ISSN 2508-755X (Print) / ISSN 2288-0178 (Online)
J. Emb. Trans. (2018) Vol. 33, No. 4, pp. 205~210 https://doi.org/10.12750/JET.2018.33.4.205

Effect of OVOIL oil on B6D2F1 Mice Oogenesis

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Abstract

This study was performed to investigate the effect of types of oil (OVOIL vs. OIL) on B6D2F1 mice oogenesis. In this study, B6D2F1 F1 mice were used in order to maximize oogenesis. The expansion rate of cumulus cells (82.0%±0.2 vs. 78.0%±0.1), *in vitro* fertilization rate (92.0%±0.1 vs. 88.0%±0.1), developmental rate (91.0%±0.1 vs. 87.0%±0.2), blastocysts formation rate (56.0%±0.1 vs. 57.0%±0.1), and zona hatched rate(41.4%±0.2 vs. 24.0%±0.1) were not different between groups (NS; $P>0.05$). However, there was a significant difference in maturation rate; the OVOIL group showed higher maturation rate compared to that of the OIL group (96.0%±0.1 vs. 87.0%±0.1; $P<0.05$). In the blastocysts cell numbers, the total cell numbers (83.9±26.1 vs. 56.9±23.9), ICM cell numbers (15.7±8.8 vs. 6.3±3.5), TE cell numbers (68.3±25.7 vs. 50.7±24.1), % ICM (21.6%±0.1 vs. 12.7%±0.1), and the ratio of ICM:TE (1:6.2±6.5 vs. 1:10.3±7.0) were significantly higher in the OVOIL group than the OIL group ($P<0.05$).

These results suggested that it is expected to achieve the more developmental ability of B6D2F1 mice depending on the type of oil (OVOIL vs. OIL). In addition, the results can provide essential information for culture condition on B6D2F1 mice. Henceforth, thus, it is expected that these results herein might be used for *in vitro* culture of human embryos.

Received : 27 November 2018
Revised : 2 December 2018
Accepted : 14 December 2018

Key Words : B6D2F1 mice, OVOIL, in vitro fertilization, inner cell mass, ogenesis

! This research was supported by the Daegu University Research Grant, 2015.

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INTRODUCTION

The culture system of animal ovum and fertilized egg is a crucial process to breeding and reproduction technologies, especially in vitro fertilization and embryo implantation. It is true, however, that there are many obstacles to overcome in technologies for *in vitro* fertilization and embryonic development inducement. Mammalian preimplantation embryos are sensitive to their environment and that conditions of culture can affect future growth and developmental potential both pre- and postnatally and culture conditions during bovine in vitro embryo production can impact somewhat on the developmental potential of the early embryo, the intrinsic quality of the oocyte is the key factor determining the proportion of oocytes developing to the blastocyst stage (Lonergan *et al*., 1999). In livestock species, it is important to devise nutritional strategies that improve reproductive efficiency and the quality of offspring but that do not add to the environmental footprint of the production system and which recognize likely changes in feedstuff availability arising from predicted changes in climate (Ashworth *et al*., 2009).

Nonbiological factors can be subdivided into environmental factors and equipment factors. Environmental factors include the types of culture medium, types of oil, and amount of culture medium. Equipment factors are experimental equipment such as incubator and microscope (Reinsberg *et al*., 2004; Sifer *et al*., 2009). The culture medium used for IVF treatment has a significant effect not only on early embryonic development, but also on subsequent fetal development and the newborn child. A period as short as 2 - 3 days of *in vitro* culture of human embryos can have a significant effect on birthweight of the resulting children (Dumoulin *et al*., 2010).

Furthermore, in several papers, high-quality sterile filtered light paraffin oil is superior to the conservative oil overlaying and co-culture system has positive effect on the in vitro development (i.e., blastocyst development, hatching development rates and cell number) of pre-implantation embryos and enhancement of vitrification methods (Tae *et al*., 2006; Van Soom *et al*., 2001). When it comes to elements influencing mouse sperms, in optimal sperm culture condition, the sperm could survived longer and were preserved sperm motility and membrane integrity (You *et al*., 2009).

