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Original article

Optimisation of in vitro culture conditions in B6CBF1 mouse embryos

Bi-ke ZHU[a]*, Simon K. WALKER[b], Simon MADDOCKS[c]

[a] Faculty of Veterinary Science, University of Sydney, Camden, NSW 2570, Australia
[b] South Australian Research & Development Institute, Turretfield Research Centre, Rosedale, SA 5350, Australia
[c] South Australian Research & Development Institute, Roseworthy Campus, Roseworthy, SA 5371, Australia

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Abstract – This study was undertaken to investigate the effects of three media, volume and type of oil and frequency of observation on the in vitro development of mouse zygotes. B6CBF1 female mice (4 to 6 wk old) were superovulated using PMSG/hCG and mated with a proven fertile male of the same strain. Putative zygotes with polar bodies were collected from the oviducts of mated mice, 25–28 h after hCG injection, and were cultured in vitro. Embryo development was evaluated at either 96 h and 120 h or every 24 h for 120 h. The results obtained showed that the CZB medium was better than the KSOM and HCO_3HTF media, and the use of 1 mL of paraffin oil was better than the use of 0.5 mL of paraffin oil. The effect of paraffin oil and mineral oil on embryo development was examined and the results indicated that the use of paraffin oil was better than the use of mineral oil. Repeated observations did not influence the proportion of embryos developing to blastocysts.

in vitro development / media / type of oil / frequency of observation / mouse embryos

1. INTRODUCTION

Culture conditions of the in vitro development of embryos have been extensively investigated [1], but the quality of the in vitro produced embryos is still inferior to that of in vivo produced embryos according to the cell counting of blastocysts and embryo transfer. Therefore, the optimising of in vitro culture conditions is an essential pre-requisite if one is to produce preimplantation embryos of high quality that are free of extraneous influences.

An M16 medium [2] was extensively used, but not employed in this study because an early study has shown that the M16 medium is inferior to the CZB medium during the culture of mouse embryos [3]. Based on the rationale that in vitro culture conditions need to be as similar as possible to those found in the oviducts, Quinn et al. [4] developed a culture medium that was biochemically very similar to that of human tubal fluid (HTF). A high proportion of mouse zygotes from (C57BL/6XCBA) F1 has been developed into blastocysts in HCO_3HTF [4]. In order to overcome the phenomenon of the in vitro "2-cell block" of mouse embryos from outbred stains, Chatot et al. [3] formulated a CZB medium on the basis of the composition of the BMOC2 medium [5] that contains a high lactate/pyruvate ratio

* Corresponding author: bzhu7534@usyd.edu.au

with glutamine, but without glucose. This medium was used to culture mouse zygotes from CF1 (CF1XB6SJLF1/J) mice and obtained satisfactory results. Subsequently, a modified version of a simplex optimized medium (SOM) [6, 7] with a high K^+ concentration (KSOM) was established [8]. As a result, most zygotes from CF1 (CF1XB6DF1/CrLBR) mice developed to blastocysts in KSOM [9]. However, the best medium for the culture of mouse zygotes from B6CBF1 mice has not been determined.

Paraffin oil [10–12], mineral oil [13–15] and silicone oil [16, 17] have been broadly used to protect culture medium from evaporation and contamination when early embryos are cultured. Further studies have indicated that the application of oil affects the embryo development either in a positive or a negative way [18, 19]. However, it is not clear which type of oil is the best for in vitro culture under various conditions and whether the volume of oil used affects the development of preimplantation embryos.

It has been reported that removing embryos from the incubator several times a day impedes embryo development [8]. However, repeated observation is required in order to score temporal development of preimplantation embryos when zygotes are cultured in vitro.

Therefore, the experiments were aimed to study the effects of three media (KSOM, CZB and HCO_3HTF), volume and type of oil, and frequency of observation on the in vitro development of mouse zygotes in the B6CBF1 strain.

2. MATERIALS AND METHODS

The procedures used for all experimental animals were approved by the Animal Ethics Committee at the University of Adelaide.

