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The Effects of Type III Antifreeze Protein and Human Heat Shock Protein 70 Added to the Vitrification Medium of Mouse Embryos on in Vitro Embryonic Development Rates

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A R T I C L E I N F O	A B S T R A C T
	The effect of antifreeze protein type III (AFPIII) and human heat shock protein 70 (HHSP70), added to the vitrification medium of mouse embryos, on post-freeze/thaw in vitro embryonic growth rates and cell numbers were investigated. In total 20 female mice were synchronized. After synchronization, 2 females and 1 male were mated in the same cage. Twenty-four h after mating, the embryos were collected at the pronuclear stage. In total 8 groups were established, including a positive control group (C+), a negative control group (C-), and treatment groups that were vitrified in a medium added with 200, 400 and 800 ng/ml of AFPIII (AFPIII200, AFPIII400, AFPIII800), and 1, 2 and 4 µg/ml of HHSP70 (HHSP70-1, HHSP70-2, HHSP70-4). Solid surface vitrification (SSV) medium was prepared for the vitrification of the embryos. Once thawed, in vitro development rates of embryos were followed at 24, 48, 72 and 96 h. Four embryos, which progressed to the full blastocyst stage, were taken from each group and stained with the Hoeschst 33258 and propidium iodine (PI) dyes to determine the inner cell mass (ICM), trophectoderm (TE) and total cell numbers. The groups showed statistically significant difference for in vitro embryonic development rates at 48, 72 and 96 h (p<0.05). Furthermore, statistically significant differences were also determined for ICM numbers, TE numbers and total cell numbers (p <0.05). It was determined that the use of 400 ng/ml of antifreeze protein type III for the vitrification of pronuclear embryos was highly effective.

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Introduction

The preservation of embryos by freezing is a very delicate process and entails some risks after freezing. During the freezing process, embryos suffer some damage from the direct effects of low temperature or physical changes caused by ice formation. The cryopreservation process can damage both cell structure and cell function. In particular, the spindle filaments formed in the metaphase stage of mitosis, ie the mitotic spindle, are very sensitive to cold shock. The severity of the damage caused by the cryopreservation process can vary with various factors such as the shape and size of the cells, and the structure and permeability of the cell membrane. All these factors differ among species (Vladimirov, Tacheva, and Dobrinov 2018). Ice crystal formation is very harmful to cells, and in such cases, damage is caused not only by the formation of crystalline ice, but also by the high solute concentration resulting from the outflow of intracellular water to the extracellular compartment (Mazur 1996). Cryoprotectant agents (CPA), on the other hand, are used for protection against cold shock, intracellular ice crystal formation, and membrane destabilization that may occur during cell

freezing. Solid-surface vitrification (SSV) is a technique performed by the direct contact of a droplet of a freezing solution containing either an embryo, an oocyte or ova with the surface of a pre-cooled metal block. The droplets frozen as such can then be transferred into cryotubes and stored in liquid nitrogen (-196°C) (Dinnyés et al. 2000).

Antifreeze proteins (AFPs) are polypeptide compounds, which are synthesized by various organisms including fish, plants and insects, and enable survival in sub-zero environments (Jinyao, Ji, and Zhang 2005). These polypeptide compounds are capable of lowering the freezing point of the blood below the freezing point of the sea without changing the osmotic pressure of the plasma (Crevel, Fedyk, and Spurgeon 2002; Hassas-Roudsari and Goff 2012). AFPs not only lower the freezing temperature, but also prevent recrystallization and are capable of preventing the formation of large ice crystals associated with recrystallization during freeze-thawing (Üstün and Turhan 2015). Ice crystals interact with threonine18 (Thr18), which is found in the structure of type III antifreeze protein. Thr18 is responsible for the recognition and interaction of the primary prism

planes of ice. AFPs cover ice surfaces that can reach water, and thus, prevent ice formation. Glutamine9 (Gln9), asparagine14 (Asn14), threonine15 (Thr15), alanine16 (Ala16), threonine18 (Thr18) and glutamine44 (Gln44) are ice-binding sites, which can significantly alter thermal hysteresis (TH) activity and ice crystal morphology (Chao et al. 1994; Jia et al. 1996; DeLuca et al. 1998). Hydrophobic interactions at the ice-binding sites are reported to be important for the antifreeze activity of proteins (Chen and Jia 1999; Graether et al. 1999).