As mentioned above, each research lab has different methods of processing eggs and sperm and there is no certain set of standards determining which method is good. Moreover, opinions are contrary to each other about the effect of environmental factors on embryo development. Unlike others, B6D2F1 mice, on the other hand, has been known for outstanding growing rate from 2 cells through blastocysts compared to other rodent models thus enables a wide range of essential experiments through effective production of blastocysts.

In this situation, no experimental result has been released yet on the effect of types of oil on B6D2F1 mice eggs and post-in vitro fertilization embryogenesis. Against this backdrop, the study utilized B6D2F1 mice and experimented on the effect of types of oil on eggs' in vitro maturation and post-in vitro fertilization embryogenesis.

MATERIALS AND METHODS

Animals

For this study, hybrid B6D2F1 mice were used. Female mice were 5~8 weeks old while male was 10~13 weeks old (Orient Bio Co., City, South Korea). For mice, lights were kept on a 12:12 h light/dark cycle. 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).

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). Lastly, the OIL (Sage, 4008-5P, USA) and the OVOIL (Vitrolife, 10029, Sweden) were used to cover culture media.

Preparation of mice oocytes and *In Vitro* Maturation (IVM)

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 hours prior to oocyte maturation experiments. After 48 hr of hormone injection, animals were killed through dislocation of the cervical vertebrae to collect ovaries. Experiments

were performed in the Infertility Clinic of Obstetrics and Genecology, Kyungpook National University Hospital. Ovaries were placed in tubes containing DPBS (Dulbecco's Phosphate-Buffered Saline, Gibco, USA) + 10% FBS to be transferred as previously described (Park *et al*., 2014).

Collected B6D2F1 mice oocytes were washed once more using PBS buffer supplemented with 20% FBS and then subjected to maturation culture. In order to compare effects of types of oils, the OVOIL or OIL was applied to 50 μL of droplet to perform in vitro maturation of Cumulus-oocyte complexes (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 (IVF) experiments.

In Vitro Fertilization (IVF)

IVF was begun 20 hours prior to the start of ovum maturity. Before commencing IVF, male B6D2F1 mice were killed in a cervical dislocation method and their tails of epididymis were collected. Fatti tissues and blood were removed from the collected epididymis tails with sterilized gauze as much as possible. Then, these were transferred to organ culture dish (Falcon, 3037 USA) containing GIVF without 0.5 ml protein source and washed once in out-well containing PBS in organ culture dish. Sperm mass inside the epididymis tails was released using dissecting tweezers and 1 ml insulin syringe where 31 gauges was attached in the IVF chamber. They were cultured in 5% CO_2 culture medium at 37°C for 15 minutes so that the coagulated sperm masses are loosened. After 15 minutes, the culture fluid with untangled sperm masses was cultured again in medium where 0.5 ml protein source was added at 37°C inside 5% or 6% CO_2 culture medium for about 90 minutes to induce capacitation. During the capacitation inducement, we assessed sperm concentration and survival rate using a sperm counter (Markler counting Chamber, Sefi medical instruments, Israel). During the capacitation, maturation cultured eggs were pipetted or cumulus cells were stripped away using 1% Hyaluronidase to utilize eggs whose polocytes were seen clear and distinct for fertilization. Mature eggs were washed in culture dish containing 1 ml culture medium for fertilization then transferred to 50 μl or 100 μl fertilization culture medium droplet to which mineral oil had been applied. Capacitation-induced sperms were injected into fertilization culture medium droplet. The injection amount was 2×10^6/ ml based on alive sperms. Fertilization was conducted for 4~6 hours.

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. Fertilization and embryonic development were judged depending upon formation of two pronucleous (male and female pronucleous) and cleavage to 2-cell stage, respectively as previously described (Lee *et al*., 2016). 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*. (2002).

Statistical analysis

Statistical significance was 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 ranges ($P<0.05$).