2.1. Superovulation of the mouse, collection and culture of mouse zygotes

B6CBF1 female mice (4 to 6 wk old) were superovulated by intraperitoneal (i.p.) injection of 6 IU PMSG (Folligon, Intervet, Australia) at about 16:00 h, followed 47 to 48 h later by an i.p. injection of 6 IU of hCG (Sigma, Australia).

After the injection of hCG, each superovulated female mouse was paired for mating with a proven male mouse of the same strain. At 25 to 28 h after the hCG injection, mated mice were euthanized by cervical dislocation and the embryos were collected from the oviducts by piercing the swollen portion of the ampulla with a 27-gauge needle or by inserting a 27-gauge needle into the ostium of the oviduct and injecting approximately 0.5 mL of a flushing Hepes-HTF medium (Hepes instead of $NaHCO_3$ in order to maintain a stable pH value outside an incubator). Cumulus cells were dispersed by a brief treatment with Hepes-HTF medium containing 300 units·mL^{-1} of hyaluronidase type IV-S (Sigma, Australia).

Zygotes with polar bodies were selected, washed 3 times in Hepes-HTF, and cultured in 4-well tissue culture dishes (Falcon, Australia) in 50 μL of medium covered with paraffin oil at 37 °C in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2. The developmental progress was recorded at either 96 h and 120 h or every 24 h for 120 h after the beginning of the culture.

2.2. Experimental designs

2.2.1. The effect of culture media (CZB, KSOM and HCO_3HTF) and the volume of paraffin oil on the in vitro development of zygotes

The composition of CZB, KSOM and HCO_3HTF media are given in Table I. Fifteen to twenty zygotes in each well were covered with either 0.5 mL or 1 mL paraffin oil. The number of blastocysts was examined using an Olympus microscope (BO61, Japan), respectively at 96 h and 120 h after the beginning of the in vitro culture. There were three replicates in this experiment.

Table I. The composition (mM) of media used for embryo culture.

	HCO_3HTF	CZB	KSOM
NaCl	101.60	82.70	95.00
KCl	4.69	4.68	2.50
KH_2PO_4	0.37	1.17	0.35
$MgSO_4$	0.20	1.18	0.20
Na Lactate	21.40	30.10	10.00
Na Pyruvate	0.33	0.26	0.20
Glucose	2.78	5.60	0.20
$NaHCO_3$	25.00	25.00	25.00
$CaCl_2$	2.04	1.71	1.71
Glutamine		1.00	1.00
EDTA		0.10	0.01
Penicillin	100 U·mL^{-1}	100 U·mL^{-1}	100 U·mL^{-1}
Streptomycin	50 μg·mL^{-1}	50 μg·mL^{-1}	50 μg·mL^{-1}
BSA	5.0 mg·mL^{-1}	5.0 mg·mL^{-1}	4.0 mg·mL^{-1}

2.2.1.1. Cell count and cell ratio between TE and ICM in in vitro produced blastocysts

The differential fluorescent labeling of TE and ICM in the blastocyst was also investigated in this experiment to further determine the effect of CZB, KSOM and HCO_3HTF on the in vitro developmental potential of the embryos. In order to obtain a great developmental potential of the embryos, 120 h of in vitro culture was defined as the final time point. Blastocysts produced in CZB, KSOM and HCO_3HTF media covered with either 0.5 mL or 1 mL of paraffin oil were stained to differentiate ICM and TE cells, using a modified method of Handyside and Hunter [20]. Briefly, the blastocysts were transferred from the above mentioned three culture media to acid Tyrode solution, under constant observation for 30–60 s, until the zonae pellucidae were completely dissolved. They were then washed 3 times in a Hepes-HTF medium and transferred into a CZB medium containing 10% rabbit anti-mouse whole serum (M5774, Sigma, Australia), and then incubated at 37 °C for 15 min. The embryos were washed 3 times in Hepes-HTF medium and transferred to CZB medium containing 10% guinea pig serum complement (51639, Sigma, Australia) and incubated at 37 °C for 5–6 min. They were washed 3 times in Hepes-HTF medium and stained in CZB medium containing 20 μg·mL^{-1} bisbenzimide (Hoechst dye) (B2883, Sigma, Australia) with 10 μg·mL^{-1} propidium iodide (PI) (P4170, Sigma, Australia) at 37 °C for 30–40 min. After that, the blastocyst was fixed in 1% paraformaldehyde in PBS for 1 min and washed 3 times in Hepes-HTF medium. Then, the embryos (3–6 embryos/slide) were transferred to a glass slide (size: 76.2 × 25.4 mm; thickness: 0.8–1.0 mm) (37101A, Livingstone International Pty. Ltd, Australia) in a droplet of less than 5 μL, covered with a microscope cover glass (size: 22 × 22) and squashed gently with the blunt end of a pen. The area under the cover glass was filled with the Hepes-HTF. Nail varnish (Supershine, Australia) was used to seal the cover glass. Finally, the differentially labeled nuclei were examined quickly using an Olympus BX60 fluorescent microscope with a range of 330 to 385 nm of UV exciting light and of 410 to 600 nm of emission in blue. The total magnification in the eyepiece and photograph was ×400 and ×250, respectively. The PI-labeled nuclei (dead cells or TE cells) appeared red while the Hoechst B2883 labeled nuclei (viable cells or ICM cells) appeared blue because

