. Reprod Fertil Dev 2008; 20:1–8.
5. Hite BH. The effects of pressure in the preservation of milk. W Va Agric Exp Station Bull 1899; 58:15–35.
6. Wemekamp-Kamphuis HH, Karatzas AK, Wouters JA, Abee T. Enhanced levels of cold shock proteins in *Listeria monocytogenes* LO28 upon exposure to low temperature and high hydrostatic pressure. Appl Environ Microbiol 2002; 68:456–463.
7. Pribenszky C, Molnár M, Cseh S, Solti L. Improving post-thaw survival of cryopreserved mouse blastocysts by hydrostatic pressure challenge. Anim Reprod Sci 2005; 87:143–150.
8. Pribenszky C, Molnár M, Ulrich P, Barbosa C, Hatamoto L, Santos C. Pressure assisted cryopreservation: a novel possibility for IVP bovine blastocyst cryopreservation. Reprod Dom Anim 2005; 40:338.
9. Pribenszky C, Siquiera E, Molnár M, Rumpf R. Improved post-warming developmental competence of open pulled straw-vitrified in vitro-produced bovine blastocysts by sublethal hydrostatic pressure pre-treatment. Reprod Fertil Dev 2008; 20:125.
10. Bogliolo L, Ariu F, Uccheddu S, Strina A, Rosati I, Zedda MT, Ledda S. High hydrostatic pressure treatment improves the quality of in vitro-produced ovine blastocysts. Reprod Fertil Dev 2010; 22:202.
11. Bock I, Mamo S, Polgar Z, Pribenszky C. Changes in gene expression of mouse blastocysts treated with high hydrostatic pressure pulse. Reprod Domest Anim 2008; 43:145–146.
12. Siqueira FM, Siqueira CE, Molnar M, Pribenszky C, Machaim FM, Rumpf R. Sublethal hydrostatic pressure pre-treatment: effect in bovine embryos' gene expression. Biol Reprod 2009; 81:629.
13. Pribenszky C, Molnár M, Kútvölgyi G, Harnos A, Horváth A, Héjja I. Sublethal hydrostatic pressure treatment improves fresh and chilled boar semen quality in vitro and in vivo. Reprod Fertil Dev 2009; 21:107.
14. Pribenszky C, Molnár M, Horváth A, Harnos A, Szenci O. Hydrostatic pressure induced increase in post-thaw motility of frozen boar spermatozoa. Reprod Fertil Dev 2006; 18:162–163.
15. Huang SY, Pribenszky C, Kuo YH, Teng SH, Chen YH, Chung MT, Chiu YF. Hydrostatic pressure affects the protein profile of boar sperm before and after freezing-thawing. Anim Reprod Sci 2009; 112:136–149.
16. Kuo YH, Pribenszky C, Huang SY. Higher litter size is achieved by the insemination of high hydrostatic pressure-treated frozen-thawed boar semen. Theriogenology 2008; 70:1395.
17. Pribenszky C, Molnár M, Horváth A, Kútvölgyi G, Harnos A, Szenci O, Dengg J, Lederer J. Improved post-thaw motility, viability and fertility are achieved by hydrostatic pressure treated bull semen. Reprod Fertil Dev 2007; 19:181–182.
18. Pribenszky C, Du Y, Molnár M, Harnos A, Vajta G. Increased stress tolerance of pig oocytes after high hydrostatic pressure treatment. Anim Reprod Sci 2008; 106:200–207.
19. Du Y, Pribenszky C, Molnár M, Zhang X, Yang H, Kuwayama M, Pedersen AM, Villemoes K, Bolund L, Vajta G. High hydrostatic pressure: a new way to improve in vitro developmental competence of porcine matured oocytes after vitrification. Reproduction 2008; 135:13–17.
20. Du Y, Lin L, Schmidt M, Bøgh IB, Kragh PM, Sørensen CB, Li J, Purup S, Pribenszky C, Molnár M, Kuwayama M, Zhang X, et al. High hydrostatic pressure treatment of porcine oocytes before handmade cloning improves developmental competence and cryosurvival. Cloning Stem Cells 2008; 10:325–330.
