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Effect of Transfer Temperature of Epididymis on Survival Rate of Semen and B6D2F1 Mice Developmental Capacity†

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ABSTRACT

This study was conducted to evaluate the effect of transfer temperature of epididymis on survival rate of semen and development ability of B6D2F1 mice embryos. No significant differences were noted in the survival rate of semen (59.0% ± 0.1 vs. 47.6% ± 0.1), in vitro fertilization rate (90.7% ± 0.1 vs. 90.7% ± 0.1), developmental rate (90.0% ± 0.1 vs. 90.0% ± 0.1), and blastocysts formation rate (53.1% ± 0.2 vs. 52.3% ± 0.2) between groups. (*NS*; $P>0.05$). However, the zona hatched rate was significantly higher in the 4°C group compared to those of the 37°C group (47.8% ± 0.1 vs. 25.6% ± 0.2; $p<0.05$). When it comes to cell numbers of blastocysts, the % ICM (/total cells) was significantly higher in the group of 4°C compared to the 37°C (27.0% ± 0.1 vs. 18.3% ± 0.1; $p<0.05$). However there were no differences in total cell numbers (72.7 ± 31.6 vs. 62.0 ± 36.6), ICM cell numbers (17.0 ± 7.8 vs. 14.6 ± 8.6), TE cell numbers (55.8 ± 29.8 vs. 64.0 ± 24.4), the ratio of ICM:TE (1:4.2 ± 4.1 vs. 1:6.4 ± 7.2) between two groups (*NS*; $P>0.05$).

Taken altogether, it is expected to achieve the best developmental ability of B6D2F1 mice embryos in the transfer temperature of epididymis. Also these results can provide fundamental data to maximize culture condition for *in vitro* fertilization on B6D2F1 mice. In future, therefore, it is expected that results herein might be applied for in vitro culture of human embryos.

(Key words: B6D2F1 mice, epididymis, *in vitro* fertilization, inner cell mass, oogenesis)

INTRODUCTION

Since it was first introduced in canine by Spallanzani in 1780, many *in vitro* fertilization studies have been carried out utilizing various animal models (Capanna, 1999). In human, it was successfully implemented in 1965 by Edwards and a test tube baby was born in Korea in 1978 for the first time (Chang et al., 1986). In case of South Korea, a total of 15,619 cases of Assisted Reproduction Techniques (ART) was performed in 2000 by 58 institutes; in the year of 2008, this was dramatically increased up to 28,029 ARTs by 78 institutes (Choi, 2011). It is important to note that several challenges of in vitro fertilization and techniques, for embryonic development, still need to be overcome. There are factors influencing on in vitro fertilization which can be further classified into biological and abiological factors; the former includes patients' age, health conditions, status of semen and oocytes (Lonergan et al. 1999; Ashworth et al., 2009) whereas the later can be further subdivided into environmental and institutional factors. Environmental factors are types of culture media, oils, as well as amount of media. On the other hand, institutional factors are mostly related with experimental instruments including incubators, and microscopes (Ashworth et al., 2009; Park et al., 1994a, 1994b; Greve et al., 1995; Meyer et al., 1999). Of note, it has been described that environmental factors influence on embryonic development, either before or after implementation, as well as fetus health (Dumoulin et al., 2010). Generally speaking, two common techniques are being utilized for animal oocytes and in vitro fertilization: either large amount of culture media or droplet of culture media. Previously, experiments were