Heat shock proteins (HSPs) are found in both prokaryotic and eukaryotic cells. Their structures are highly protected, which suggests that they are involved in basic cellular processes. The production of HSPs is induced in cells exposed to heat shock. However, HSP synthesis is induced not only by heat shock (Kiang and Tsokos 1998), but also by amino acid analogues (Kelley and Schlesinger 1978; Li and Laszlo 1985), glucose analogues (Pouysségur, Shiu, and Pastan 1977), heavy metals (Levinson, Oppermann, and Jackson 1980), protein kinase C (PKC)-stimulants, Ca^{+2}-enhancing agents (Ding et al. 1996), ischemia, sodium arsenite (Johnston et al. 1980), microbial infections, nitric oxide, and exposure to hormones and antibiotics. HSP70 is reported to protect cells from thermal and oxidative stress (Ikwegbue et al. 2018). Stress factors can normally cause protein buildup by damaging proteins. HSP70 temporarily binds hydrophobic amino acid sequences exposed to stress, preventing the accumulation of these partially denatured proteins. In a study, in which HSP70 and HSP60 antibodies were used for mouse embryos, both apoptotic trophectoderm and apoptotic inner cell mass were determined to have increased, suggesting the involvement of these proteins in the modulation of apoptosis (Esfandiari et al. 2007). During apoptosis, cytochrome-C binds to apoptotic protease activating factor-1 (Apaf-1) and dATP/ATP (deoxyadenosine triphosphate/adenosine triphosphate) to form the apoptosome complex. Later, this complex activates procaspase-9 by converting it to caspase-9, and caspase-9 causes apoptosis by activating caspase-3. HSP70 is reported to inhibit this process by preventing the conversion of this complex to procaspase-9 and caspase-9 (Beere et al. 2000). In addition, it has been stated by researchers that HSPs take place in the antioxidant system by inhibiting or neutralizing the cellular effects of reactive oxygen species (ROS) (Trott et al. 2008; Ikwegbue et al. 2018). The aim of the present study was to increase the post-thaw in vitro embryonic development rate by adding HHSP70 to the freezing medium of the embryos, taking advantage of the antiapoptotic properties of this protein, similar to the case in studies on the cryopreservation of semen (Alvarez-Rodríguez et al. 2013; Holt, Del Valle, and Fazeli 2015).

Materials and Methods

In the present study, 6 to 8-week-old 20 female and 16-week-old 10 male mice, all of the CB6 breed and with proven fertility, were used. The animals were housed individually in ventilated hepafilter cages. In the rooms, where the cage systems were located, the lighting intensity was set as 330 lux and the animals were maintained under a 12 h light/12 h dark cycle. The in-room temperature was 20-24°C and relative humidity was 45-65%.

Ethics Approval

The present study was conducted pursuant to the 2020.HADYEK.015 numbered approval of the Local Ethics Committee for Animal Experiments of Koç University. The experimental animals used in this study were obtained from the Experimental Animal Laboratory of Koç University Research Center for Translational Medicine, and the embryo experiments were carried out in the Embryo Manipulation Laboratory.

Preparation of the Washing Medium

A HEPA-filter cabinet was used for the preparation of all media. The working cabinet was sterilized just before use. The Global w/HEPES medium was used for the in vitro manipulations of the embryos. This medium is characterized by not requiring CO_2 for manipulations. The Global w/HEPES medium was added 3 mg/ml of bovine serum albumin (BSA, SIGMA A4378), and after BSA was dissolved, the mixture was passed through a 0.22 μm-filter. The medium was prepared by keeping it in a 37°C heating plate for 10 minutes.

Preparation of the Culture Medium

Global® Total® LP (Cooper Surgical Fertility Solutions, Denmark) was used as the culture medium, and preparations were started 12-16 h before embryo acquisition to ensure the availability of ready-to-use medium. The refrigerated embryo culture medium was added 4 mg/ml of BSA (SIGMA A4378) as a protein support, and then passed through a 0.22 μm-filter. After being filtered, the embryo culture medium was distributed in 10 μl-droplets with an automatic pipette onto the surface of 35 mm-embryo culture plates, which were later transferred to an incubator (37°C, 5% CO_2, 90% humidity).

Preparation of the Equilibration Medium

The equilibration medium was prepared by mixing 0.2 ml of ethylene glycol (Sigma EG E-9129), 4.8 ml of HTF-HEPES, and 20 mg of BSA. After being filtered, the solution was distributed in 100 μl-droplets onto a 60 mm-plate (Falcon 353004) with a 200 μl-automatic pipette and kept at 37°C in a heating plate until being frozen.

Experimental Groups

Positive Control Group: Pronuclear-stage embryos were maintained in the culture medium only until the hatched blastocyst stage without being frozen.

Negative Control Group: Pronuclear-stage embryos were maintained in the culture medium until the hatched blastocyst stage after undergoing freeze-thawing with no protein added to the SSV medium.