21. Pribenszky C, Du Y, Molnar M, Vajta G. Sublethal stress on porcine oocytes enhances the efficacy of ART procedures. Human Reprod 2008; 23:161.
22. Lin L, Pribenszky C, Molnár M, Kragh PM, Du Y, Zhang X, Yang H, Bolund L, Callesen H, Macháty Z, Vajta G. High hydrostatic pressure treatment of porcine oocytes induces parthenogenetic activation. Cell Reprog 2010; 12:475–480.
23. Pribenszky C, Mátyás S, Losonczi E, Stanca C, Bock I, Vajta G. Stress for stress tolerance: Improving cell survival by sublethal stress treatment of eggs before vitrification pilot study. Fertil Steril (Scientific Abstracts from the 2010 Annual Meeting of the American Society for Reproductive Medicine) 2010; 94:O-106.
24. Dinnyes A, Polgar Z, Pribenszky C, Pirity MK. Improved embryoid body cryopreservation and cardiomyocyte differentiation following high hydrostatic pressure treatment. Proceedings of The 1st International Congress on Controversies in Cryopreservation of Stem Cells, Reproductive Cells, Tissue and Organs, Valencia, Spain (CometMed, Israel), 2010; A-7.
25. Lin L, Du Y, Liu Y, Kragh PM, Li J, Purup S, Kuwayama M, Zhang X, Yang H, Bolund L, Vajta G. Elevated NaCl concentration improves cryotolerance and developmental competence of porcine oocytes. Reprod Biomed Online 2009; 18:360–366.
26. Lin L, Kragh PM, Purup S, Kuwayama M, Du Y, Zhang X, Yang H, Bolund L, Callesen H, Vajta G. Osmotic stress induced by sodium chloride sucrose or trehalose improves cryotolerance and developmental competence of porcine oocytes. Reprod Fertil Dev 2009; 21:338–344.
27. Agca Y, Liu J, Rutledge JJ, Critser ES, Critser JK. Effect of osmotic stress on the developmental competence of germinal vesicle and metaphase II stage bovine cumulus oocyte complexes and its relevance to cryopreservation. Mol Reprod Dev 2000; 55:212–219.
28. Mullen SF, Agca Y, Broermann DC, Jenkins CL, Johnson CA, Critser JK. The effect of osmotic stress on the metaphase II spindle of human oocytes, and the relevance to cryopreservation. Hum Reprod 2004; 19:1148–1154.
29. Vandaele L, Thys M, Bijttebier J, Van Langendonckt A, Donnay I, Maes D, Meyer E, Van Soom A. Short-term exposure to hydrogen peroxide during oocyte maturation improves bovine embryo development. Reproduction 2010; 139:505–511.
30. Ju JC, Parks JE, Yang X. Thermotolerance of IVM-derived bovine oocytes and embryos after short-term heat shock. Mol Reprod Dev 1999; 53:336–340.
31. Tseng JK, Tang PC, Ju JC. In vitro thermal stress induces apoptosis and reduces development of porcine parthenotes. Theriogenology 2006; 66: 1073–1082.
32. Rivera RM, Dahlgren GM, De Castro E, Paula LA, Kennedy RT, Hansen PJ. Actions of thermal stress in two-cell bovine embryos: oxygen metabolism, glutathione and ATP content, and the time-course of development. Reproduction 2004; 128:33–42.
33. Isom SC, Lai L, Prather RS, Rucker EB. Heat shock of porcine zygotes immediately after oocyte activation increases viability. Mol Reprod Dev 2009; 76:548–554.
34. Selye HA. Syndrome produced by diverse nocuous agents. Nature 1936; 138:32–32.
35. Kültz D. DNA damage signals facilitate osmotic stress adaptation. Am J Physiol Renal Physiol 2005; 289:504–505.
36. Hansen PJ. To be or not to be—determinants of embryonic survival following heat shock. Theriogenology 2007; 68:40–48.