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carried out to compare volumes of culture media (<0.05 ml vs. 0.5~1 mL culture media) after coating with mineral oils (Gwatkin, 1963; Brinster, 1963). Application of micro culture media provides several benefits. First, it prevents water evaporation, microbial contamination, and lowers temperature fluctuation as well as culture gas production. Further, it can be easily performed for animal oocytes and incubation of fertilized eggs (Brinster, 1963; Kane, 1987). It has been described that application of oils in in vitro fertilization of animal oocytes may neutralize culture media toxicity and protect embryos (Lee et al., 2004), In addition, in multiple studies, the quality of oils plays an important role in development of mammal fertilized eggs to blastocysts and improvement of cryopreservation methods (Tae et al., 2006; Van Langendonckt, et al., 1996; Van Soom et al., 2001). When it comes to factors influencing mouse sperms, the condition of sperms, one of biological factors as aforementioned, can significantly impact on in vitro fertilization and embryonic development thus, it is important to maintain its mobility, survival rate and sperm concentrations (You et al., 2009). Utilizing experimental animals, sperm conditions as well as in vitro fertilization rate can differ depending upon transfer methods, from animal breeding rooms to laboratories. Often, mouse sperms are subjected to liquid nitrogen freezing or can be transported in different temperature conditions. Due to a need of special apparatus for freezing transport method (e.g., liquid nitrogen tank) in addition to technical difficulties, it is not easy to implement for many cases (Fuller et al., 1996). Therefore, easier and simpler cold transport method, which stores capacitation sperm samples in paraffin oils at 4°C, has been more widely used in various applications. This method also has limitations since low temperature induces cold shock thus most sperms lost their mobility and became infertalitas (Jishage and Suzuki, 1993). These limitations, however were improved when epididymis was stored at 4°C over 24 hrs; higher mobility and survival rates of sperms were observed (Jishage et al., 1997). Hence, it might be better to transfer epididymis for sperm transportations yet few studies investigated with regards to optimal temperature conditions. The B6D2F1 mouse strain has been known for excellent developmental rate from 2 cell stage through blastocysts compared to other mouse models thus enables a wide range of fundamental experiments through effective production of blastocysts. In the present study, therefore, in B6D2F1 mice, we investigated effects of transfer temperature of epididymis on semen survival rate, *in vitro* fertilization rate and embryonic development.

MATERIALS and METHODS

1. Animals

In the current study, hybrid B6D2F1 mice (maternal line: C57BL/6NCrIBR; paternal line: DBA/2CrIBR) were used. Female mice were 5~8 weeks old whereas male was 9~13 weeks old (OrientBio Co., City, South Korea). Upon arrival, experimental animals were acclimated to breeding conditions over a week to minimize stress in advance to implement experiments. For animals, lights were maintained on a 12:12 h light/dark cycle (Light: 7:00~19:00, Dark: 19:00~7:00). Temperature and humidity were maintained at 22~24°C and 40~50%, respectively. Mice were fed ad libitum and animal handling and experiments followed the guideline of Daegu University Animal Experiment Ethics Committee (DUIACC-2014-02-0509-101).

2. B6D2F1 mice culture media and oil

The G1 plus culture media (Vitrolife, Sweden) and the Cleavage medium (CM) (COOk Medical, Austraila) were used up to 2PN~8 cell stages. After, the G2 plus culture media (Vitrolife, Sweden) and the Blastocyst medium (BM) (COOk Medical, Australia) were utilized from 8 cell stage through blastula stage. Suspension culture media for sperms was the GIVF (Vitrolife, Sweden), while capacitation sperm and in vitro fertilization culture media was the fertilization medium (FM, COOk Medical, Australia). Last, the OVOIL (Vitrolife, 10029, Sweden) and the OIL (Sage, 4008-5P, USA) were used to coat culture media.

3. Preparation of mice oocytes

To achieve more oocytes, animals were subjected to superovulation in which female B6D2F1 mice (5~8 weeks old) were injected with 7.5 IU of pregnant mare's serum gonadotropin (PMSG, SIGMA, USA) 48 hr prior to oocyte maturation experiments. In order to improve experimental accuracy and minimize variations, the hormone was injected at 4:00 in the afternoon. After 48 hr of hormone injection, animals were killed through dislocation of the cervical vertebrae to collect ovaries. Upon collection, excessive blood and adipose tissues of collected ovaries (or epididymis) were removed using