RESULTS

In order to investigate the effect of types of oil (OVOIL vs. OIL) on B6D2F1 mice's eggs and embryogenesis, immature oocytes were maturated in vitro for the experiment. For each experiment group, 10~15 embryos were cultured and observed. Table 1 shows the results of B6D2F1 mice ovum maturity rate. B6D2F1 mice's cumulus cell expansion rate in OVOIL group and OIL group was 82.0%±0.2 and 78.0%±0.1, respectively with no significant difference. However, B6D2F1 mice's ovum maturity rate of OVOIL group and OIL group was 96.0%±0.1 and 87.0%±0.1, respectively. The OVOIL group had a significantly higher result than OIL group ($P<0.05$). In addition, to examine the effect of types of oil (OVOIL vs. OIL) on B6D2F1 mice's fertilization rate, development rate and blastocysts formation rate, we maturated immature ova in vitro and utilized only 2-cell stage embryos

Table 1. Effect of different oil on expansion rate and maturation rate of B6D2F1 mice

Variables	Groups		*P*-value
	OVOIL[Producto Ⅰ]	OIL[Producto Ⅱ]	
No. of used oocytes (n)	100	100	-
Expansion rate (%)	82.0±0.2	78.0±0.1	*NS*
Maturation rate (%)	96.0±0.1	87.0±0.1	< 0.05

Each value is mean ± standard deviation.
NS: means no significant difference between groups.
[Producto Ⅰ]: Come from vitro life.
[Producto Ⅱ]: Come from sage.

Table 2. Effect of different oil on fertilization rate, cleavage rate, and blastulation rate of B6D2F1 mice oocytes

Variables	Groups		*P*-value
	OVOIL[Producto Ⅰ]	OIL[Producto Ⅱ]	
No. of used oocytes (n)	100	100	-
Fertilization rate (%)	92.0±0.1	88.0±0.1	*NS*
Cleavage rate (%)	91.0±0.1	87.0±0.2	*NS*
Blastulation rate (%)	56.0±0.1	57.0±0.1	*NS*

Each value is mean ± standard deviation.
NS: means no significant difference between groups.
[Producto Ⅰ]: Come from vitro life.
[Producto Ⅱ]: Come from sage.

Table 3. Effect of different oil on cell numbers of B6D2F1 mice blastocysts

Variables		Groups		*P*-value
		OVOIL[Producto Ⅰ]	OIL[Producto Ⅱ]	
Blastocysts (n)		31	27	-
Cell numbers (n)	ICM[1]	15.7±8.8	6.3±3.5	< *0.05*
	TE[2]	68.3±25.7	50.7±24.1	< 0.05
	Total	83.9±26.1	56.9±23.9	< 0.05
% ICM of total cells		21.6±0.1	12.7±0.1	< 0.05
ICM:TE ratio (1:X)		6.2±6.5	10.3±7.0	< 0.05

Each value is mean ± standard deviation.
NS: means no significant difference between groups.
[Producto Ⅰ]: Come from vitro life.
[Producto Ⅱ]: Come from sage.
[1]: Inner cell mass.
[2]: Trophectoderm.

for the experiment, which had complete blastomere separation after 24 hours from fertilization. One-hundred embryos were cultured for each group and observed. Table 2 shows the results of B6D2F1 mice's fertilization rate, development rate and blastocysts formation rate. As in Table 2, the OVOIL group and OIL group were found to have B6D2F1 mice fertilization rate of 92.0%±0.1 and 88.0%±0.1; development rate, 91.0%±0.1 and 87.0%±0.2; and blastocysts formation rate, 56.0%±0.1 and 57.0%±0.1, respectively without significant differences. But, the OVOIL group had slightly better results than those of the OIL group in terms of B6D2F1 mice's fertilization rate and development rate. To see the effect of types of oil (OVOIL vs. OIL) on B6D2F1 mice's number of blastula cells, we performed dual fluorescent staining on the 5th day of B6D2F1 mice fertilization. The results are presented in Table 3. With respect to the number of ICM cells of B6D2F1 mice's blastulae, the OVOIL group and OIL group were found to have 15.7±8.8 and 6.3±3.5; their number of TE cells was 68.3±25.7 and 50.7±24.1; number of total cells, 83.9±26.1 and 56.9±23.9; % ICM was 21.6%±0.1 and 12.7%±0.1; and the ratio of ICM:TE was 1:6.2±6.5

and 1:10.3±7.0, respectively. These results show that the OVOIL group had higher results than those of the OIL group in all cases (P<0.05). To investigate the effect of OVOIL and OIL on B6D2F1 mice's zona pellucida herniation, B6D2F1 mice's embryos were fertilized and cultured for 7 days. The results are in Figure 1. According to the types of oil, the OVOIL group showed 41.4%±0.2 for the zona hatched rate of blastulae of B6D2F1 mice; and the OIL group, 24.0%±0.1. Though the groups had no significant difference, the OVOIL group showed a higher zona hatched rate than the OIL group.