of different emission spectra of Hoechst B2883 and PI.

2.2.1.2. Transfer of embryos

Embryo transfer was finally employed to determine the in vitro developmental potential of mouse embryos. The proportion of embryos developing to blastocysts and the cell number of in vitro produced blastocysts have shown that CZB covered with 1 mL of paraffin oil produced the best results. Blastocysts cultured for 120 h in CZB and KSOM covered with 1 mL of paraffin oil were only used in this experiment. Briefly, B6CBF1 females were made pseudopregnant by mating in natural cycles with vasectomized B6CBF1 males. The embryos produced in KSOM and CZB covered with 1 mL of paraffin oil were transferred to the uteri of Day 4 pseudopregnant mice, with 5 embryos to each uterine horn. Day 4 embryos flushed from the uteri of the same strain of mice were also transferred into Day 4 pseudopregnant recipients as the control. Fetuses were collected from pregnant mice on Day 18 postfertilization for gross examination.

2.2.2. The effect of paraffin oil and mineral oil on the in vitro development of mouse zygotes

To compare the effect of paraffin and mineral oil on the in vitro development of mouse zygotes, 15 to 20 zygotes in 50 μL of CZB medium within each well of the 4-well tissue culture dish were covered with either 1 mL of paraffin (BDH, Australia) or light mineral oil (M8410, Sigma, Australia) and then cultured using the methods as described in Section 2.1. The number of blastocysts was examined using the previously mentioned microscope, respectively at 96 h and 120 h after in vitro culture. There were two replicates in this experiment.

2.2.3. The effect of the observation frequency on the in vitro development of mouse zygotes

To determine the effect of observation frequency on the in vitro development of mouse embryos, 20 zygotes in 50 μL of CZB medium within each well of the 4-well tissue culture dish were covered with 1 mL of paraffin oil and cultured using the methods as described in Section 2.1. The developmental progress was checked using a microscope previously mentioned at either 96 h and 120 h or every 24 h. There were four replicates in this experiment.

2.3. Data analysis

Comparisons and differences between groups were made using the Chi-square test, T-test and analysis of variance. A significant difference was assumed to exist when the probability due to chance was less than 5%.

3. RESULTS

3.1. The effect of the composition of culture media and volume of paraffin oil on the in vitro development of mouse zygotes

3.1.1. The in vitro development of mouse zygotes in media covered with 0.5 mL of paraffin oil

The results obtained with these three media (KSOM, CZB and HCO_3HTF) covered with 0.5 mL of paraffin oil on the in vitro development of mouse embryos are shown in Table II.

During 96 h of in vitro culture and using 0.5 mL of paraffin oil that just covered the media, the number of embryos developing to blastocysts in CZB and KSOM was much higher than in HCO_3HTF (Tab. II). More embryos developed to blastocysts in either KSOM or in CZB than in HCO_3HTF ($P < 0.001$), but there was no significant difference between KSOM and CZB although the number of blastocysts was higher in CZB than in KSOM (Tab. II).