37. Garrido C, Brunet M, Didelot C, Zermati Y, Schmitt E, Kroemer G. Heat shock proteins 27 and 70: anti-apoptotic proteins with tumorigenic properties. Cell Cycle 2006; 5:2592–2601.
38. Arya R, Mallik M, Lakhotia SC. Heat shock genes—integrating survival and death. J Biosci 2007; 32:595–610.
39. Csermely P, Schnaider T, Soti C, Prohaszka Z, Nardai G. The 90-kDa molecular chaperone family: structure, function, and clinical applications. A comprehensive review. Pharmacol Ther 1998; 79:129–168.
40. Kültz D. Evolution of the cellular stress proteome: from monophyletic origin to ubiquitous function. J Exp Biol 2003; 206:3119–3124.
41. Söti CS, Csermely P. Protein stress and stress proteins: implications in aging and disease. J Biosci 2007; 32:511–515.
42. Kaarniranta K, Elo M, Sironen R, Lammi MJ, Goldring MB, Eriksson JE, Sistonen L, Helminen HJ. Hsp70 accumulation in chondrocytic cells exposed to high continuous hydrostatic pressure coincides with mRNA stabilization rather than transcriptional activation. Proc Natl Acad Sci U S A 1998; 95:2319–2324.
43. Ritossa FA. A new puffing pattern induced by a temperature shock and DNP in Drosophila. Experientia 1962; 18:571–573.
44. Tissiere A, Mitchell HK, Tracy U. Protein synthesis in salivary glands of *Drosophila melanogaster*: relation to chromosomal puffs. J Mol Biol 1974; 84:389–398.
45. Hörmann S, Scheyhing C, Behr J, Pavlovic M, Ehrmann M, Vogel RF. Comparative proteome approach to characterize the high-pressure stress response of *Lactobacillus sanfranciscensis* DSM 20451(T). Proteomics 2006; 6:1878–1885.
46. Neuer A, Spandorfer SD, Giraldo P, Dieterle S, Rosenwaks Z, Witkin SS. The role of heat shock proteins in reproduction. Hum Reprod Update 2000; 6:149–159.
47. Luft JC, Dix DJ. Hsp70 expression and function during embryogenesis. Cell Stress Chaperones 1999; 4:162–170.
48. Edwards JL, Hansen PJ. Differential responses of bovine oocytes and preimplantation embryos to heat shock. Mol Reprod Dev 1997; 46:138–145.
49. Christians ES, Zhou Q, Renard JP, Benjamin IJ. Heat shock proteins in mammalian development. Semin Cell Dev Biol 2003; 14:283–290.
50. Edwards JL, King WA, Kawarsky SJ, Ealy AD. Responsiveness of early

embryos to environmental insults: potential protective roles of HSP70 and gluthatione. Theriogenology 2001; 55:209–223.
51. Bell CE, Larivière NMK, Watson PH, Watson J. Mitogen-activated protein kinase (MAPK) pathways mediate embryonic responses to culture medium osmolarity by regulating Aquaporin 3 and 9 expression and localization, as well as embryonic apoptosis. Hum Reprod 2009; 24:1373–1386.
52. Baltz JM, Tartia AP. Cell volume regulation in oocytes and early embryos: connecting physiology to successful culture media. Hum Reprod Update 2010; 16(2):166–176.
53. Xie Y, Zhong W, Wang Y, Trostinskaia A, Wang F, Puscheck EE, Rappolee DA. Using hyperosmolar stress to measure biologic and stress-activated protein kinase responses in preimplantation embryos. Mol Hum Reprod 2007; 13:1–9.
54. Welch TJ, Farewell A, Neidhardt FC, Bartlett DH. Stress response of *Esherichia coli* to elevated hydrostatic pressure. J Bacteriol 1993; 175: 7170–7177.
55. Lammi MJ, Elo MA, Sironen RK, Karjalainen HM, Kaariranta K, Helminene HJ. High hydrostatic pressure-induced changes in cellular protein synthesis. Biorheology 2004; 41:309–313.
56. Diller KR. Stress protein expression kinetics. Annu Rev Biomed Eng 2006; 8:403–424.