- For the preparation of the SSV medium, 0.875 ml EG + 0.3785 g trehalose (Sigma-T0167) + 125 mg polyvinyl pyrrolidone (PVP, Sigma P0930) + 10 mg BSA were mixed, added 1.625 ml of HTF-HEPES, and filtered.

Group Antifreeze Protein III-200: Pronuclear-stage embryos were frozen by adding 15 μl of antifreeze protein III (200 ng/ml) to the SSV medium, and cultured until the hatched blastocyst stage after being thawed.

Group Antifreeze Protein III-400: Pronuclear-stage embryos were frozen by adding 30 μl of antifreeze protein III (400 ng/ml) to the SSV medium, and cultured until the hatched blastocyst stage after being thawed.

Group Antifreeze Protein III-800: Pronuclear-stage embryos were frozen by adding 60 µl of antifreeze protein III (800 ng/ml) to the SSV medium, and cultured until the hatched blastocyst stage after being thawed.

Group Human Heat Shock Protein 70-1: Pronuclear-stage embryos were frozen by adding 25 µl of human heat shock protein 70 (1 µg/ml) to the SSV medium, and cultured until the hatched blastocyst stage after being thawed.

Group Human Heat Shock Protein 70-2: Pronuclear-stage embryos were frozen by adding 50 µl of human heat shock protein 70 (2 µg/ml) to the SSV medium, and cultured until the hatched blastocyst stage after being thawed.

Group Human Heat Shock Protein 70-4: Pronuclear-stage embryos were frozen by adding 100 µl of human heat shock protein 70 (4 µg/ml) to the SSV medium, and cultured until the hatched blastocyst stage after being thawed.

Preparation of the Thawing Solution

The thawing solution was prepared by adding 0.5675 g of trehalose and 20 mg of BSA to 5 ml of HTF-HEPES and filtering the solution.

Superovulation of the Female Mice

For the synchronization of the female mice, 10 IU of pregnant mare serum gonadotropin (PMSG, Folligon) was administered intraperitoneally at 13:00 h on the day of administration. Forty-eight h after this treatment, 10 IU of human chorionic gonadotropin (hCG, Chorulon) was administered intraperitoneally at 13:00 h to ensure ovulation. Sixteen-week-old male mice were used for breeding purposes, and 2 females and 1 male were placed in the same cage. Vaginal plug controls were performed 22-24 h after mating. Female mice with a vaginal plug were taken to the operating room.

Embryo Collection

The female mice taken to the operating table were euthanized by cervical dislocation. Their abdomen was sterilized with alcohol and incised with pointed scissors along the midline. After the incision, organs and tissues were removed to reach the ovaries. The fat tissue surrounding the ovaries was stripped and the oviducts were collected with care. The oviducts were washed in a solution prepared by adding 4 mg/ml of BSA to HTF-HEPES (Cooper Surgical Fertility Solutions, Denmark). HTF-HEPES was placed in one well of a 4-well plate and 300 µg/ml of hyaluronidase was added. The other 3 wells of the plate were filled with HTF-HEPES alone for the washing of the pronuclear embryos. An incision was made in the ampulla region of the oviducts, which were transferred into HTF-HEPES-hyaluronidase solution, using a sterile toothed forceps. The pronuclear-stage embryos, which were surrounded by cumulus cells and recovered from the incision made in the ampulla, were maintained in this solution for 3 minutes. Immediately afterwards, they were washed in sequence in the 3 wells containing HTF-HEPES. The best quality embryos were selected in the last well. The culture medium was added to another 4-well plate. The embryos recovered in HTF-HEPES medium were transferred into the culture medium and the pipettes were

changed between embryos. The embryos were then washed in 3 different wells and again the glass pipettes were changed between embryos. A total of 308 high-quality pronuclear embryos were obtained. Single-cell embryos were cultured in the culture medium (Global® Total® LP, Cooper Surgical Fertility Solutions, Denmark) in an incubator under 95% humidity at 37°C with 5% CO_2 until the vitrification stage.

Embryo Freezing by the SSV Method

The embryos were transferred into 100 µl of equilibration medium and maintained therein for 12-14 minutes. Next, they were transferred into droplets of freezing medium and washed 3 times. The last droplet containing embryos of the trial groups was drawn with a glass pipette. Subsequently, the embryos were introduced into liquid nitrogen by dropping the droplets on a flat tray covered with aluminum foil. The frozen droplets were collected with a forceps and transferred into cryotubes, which were immediately immersed in liquid nitrogen. This experiment was repeated 4 times.

Thawing and Culturing of the Embryos

The embryos were thawed in the thawing solution on the same day they were frozen. For this purpose, the embryos taken from the cryotubes were placed in the thawing solution, maintained therein for 3 minutes, and then washed in 3 wells containing HTF. After being washed at least 3 times in the culture medium, the embryos were cultured.