sterilized gauze and then transferred to the laboratory. Experiments were performed in the Infertility Clinic of Obstetrics and Genecology, Kyungpook National University Hospital. Ovaries were placed in Eppendorf test tubes containing DPBS (Dulbecco's Phosphate-Buffered Saline, Gibco, USA) + 10% FBS to be transferred. The test tubes were covered with aluminum foil to block light and then further covered with sterilized gauze; to maintain the ovaries at specific temperature (i.e., 37°C), the test tubes were directly attached to body while transporting. On the other hand, epididymis samples were stored in Eppendorf test tubes containing mineral oil (Sigma, USA) and transferred at either at 4 °C or 37°C. To keep the temperature at 4°C, test tubes were stored in a Styrofoam box containing ice packs (Fig. 1) whereas the other case, 37°C, was similar to that of ovary transport. Transferred ovaries were moved to culture dishes (Falcon, USA) containing 2 ml of PBS + 10% FSB and folliculus were ruptures using a syringe needle under a dissecting microscope to collect immature oocytes. Likewise, release of sperm pellet was induced from epididymis in GIVF culture media.

4. *In Vitro* Maturation (IVM)

Collected B6D2F1 mice oocytes were washed once more using PBS buffer supplemented with 20% FBS and then subjected to maturation culture. As depicted in the Figure 1, oocytes were classified into 1) Cumulus-oocyte complexes (COC), 2) Cumulus-oocyte partial contact (CO), or 3) Denuded oocyte (O). The COC was defined if oocytes were densely and uniformly covered by cumulus cells. The CO and O were defined in which oocytes were either partially covered or not covered by the cumulus cells, respectively. In order to compare effects of types of oils, the OVOIL or OIL was applied to 50 uL of droplet to perform in vitro maturation of COC. Approximately after 19 hours of in vitro maturation, dilated cumulus cells around oocytes were removed and the maturation degree was determined as follow: 1) metaphase II (MII) where the first polar body was released, 2) anaphase I (AI) where the first polar body has not been released, or 3) metaphase I (MI) where the first polar body has not been formed. Selected COC were subjected to further in vitro fertilization experiments.

5. *In Vitro* Fertilization (IVF)

As shown in the Figure 1, remaining mineral oil on transferred epididymis was removed using sterilized gauze. In order to induce suspension, sperms were transferred in 100 uL of sperm suspension culture media (GIVF), spread with the OVOIL; and, under a dissecting microscope, epididymis was ruptured using a dissecting forceps and syringe needle to release sperm pellets. After, sperm suspension was induced by placing samples in an incubator maintaining 37°C and 6% CO_2 (Heraeus BB6620, Germany) for 15 minutes. Suspension solution (100 uL) was then transferred to the capacitation culture media (FM) and incubated over 1.5 hours to perform sperm capacitation. While capacitating, sperm concentrations and their activity were monitored using a sperm counter (Makler counting chamber, Sefi Medical Instruments, Israel) and inverted microscope (Olympus, BX-50, Japan), respectively. To investigate effects of transfer temperature of epididymis, sperms acquired from different temperature conditions (i.e., 4°C vs 37°C) were compared. The sperm was applied into fertilization culture media

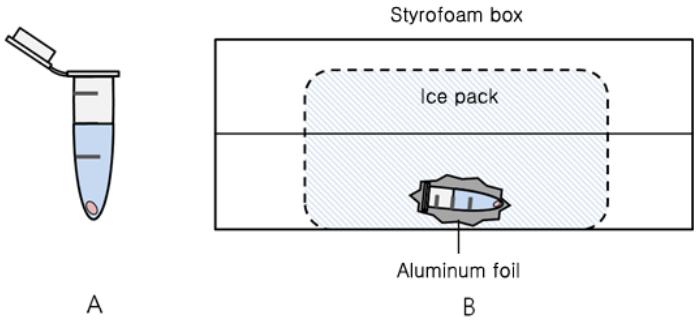

Fig. 1. Prepare of organ transport.
A: Preparing for the transport eppendorf tube.
B: 4℃ epididymis transportation methods by using styrofoam box.

at a concentration of 2 × 10^6/mL and the fertilization experiment was carried out over 4 ~ 6 hours.