Compared with 96 h of culture, the total number of embryos developing to blastocysts did not change in CZB and KSOM in

Table II. The effect of three media covered with 0.5 mL of paraffin oil on the development of zygotes.

Developmental stages	Culture media			Time of examination[b] (h)
	KSOM	CZB	HCO_3HTF	
No. zygotes	17.3 ± 1.33	21.7 ± 0.88	24.0 ± 1.53	
M[a]	2.0 ± 0.58	2.0 ± 1.00	6.7 ± 1.76	
B	**9.7 ± 1.76**	**14.0 ± 1.54**	**1.0 ± 0.58*****	96
Others	5.7 ± 1.20	5.7 ± 1.33	16.3 ± 1.20	
M	0.0 ± 0.00	0.0 ± 0.00	0.7 ± 0.67	
B	6.0 ± 1.54	9.3 ± 0.88	5.0 ± 1.15	
HB	3.7 ± 0.67	5.0 ± 0.58	0.0 ± 0.00	120
B+HB	**9.7 ± 1.76**	**17.7 ± 4.70**	**5.0 ± 1.15*****	
Others	5.7 ± 1.20	6.3 ± 1.67	18.3 ± 0.88	

[a] M: morulae, B: blastocysts, HB: hatched blastocysts, others including 2 to 4 cells, abnormal and dead embryos.
[b] Time of examination is indicated as the time after the beginning of in vitro culture.
Values given are mean ± SEM; Values with *** superscripts differ significantly ($P < 0.001$) between KSOM, CZB and HCO_3HTF media, at the blastocyst stage, 96 h after the beginning of culture and at both blastocyst and hatched stages, 120 h after the beginning of culture (the Chi-square test).

120 h of culture. But 21.2% and 30.8% of the embryos cultured in KSOM and CZB developed respectively to hatched blastocysts. In the HCO_3HTF, no zygotes developed to hatched blastocysts; only 20.8% of the embryos developed to blastocysts in 120 h of culture compared with 4.2% of the embryos in 96 h of culture. Further analysis showed that there were highly significant differences ($P < 0.001$) in the proportion of embryos developing to blastocysts plus hatched blastocysts in both KSOM or CZB than in HCO_3HTF ($P < 0.001$) (Tab. II).

3.1.2. The in vitro development of mouse zygotes in three media covered with 1 mL of paraffin oil

During 96 h and 120 h of in vitro culture, and using 1 mL of paraffin oil, no embryos developed to blastocysts in HCO_3HTF (0.0%) (Tab. III). This showed directly that the HCO_3HTF medium was inferior to CZB and KSOM. Therefore, we only analysed and compared the results between CZB and KSOM. During 96 h of in vitro culture, 91.7% and 65.8% of the zygotes developed respectively to blastocysts in CZB and KSOM. These differences in the development of blastocysts between CZB and KSOM were highly significant and the proportion of embryos developing to the blastocyst stage was greater in CZB than KSOM ($P < 0.001$) (Tab. III).

During 120 h of in vitro culture, the blastocyst rate was significantly higher ($P < 0.02$) in CZB than in KSOM although there were no significant differences in the development to hatched blastocysts between KSOM and CZB (Tab. III).

3.1.3. The effect of volumes of paraffin oil on the in vitro development of mouse zygotes

When using CZB and 96 h of in vitro culture, the number of embryos developing to blastocysts was significantly higher ($P < 0.001$) when covered with 1 mL of paraffin oil than in the medium covered with 0.5 mL of paraffin oil (Tab. IV), but using KSOM, this difference was not significant (Tab. V).

During 120 h of in vitro culture, more zygotes developed to blastocysts plus hatched blastocysts in KSOM ($P < 0.05$) or CZB

Table III. The effect of three media covered with 1 mL of paraffin oil on the development of zygotes.