Evaluation of the In vitro Culture Rates

All pronuclear-stage embryos, which were transferred into the culture medium after being thawed, were cultured for 96 h and their in vitro growth rates were evaluated at 24, 48, 72 and 96 h in each group. The quality of the blastocysts was examined under an stereo microscope according to the International Embryo Transfer Society standards for embryo evaluation.

Determination of the Cell Counts by Differential Staining

In order to evaluate the quality of the embryos at the full blastocyst stage, differential staining was performed and the trophectoderm and inner cell mass cell numbers were calculated.

Staining of the embryos with the propidium iodine (PI) dye

Twenty-five mg of PI dye was brought to a volume of 10 mL with PBS/PVP, such that a 2.5 mg/ml stock solution was obtained. Ten µL of commercial Triton-100x was mixed with 10 ml of PBS/PVP to prepare 0.1% TritonX (as Triton X is very sticky, these processes should be performed at 37°C, otherwise Triton X will clog the tip of the automatic pipette). Next, Triton X was diluted with PBS/PVP to a final concentration of 100 µg/ml. Two µl of this dye was transferred into 998 µl of HTF medium. Subsequently, 500 µl of the prepared PI dye was transferred into a 4-well plate. Embryos were transferred into a droplet of the PI dye and stained for 10-15 seconds.

Staining of the embryos with the Hoeschst 33258 dye

Forty µL of Hoeschst 33258 dye was mixed with 780 µL of 100% ethanol to prepare the Hoeschst 33258 dye. Next, 500 µL of this dye was placed into one well of a 4-

well plate. Each of the remaining 3 wells were filled with 1 ml of 100% ethanol. Also, small pieces of paper towel were placed in these 3 wells to prevent the evaporation of the alcohol content. For staining, the embryos were transferred into droplets of Hoeschst 33258 after being stained with PI, and the entire plate was covered with cling film and aluminum foil and kept at +4ºC for 24 h. Once stained, the embryos were transferred to glass slides. All of these procedures were performed in the dark.

Screening of the Embryo Stainings

In order to visualize the stained embryos, firstly, circular coverslips were cut into small pieces. These small pieces of coverslip were placed on a glass slide to form a gap in the middle, and were fixed onto the slide with clear nail polish (Caglar et al. 2020). Thereby, heightened slides were prepared. Next, 20 µL of glycerol was dropped on the slides, and embryos from the droplets were transferred into glycerol with a very small amount of liquid. A coverslip was placed, enclosed with nail polish and the remaining gaps were filled with glycerol. All procedures were performed in a dark environment. The preparations were visualized under a confocal microscope with red and blue fluorescent attachments, and the trophectoderm (TE) and inner cell numbers (ICM) were calculated. The inner cell mass cells of the stained blastocysts were of red color, whilst the trophectoderm cells were of blue color.

Statistical Analysis

Differences between groups were determined with one-way analysis of variance (ANOVA) using the IBM SPSS Statistics 25.0 software. The in vitro development rate (%)

of a group was calculated by dividing the number of embryos that could develop in vitro in that group by the total number of embryos in the same group, and this calculation was applied to all groups and hours. An arcsin transformation was performed on the percentile data to equalize variance across different development levels, and then statistical analysis was performed with one-way ANOVA using the IBM SPSS Statistics 25.0 software.

Results

In the present study, the in vitro embryonic development rates determined at 0, 24, 48, 72, and 96 hr in Groups C+, C- AFP200, AFP400, AFP800, HHSP70-1, HHSP70-2 and HHSP70-3 are shown in Figure 1.

As can be seen in Figure 1, the live embryo rates (%), statistically calculated after the standardization of the data with the arcsin transformation method, in the positive control (C+), negative control (C-), Antifreeze Protein III 200 (AFPIII200), Antifreeze Protein III 400 (AFPIII400), Antifreeze Protein III 800 (AFPIII800), Human Heat Shock Protein 70-1 (HHSP70-1), Human Heat Shock Protein 70-2 (HHSP70-2) and Human Heat Shock Protein 70-4 (HHSP70-4) groups were 92.50 (59/61), 93.93 (32/35), 94.72 (31/33), 100 (43/43), 91.67 (31/32), 100 (27/27), 94.10 (31/33), and 91.94 (41/44), respectively.