6. *In Vitro* Culture (IVC) and differential fluorescence staining of blastocysts

In vitro fertilization of B6D2F1 mice oocytes was implemented and then confirmed after 4 ~ 6 hours later. First, oocytes were washed and then sperms and cumulus cells were removed on a tissue culture dish containing washing culture media. Washed oocytes were transferred into *in vitro* culture media and fertilization was determined based on formation of male and female pronucleus an inverted differential interference microscope (BX-71, Olympus, Japan). Fertilization and embryonic development were judged depending upon formation of two pronucleous (male and female pronucleous) and cleavage to 2-cell stage, respectively. On the 5th day, differential fluorescence staining of blastocysts was performed to monitor inner cell mass (ICM) and trophectoderm (TE). After 7 days of culture, zona hatched rate of blastocysts as well as their attachment were measured to indirectly assess implantation. During the culture, culture media was changed once every other day and the staining method of B6D2F1 mouse blastocysts was performed according to the method previously described by Park et al. (2014). In brief, following a 7 days incubation period, solution 1 (a mixture of Ham's F-10, 1% Triton X-100 [T9254, Sigma], and 100 μg/mL propidium iodide [P4170, Sigma]) and solution 2 (a mixture of 99.9% ethanol [Merck, Germany], 25 μg bisbenzimide [B2883, Sigma]) were used for staining. The blastocyst embryo was stained following exposure to solution 1 for 15 second, and to solution 2 in 4°C atmosphere for 2 hours. The stained blastocyst was washed in glycerol (G2025, Sigma) and transferred to a slide glass for inspection under a fluorescent microscope (BX50, Olympus, Tokyo, Japan) (Fig. 2). Cultured embryos were checked every day and abnormally developed or dead embryos were removed.

7. Statistical analysis

In order to remove any potential bias, experiment batches and observations were planned in a completely randomized design. Experimental results were expressed as percentage and standard deviation of discontinuous variables and statistical significance were tested using the SAS program (Statistics Analytical System, version 9.4, USA). Statistical significance between groups was examined using the LSD and Duncan's multiple range tests and p-value less than 0.05 was considered as statistically significant.

RESULTS

1. Effects of transfer temperature of B6D2F1 mouse epididymis on survival rate of sperm

This experiment was done to confirm the survival rate of sperm in response to different transfer temperature of epididymis (4°C vs. 37°C). The survival rates of B6D2F1 mouse sperms are summarized in the Table 1. It was 59.0% ± 0.1 and 47.6% ± 0.1 in the 4°C and 37°C group, respectively. Although the survival rate was slightly higher in the 4°C group yet no statistical significance was noted between groups.

2. Effects of transfer temperature of B6D2F1 mouse epididymis on fertilization rate, cleavage rate, and blastocyst rate

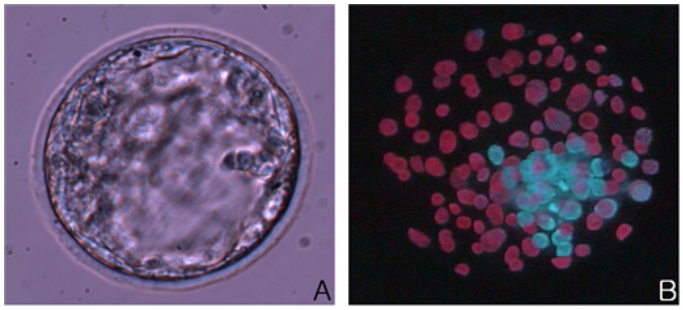

Fig. 2. Differential fluorescence staining of B6D2F1 mouse blastocyst (x200).
A: blastocyst from B6D2F1 mouse; B: stained blastocyst by propidium iodide and bisbenzimide, TE cells stained by propidium iodide (PI, Red), ICM cells stained by bisbenzimide (Blue).