Developmental stages	Culture media			Time of examination[b] (h)
	KSOM	CZB	HCO_3HTF	
No. zygotes	37.0 ± 2.00	36.0 ± 1.00	22.3 ± 0.58	
M[a]	5.3 ± 1.53	0.3 ± 0.58	0.3 ± 0.58	
B	**27.7 ± 3.79**	**33.0 ± 2.65*****	**0.0 ± 0.00**	96
Others	7.3 ± 3.21	2.7 ± 1.53	22.0 ± 0.00	
M	0.7 ± 1.15	0.0 ± 0.00	0.0 ± 0.00	
B	**21.7 ± 3.51**	**26.7 ± 1.15**	0.0 ± 0.00	
HB	**6.3 ± 2.89**	**6.3 ± 1.53**	**0.0 ± 0.00**	120
B+HB	**28.0 ± 6.24**	**33.0 ± 2.66****	**0.0 ± 0.00**	
Others	8.3 ± 3.61	3.0 ± 1.73	22.3 ± 0.58	

[a,b] M, B, HB, others and time of examination are the same as in Table II.
Values given are mean ± SEM; Values with ** and *** superscripts differ significantly ($P < 0.01$ and $P < 0.001$) between the KSOM and CZB media (the Chi-square test), respectively at 120 h and 96 h after the beginning of in vitro culture.

Table IV. The effect of the volume of paraffin oil on the development of zygotes in CZB medium.

	Volume of paraffin oil (mL)		Time of examination[b] (h)
	0.5	1.0	
No. zygotes	21.7 ± 0.88	36.0 ± 1.00	
M[a]	2.0 ± 1.00	0.3 ± 0.58	
B	**14.0 ± 1.54**	**33.0 ± 2.65*****	96
B	**9.3 ± 0.88**	**26.7 ± 1.15*****	
HB	**5.0 ± 0.58**	**6.3 ± 1.53***	120
B+HB	**17.7 ± 4.70**	**33.0 ± 2.66****	

[a,b] M, B, HB and time of examination are the same as in Table II.
Values given are mean ± SEM; Values with *, ** and *** superscripts differ significantly ($P < 0.05$–0.001) between CZB covered with 0.5 mL of paraffin oil and CZB covered with 1 mL of paraffin oil (the Chi-square test).

($P < 0.01$) covered with 1 mL of paraffin oil than in the media covered with 0.5 mL of paraffin oil (Tabs. IV and V). However, the number of embryos developing to hatched blastocysts only in CZB was higher ($P < 0.05$) if 0.5 mL oil was used (Tab. IV). In addition, 20.8% zygotes developed to blastocysts in culture of 120 h in HCO_3HTF covered with 0.5 mL of paraffin oil, while no embryos developed to blastocysts in HCO_3HTF covered with 1 mL of paraffin oil.

3.1.4. The effect of three media on the cell number of blastocysts

The effect of three media on the cell number of blastocysts, 120 h after the beginning of in vitro culture was also determined in this experiment.

When using 0.5 mL of paraffin oil, the number of TE, ICM and TE + ICM in blastocysts produced in either CZB or KSOM were highly significant ($P < 0.001$) than in HCO_3HTF (Tab. VI). The number of TE

Table V. The effect of the volume of paraffin oil on the development of zygotes in KSOM medium.

	Volume of paraffin oil (mL)		Time of examination[b] (h)
	0.5	1.0	
No. zygotes	17.3 ± 1.33	37.0 ± 2.00	
M[a]	2.0 ± 0.58	5.3 ± 1.53	
B	9.7 ± 1.76	27.7 ± 3.79	96
B	**6.0 ± 1.54**	**21.7 ± 3.51****	
HB	3.7 ± 0.67	6.3 ± 2.89	120
B+HB	**9.7 ± 1.76**	**28.0 ± 6.24***	

[a,b] M, B, HB and time of examination are the same as in Table II.
Values given are mean ± SEM; Values with * and ** superscript differ significantly ($P < 0.05$–0.01) between KSOM covered with 1 mL of paraffin oil and KSOM covered 0.5 mL of paraffin oil (the Chi-square test).

Table VI. The cell number of blastocysts obtained in three media covered with 0.5 mL of paraffin oil.