While there was no statistically significant difference between the groups for in vitro embryonic growth rates at 0 and 24 h (p> 0.05), the differences observed between the groups for the growth rates at 48, 72 and 96 h were statistically significant (P<0.05).
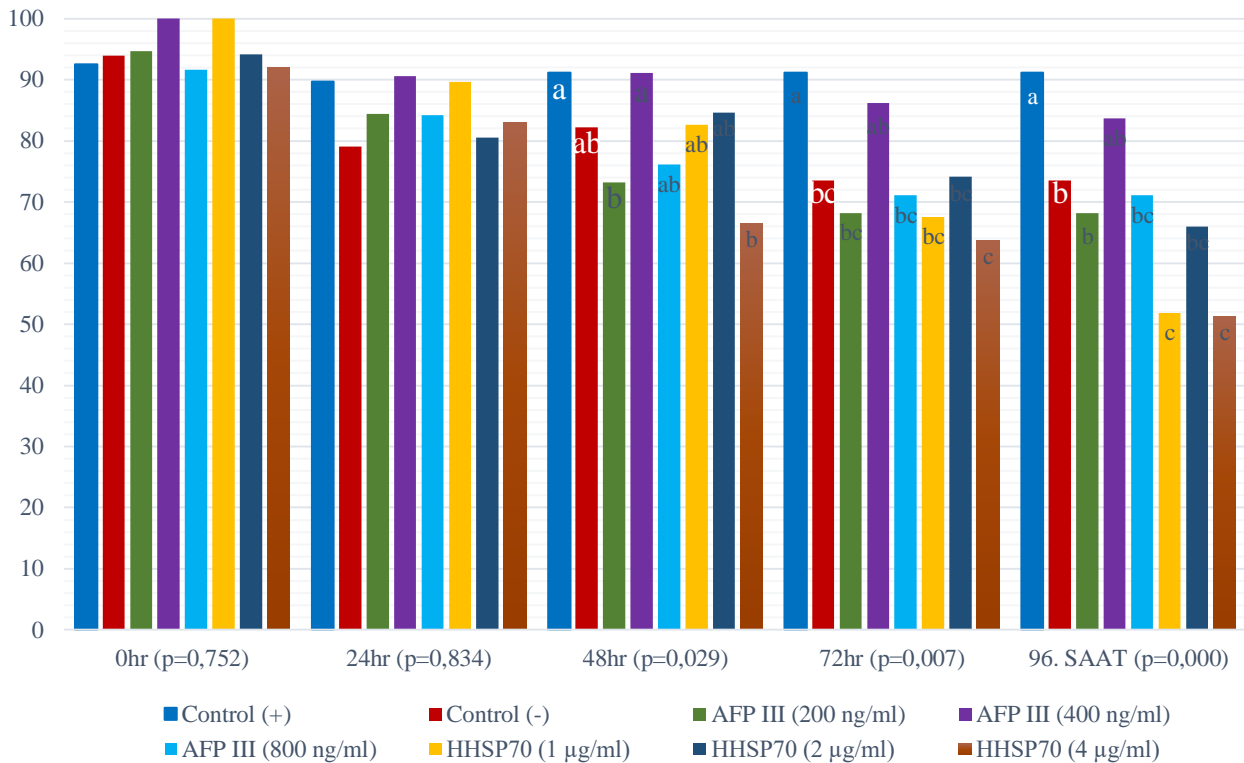

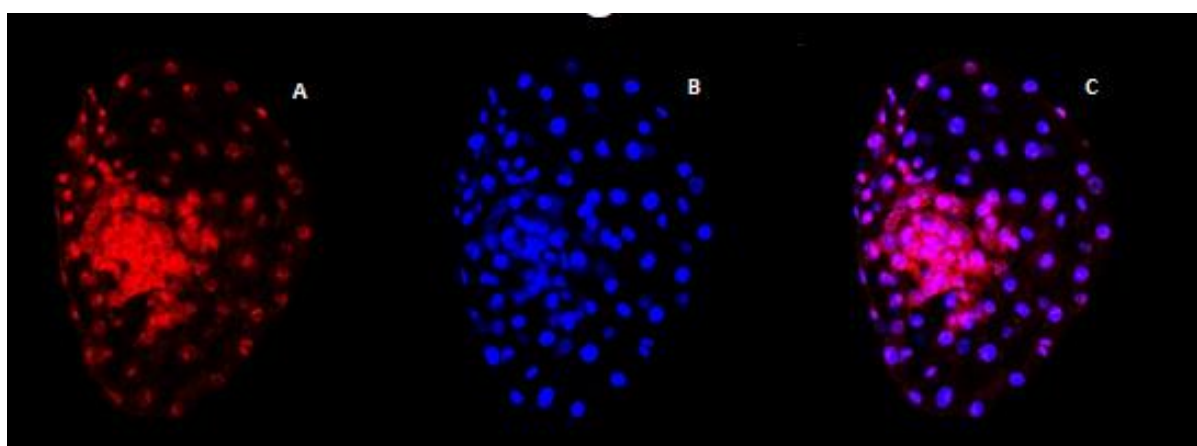Figure 1. Post-freeze/thawing In Vitro Growth Rates