In order to find if transfer temperature of B6D2F1 mouse epididymis influence on fertilization rate, cleavage rate, and blastocyst rate, immature oocytes were matured in vitro and, after 24 hr of fertilization, completely cleaved 2-cell stage embryos were used for the experiment. In each group, a total of 100 embryos were included and respective effects on fertilization rate, cleavage rate, and blastocyst rate are shown in the Table 2. As shown, depending on the transfer temperature (4°C vs. 37°C), the fertilization rate and cleavage rate were 90.7% ± 0.1 vs. 90.7% ± 0.1 and 90.0% ± 0.1 vs. 90.0% ± 0.1, respectively. Further the blastocyst rate was 53.1% ± 0.2 and 52.3% ± 0.2 in the 4°C and 37°C group respectively. There was no statistical significance noted between groups. Even though it was not statistically significant, the blastocyst rate in the 4°C group tends to be slightly higher compared to that of 37°C group.

3. Effects of transfer temperature of B6D2F1 mouse epididymis on cell number of B6D2F1 mice blastocysts after 5 days of *in vitro* fertilization

On the 5th day of in vitro fertilization, blastocysts were subjected to differential fluorescence staining and then cell numbers were compared between groups (i.e., 4°C vs. 37°C; Table 3). Regardless of the transfer temperature, there was no

Table 1. Effect of epididymis transport temperatures on sperm survival rate of B6D2F1 mice

Variables	Groups		*P*-value
	4℃	37℃	
Examination (n)	9	9	-
Sperm concentration (n)	24×10⁶ ±11.8	31×10⁶ ±14.6	-
Sperm survival rate (%)	59.0±0.1	47.6±0.1	*NS*

Each value is mean±standard deviation.
NS: means no significant difference between groups.

Table 2. Effect of epididymis transport temperatures on fertilization rate, cleavage rate, and blastocyst rate of B6D2F1 mice oocytes

Variables	Groups		*P*-value
	4℃	37℃	
No. of used oocytes (n)	130	130	-
Fertilization rate (%)	90.7±0.1	90.7±0.1	*NS*
Cleavage rate (%)	90.0±0.1	90.0±0.1	*NS*
Blastocyst rate (%)	53.1±0.1	52.3±0.1	*NS*

Each value is mean±standard deviation.
NS: means no significant difference between groups.

Table 3. Effect of epididymis transport temperatures on cell number of B6D2F1 mice blastocysts after 5days *in vitro* fertilization

Variables		Groups		*P*-value
		4℃	37℃	
Blastocysts (n)		22	23	-
Cell numbers (n)	ICM[1]	17.0±7.8	14.6±8.6	*NS*
	TE[2]	55.8±29.8	64.0±24.4	*NS*
	Total	72.7±31.6	60.0±36.6	*NS*
% ICM of total cells		27.0±0.1	18.3±0.1	<0.05
ICM:TE ratio (1:X)		4.2±4.1	6.4±7.2	*NS*

Each value is mean±standard deviation.
NS: means no significant difference between groups.
[1]: Inner cell mass.
[2]: Trophectoderm.

difference in ICM of blastocysts, developed from fertilized B6D2F1 mouse oocytes (17.0 ± 7.8 vs. 14.6 ± 8.6), TE cells (55.8 ± 29.8 vs. 64.0 ±24.4), total cell numbers (72.7 ± 31.6 vs. 62.0 ± 36.6) and ICM:TE ratio (1:4.2 ± 4.1 vs. 1:6.4 ± 7.2). Although ICM of blastocysts were higher in the 4°C group TE cell numbers were higher in the other group. On the other hand, % ICM was significantly higher in the 4°C group (27.0% ± 0.1) compared to the 37°C group (18.3% ± 0.1; $p<0.05$; Table 3).

4. Effects of transfer temperature of B6D2F1 mouse epididymis on zona hatched rate of blastocysts after 7 days of *in vitro* fertilization

To find effects of different transfer temperature of B6D2F1 mouse epididymis on zona hatched rate, B6D2F1 mouse embryos were fertilized and then cultured for 7 days. Results are summarized in the Figure 3. As shown, the hatched rate of B6D2F1 mouse blastocysts was higher in the 4°C group compared to the 37°C group (47.8% ± 0.1 vs. 25.6% ± 0.1; $p<0.05$).