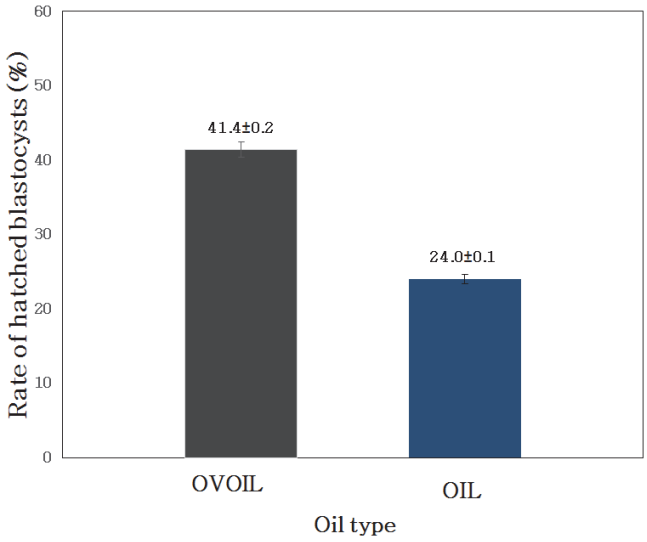

Figure 1. Effect of different oil on zona hatched rate of B6D2F1 mice blastocysts.
Each value is mean ± standard deviation.
OVOIL : Come from vitro life.
OIL : Come from sage.

DISCUSSION

In the last few centuries, *in vitro* fertilization and embryos culture techniques of animals have been fast developed including its related culture skills, culture tools 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 constantly exposed to covered oil and plastic dishes. For example, 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; Reinsberg *et al*., 2004; Yoon *et al*., 2006). Additionally, other studies have renowned that steroid concentrations particularly contribute to acquisition of development ability of oocytes that is directly related with in vitro maturation division (Xu *et al*., 1988; Zhang and Armstrong, 1989; Erbach *et al*., 1995). Hence, constant efforts are being made for materials to minimize such harmful effects on oocytes culture and *in vitro* fertilization of mammal oocytes (Xu *et al*., 1988; Zhang and Armstrong, 1989). A batch of silicone oil (dimethylpolysiloxane) had differential effects on the development of 1- and 2-cell preimplantation mouse embryos in vitro when used as a microdrop overlay over two culture media and the higher concentrations of ethylene diamine tetraacetic acid (EDTA) and bovine serum albumin (BSA) in CZB medium protect against the toxic component in the oil (Erbach *et al*., 1995). The oil is commonly applied to culture medium. Such a method is helpful for osmotic pressure and pH maintenance in culture medium, rapid temperature change prevention and microorganism pollution prevention (Van Soom *et al*., 2001). Also, oil is useful in preventing medium moisture evaporation and facilitating embryo observation, it could sometimes negatively impact culture medium depending upon its raw materials (Van Soom *et al*., 2001). It is also possible that toxic substances are accumulated in culture medium to undermine ovular and embryonic generation. As shown before, oil is one of the important factors in in vitro culture. But each researcher uses various kinds of different oils and their effects on embryogenesis have been reported differently.

In this experiment, the effect of types of oil (OVOIL vs. OIL) on B6D2F1 mice's ovum maturity and post-*in vitro* fertilization embryogenesis *in vitro* was examined. As a result, as mentioned above, OVOIL was found to show better results of ovular and embryonic generation than other types of oil. Therefore, the results of this experiment are expected to be useful fundamental study data for maximizing culture conditions of B6D2F1 mice's *in vitro* fertilization as well as human *in vitro* fertilization and embryogenesis.

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