Figure 2. Demonstration of TE and ICM Cells with Hoechst 33285 and Propidium iodine (PI). A) ICM cells B) TE Cells, C) Total Cell Number.
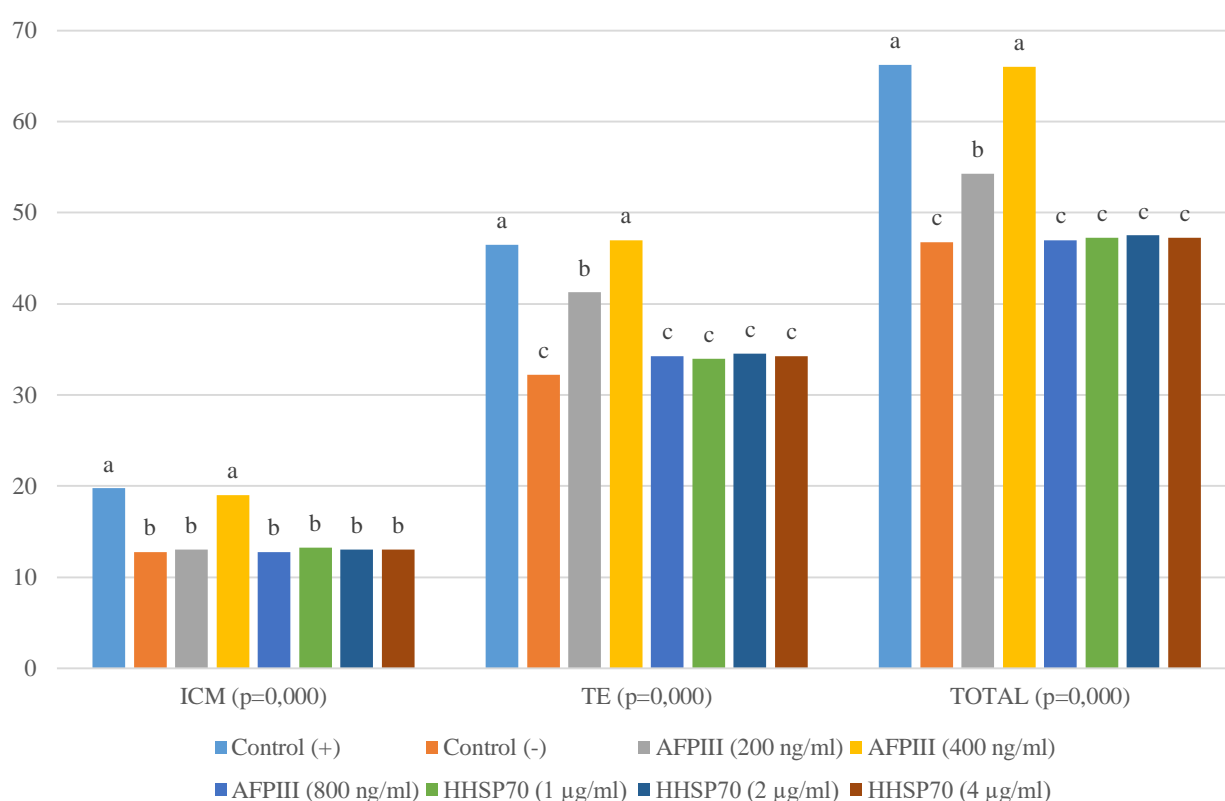

Figure 3. ICM, TE and Total Cell Numbers Determined in Blastocysts by Differential Staining

The in vitro development rates (%) determined in the groups were 89.72 (55/61), 79.11 (28/35), 84.45 (27/33), 90.58 (39/43), 84.17 (28/32), 89.63 (24/27), 80.48 (27/33), and 83.09 (38/44), respectively, at 24 h; 91.11 (57/61), 82.23 (29/35), 73.20 (24/33), 94.16 (39/43), 76.11 (25/32), 82.59 (22/27), 84.65 (28/33), and 66.50 (31/44), respectively, at 48 h; 91.11 (57/61), 73.48 (26/35), 68.19 (22/33), 86.15 (37/43), 71.11 (23/32), 67.59 (18/27), 74.10 (24/33), and 63.73 (28/44), respectively, at 72 h; 91.11 (57/61), 73.48 (26/35), 68.20 (22/33), 83.65 (36/43), 71.11 (23/32), 51.85 (14/27), 65.97 (21/33), and 51.31 (24/44), respectively, at 96 h.