DISCUSSION

Over last several decades, human in vitro fertilization and embryos culture techniques have been rapidly improved including its related culture techniques, culture apparatus as well as culture materials. However, origins of raw materials and their quality, used for human in vitro fertilization and embryo culture can slightly vary depending on manufacturers. And, oocytes and embryos are continuously exposed to coated oil and plastic dishes. For instance, it was addressed in multiple previous reports that negative effects might be shown by toxic substances from culture media; further secreted oil from cumulus cells may influence on absorptivity as well (Erbach et al., 1995; Miller and Pursel, 1987; Reinsberg et al., 2004; Shimada et al., 2002; Yoon et al., 2006). In addition, other studies have noted that steroid concentrations particularly contribute to acquisition of development ability of oocytes that is directly related with in vitro maturation division (Erbach et al., 1995; Xu et al., 1988; Zhang and Armstrong, 1989). Therefore, continuous efforts are being made for materials to minimize such negative effects on oocytes culture and *in vitro* fertilization of animal oocytes.

In the present study, the authors examined effects of transfer temperature (4°C vs. 37°C) of B6D2F1 mouse epididymis on sperm survival rate, and embryonic development after in vitro fertilization. As aforementioned, even though the survival rate of sperms was slightly higher in the 4°C group compared to the 37°C group yet no statistical significance was shown. In contrast, the blastulation rate was shown to be slightly higher in the 37°C group than the 4°C group. Jishage and Suzuki (1993) demonstrated that there was no difference in fertilization rate

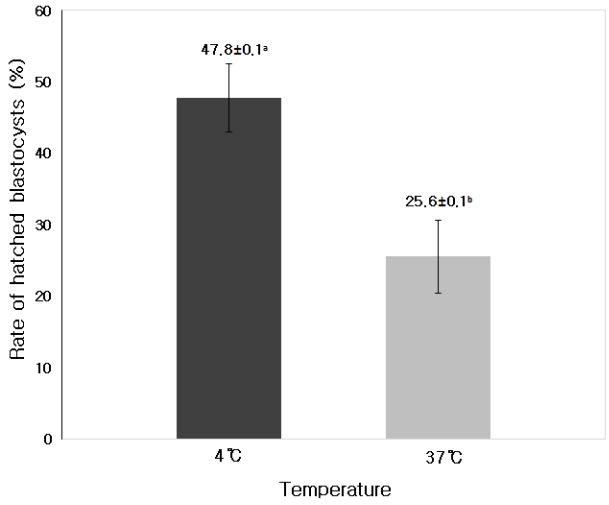

Fig. 3. Effect of epididymis transport temperatures on zona hatched rate of B6D2F1 mice blastocysts. Each value is mean±standard deviation.
[a,b]: Means with different superscript within a row were significantly

when ICR mouse sperms were stored at 4°C and 37°C and then subjected to the fertilization on following day. Nichi et al. (2006), however, noted that higher blastulation rate was achieved in which cow epididymis was stored at 4°C thus temperature that sperms are exposed to might be a significant factor. Sankai et al. (2001), on the other hand, described that mobility of ICR mouse sperms tends to decrease if storage temperature was elevated from 5°C up to 20°C, possibly due to changes in metabolic activity in response to temperature alteration. Taken together, it seems that there are inconsistent results reported with regards to effects of temperature.

No differences in ICM cell numbers of blastocysts, TE cells, total cell counts, and ICM:TE ratio were demonstrated between difference temperature, tested in the study. Of note, although ICM cell numbers of blastocysts were slightly higher in the 4°C group yet TE cells were higher in the other group. Lastly, %ICM and zona hatched rate of blastocysts were higher in the 4°C group ($p<0.05$). ICM cells are developed to fetus after implantation while TE cells become amnions. Thus higher ICM cell numbers and ratio are considered as fetus with excellent development ability (Park et al., 2002). In this regards, it seems that transport epididymis at 4°C may provide better outcomes given its higher % ICM.

Taken altogether, transportation of epididymis at 4°C might be better in later development of mouse blastocysts as opposed to 37°C. Given our results herein, it might be also applicable for long-distance transportation of epididymis spermatozoa as well. Further, our results may provide important preliminary data for not limited to optimizing B6D2F1 mouse in vitro fertilization yet also human in vitro fertilization and embryonic development.

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