As can be seen in Figure 2, in the positive control (C+), negative control (C-), Antifreeze Protein III 200 ng/ml (AFPIII200), Antifreeze Protein III 400 ng/ml (AFPIII400), Antifreeze Protein III 800 ng/ml

(AFPIII800), Human Heat Shock Protein 70 1 µg/ml (HHSP70-1), Human Heat Shock Protein 70 2 µg/ml (HHSP70-2) and Human Heat Shock Protein 70 4 µg/ml (HHSP70-4) groups, the inner cell mass (ICM) numbers were 19.75, 12.75, 13.00, 19.00,; 12.75, 13.25, 13.00, and 13.00, respectively; the trophectoderm (TE) numbers were 46.5, 32.25, 41.25, 47.00, 34.25, 34.00, 34.50 and 34.25, respectively; and the total cell numbers were 66.25, 46.75, 54.25, 66.00, 47.00, 47.25, 47.50, and 47.25, respectively. ICM, TE and Total Cell numbers were visualized under a confocal microscope with red and blue fluorescent attachments, and the trophectoderm (TE) and inner cell numbers (ICM) were calculated (Figure 3). The differences observed between the groups for the ICM, TE and total cell counts were statistically significant (P<0.05).

Discussion

The cryopreservation of oocytes and embryos is highly important for the dissemination and protection of animal resources that have superior genetic characteristics or are endangered. Although many researches have been conducted on embryo freezing in recent years and progress has been achieved, an effective protocol has not yet been established. Embryos are very sensitive to cooling and freezing. All embryos suffer significant morphological and functional damage during freezing. However, the magnitude of these damages, the survival of embryos against these damages, and embryonic development rates may vary with species, as well as the type and origin of the damage. One of the most important purposes of freezing is the long-term preservation of embryos of animals, which are genetically superior and used as breeders, endangered, and forced out of the breeding program for any reason. By successfully freezing and storing these embryos, embryo transfer can be performed at any time.

Most of the damage to chromosomes or microtubules (structures responsible for forming spindle filaments) occurs during the cooling or freezing of embryos (Succu et al. 2007). In addition, increased polyspermia, displacement of the cortical granules, or hardening of the zona pellucida may also take place (Morató et al. 2008). In some studies, fractures and embryonic cell death were observed in the zona pellucida or cytoplasmic membrane after the freeze-thawing of embryos (Lim, Fukui, and Ono 1992; Cuello et al. 2007). In this context, the present study is based on the postulate that embryonic damage associated with freeze-thawing can be prevented by the addition of certain agents to the freezing medium.

Jo et al. (2011) administered 6-week-old BDF-1 mice with 5 IU of PMSG, followed by an injection of 5 IU of hCG 48 hours later. They collected metaphase II-ova from the oviducts and removed the cumulus in a solution containing 85 IU/ml of hyaluronidase. The ova were assigned to two groups and frozen in vitrification media with and without AFPIII (500 ng/ml). The researchers also determined the total cell numbers by staining the blastocyst-stage embryos. After thawing, the percentages of live ova were 84.5% and 94.6% in the control group and AFPIII group, respectively. Following the in vitro fertilization of the live ova, the blastocyst rate was 68.9% in the control group and 89.1% in the AFPIII group. Compared to the growth rate reported by Jo et al. (2011) for the AFPIII500 group, lower growth rates were obtained in the present study (C-/73.48%, AFPIII200/68.20%, AFPIII400/83.65%, AFPIII800/71.11%, HHSP70-1/51.85%, HHSP70-2/65.97%, and HHSP70-4/51.31%). After staining, the researchers obtained a total cell count of 57.2 in the control group and 66.2 in the AFPIII (500 ng/ml) group. The total cell number obtained by the researchers in the AFPIII group (66.2) was similar to the rates determined in the the C+ (66.25) and AFPIII400 (66) groups in the present study, whilst the rate obtained by the researchers in the control group (57.2) was higher than the rates determined in Groups C- (46, 75), AFPIII200 (54.25), AFPIII800 (47), HHSP70-1 (47.25), HHSP70-2 (47.5) and HHSP70-4 (47.25) in the present study. Compared to the present study, the differences observed in the in vitro development rates reported by Jo et al. (2011) are attributed to the use of a different dose of AFPIII and in vitro-fertilized embryos having been obtained with the use of frozen oocytes instead of frozen embryos. It has been reported by some researchers that the mitochondrial membrane potential ($\Delta\Psi$m) is lower in pronuclear embryos after freeze-thawing and that these embryos are more sensitive to the freeze-thawing process (Leibo et al. 1996; Zhao et al. 2009). In terms of total cell numbers, based on the findings obtained in previous research (Jo et al. 2011; Nishijima et al. 2014) and the results obtained in the present study, it can be said that AFPIII preserves the integrity of the oocyte and embryo membrane by lowering the freezing point.

Sun et al. (2020) obtained primary oocytes from bovine slaughterhouse material, and after undergoing in vitro maturation, these oocytes reached the metaphase II stage. These ova were assigned to a control group and treatment groups, in which the freezing medium was added with 0.1 mg/ml AFPIII (AFPIII-0.1), 1 mg/ml AFPIII (AFPIII-1), 10 mg/ml AFPIII (AFPIII-10), 0.1 mg/ml *Leucosporidium*-derived ice-binding protein (LeIBP) (L-0.1), 1 mg/ml LeIBP (L-1), and 10 mg/ml LeIBP (L-10), for vitrification. After the vitrified ova were thawed, in vitro fertilization was performed and blastocyst development was observed. Following in vitro culture, the blastocyst rates determined in the control group and Groups AFPIII-0.1; AFPIII-1, AFPIII-10, L-0.1; L-1 and L-10 were 58.13%, 54.55%, 56.07%, 58.34%, 74.09%, 63.69%, and 55.95%, respectively. Sun et al. (2020) and Do et al. (2012) have similarly reported that LeIBP-type proteins protect embryos against cold shock after freeze-thawing, prevent ice crystal formation and reduce ROS production owing to their high thermal hysteresis activity. In the present study, it was ascertained that while AFPIII showed an activity similar to that of LeIBP and indirectly increased in vitro embryonic development rates, its high doses suppressed embryonic development by showing toxic effects.

The human heat shock protein 70 (HHSP70) used in our study has not been used in embryo or oocyte vitrification studies before, and the doses tested in the present study were chosen on the basis of sperm studies (Alvarez-Rodríguez et al. 2013; Holt, Del Valle, and Fazeli 2015). In the present study, there was a decrease in the cleavage rates and an increase in the degeneration rates from 0 h to 96 h in the HHSP70 groups. Although heat shock protein 70 is produced in the cytosol, mitochondria, endoplasmic reticulum and nucleus, and reported to act as a chaperone (Kiang and Tsokos 1998) and an anti-apoptotic factor (Esfandiari et al. 2007), it is considered that the degenerations associated with its use may be related to this protein undertaking different roles in the extracellular environment. HSP70 contains sulfurous amino acids (cysteine and methionine) (Arakawa et al. 2011; Yang et al. 2020). Cysteines form disulfide bonds between thiol groups of the same protein or with the thiol group of the cysteine amino acid of another protein ($RSH + RSH = RS-RS + 2H^+ + 2e^-$). While forming disulfide bonds, they give the hydrogen ion (H^+) to the environment (Witt 2008). H^+ forms a hydronium ion (H_3^+O) by binding to the water molecule (H_2O) in an aqueous environment. The hydronium ion is acidic and lowers the pH of the environment (Başpınar 2010). It can be said that the gradually decreasing rates of cleavage were caused by the decrease in the pH of the environment.

Some researchers have stated that freezing with the vitrification method increases mitochondrial damage, apoptosis, ROS generation, and blocking (Somfai et al. 2007; Gupta, Uhm, and Lee 2010). Furthermore, it is also emphasized that ROS reduce embryonic development and stimulate apoptosis in the early embryonic period (Evans, Dizdaroglu, and Cooke 2004). As can be understood from research data, by using AFPIII, the oocyte and embryo membranes are protected, damage to chromosomes and myotic spindle strands is prevented, the formation of apoptotic cells is reduced, and embryos are frozen such that their quality is preserved. The results of previous research and the present study suggest that the effects of AFPIII are dose-dependent. The use of 400-500 ng/ml in mice can produce higher in vitro growth rates and total cell numbers. However, further dose studies are needed in other animal species, including cattle and sheep. The HHSP70 protein used in the present study was tested for the first time, and this protein needs to be further investigated.

Conclusion

In the present study, statistically significant differences were determined between the groups, in which AFPIII and HHSP70 were added to the vitrification medium, in terms of in vitro embryonic growth rates, and inner cell mass, trophectoderm and total cell numbers. In result, it was determined that 400 ng/ml of antifreeze protein type III was more effective in the freezing of murine pronuclear embryos and preserved embryo membrane integrity. It was observed that the effects of antifreeze protein type III were dose-dependent, such that high doses could reduce embryo development. Further dose studies in cattle and sheep would provide input on how to safely use this protein. This study investigated for the first time the use of human heat shock protein 70 in the freezing of embryos, and furthermore detailed research is needed with emphasis on the investigation of the energy metabolism of embryos after the use of this protein. It is considered that embryos of superior animals can be safely vitrified with these proteins -on the basis of the results of future dose studies.

Acknowledgement

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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