Media	No.B[a]	TE	ICM	Ratio (ICM/TE)	Total No. TE + ICM
CZB	12	34.7 ± 6.14	21.4 ± 3.5	0.63 ± 0.12	56.1 ± 8.33
KSOM	11	28.9 ± 4.89*	19.6 ± 1.57	0.69 ± 0.11	48.5 ± 5.17*
HCO_3HTF	10	21.4 ± 4.12***	16.1 ± 2.51***	0.83 ± 0.43	37.2 ± 3.49***

[a] B: blastocysts produced, 120 h after the beginning of culture in vitro; TE: trophectoderm; ICM: inner cell mass.
The values given are mean ± SEM; the values given in the ratio are the sum of addition of ICM/TE ratio in each embryo divided by the total number of blastocysts used.
Values with * superscript differ significantly ($P < 0.05$) between either the number of TE or TE + ICM in CZB and those in KSOM, with *** superscripts differ significantly ($P < 0.001$) between the number of TE, ICM and TE + ICM in either CZB or KSOM and those in HCO_3HTF (ANOVA analysis).

and TE + ICM cells in the embryos produced in CZB was higher ($P < 0.05$) than in KSOM (Tab. VI). However, the ratio of ICM/TE was not significantly different among the three media (Tab. VI).

The results given in Table VII indicate that the number of TE, ICM and TE + ICM cells in embryos cultured in the CZB medium covered with 1 mL of paraffin oil was higher than in the KSOM medium, but the ratio of ICM/TE in the CZB medium was lower than in KSOM. Further analyses showed that there were significant differences between the number of ICM ($P < 0.05$) and TE + ICM ($P < 0.01$) in CZB and those in KSOM. Furthermore, there were no significant differences between the cell number of embryos produced in CZB covered with 1 mL and 0.5 mL of paraffin oil, although the number of TE, ICM and TE + ICM was higher in CZB covered with 1 mL of paraffin oil than with 0.5 mL of paraffin oil.

3.1.5. Embryo transfer

Embryo transfer was conducted to further determine the development potential of blastocysts produced in CZB and KSOM only covered with 1 mL of paraffin oil, 120 h after the beginning of in vitro culture because the number of blastocysts and cell number in the blastocyst were lower in the three media used, covered with 0.5 mL of

Table VII. The cell number of blastocysts obtained in two media covered with 1 mL of paraffin oil.

Media	N.B[a]	TE	ICM	Ratio (ICM/TE)	Total No. TE + ICM
CZB	16	40.25 ± 17.91	24.94 ± 8.10	0.72 ± 0.32	65.31 ± 21.25
KSOM	33	30.92 ± 16.32	17.82 ± 10.27*	0.85 ± 0.83	49.09 ± 17.08**

[a] B, TE, ICM and Ratio (ICM/TE) are the same as in Table VI.
Values given are mean ± SEM; Values with* and ** superscripts differ significantly ($P < 0.05$ and $P < 0.01$, respectively) between the number of ICM in CZB and that in KSOM and between the total number of TE + ICM in CZB and that in KSOM, respectively (ANOVA analysis).

Table VIII. Transfer of blastocysts produced in vitro in media covered with 1 mL of paraffin oil and those produced in vivo (the control).

Embryo type	No. embryos	Embryo No/recipient	No. implantation failure[c]	No. resorption	No. normal fetuses
Control	60	10.0 ± 0.00*	1.7 ± 0.33	0.0 ± 0.00	8.3 ± 0.33
KSOM	60	10.0 ± 0.00	5.0 ± 0.58	2.0 ± 0.58	3.0 ± 1.30[a,b]
CZB	60	10.0 ± 0.00	4.0 ± 0.58	1.0 ± 0.58	5.0 ± 1.20[a]

[c] Implantation failure was determined based on the presence of placenta or embryo trace at the implantation site.
* Sixty blastocysts were used in each group, each recipient mouse received 10 embryos and the values given are mean ± SEM in each recipient mouse.
Values with [a] superscript differ significantly ($P < 0.001$) between the control and KSOM and CZB; values with [b] superscript differ significantly ($P < 0.05$) between KSOM and CZB (ANOVA analysis).

paraffin oil than with 1 mL of paraffin oil. The results presented in Table VIII showed that the number of viable fetuses after transfer of the embryos produced in CZB was higher ($P < 0.05$) than in KSOM.

3.2. The effects of the types of oil on the in vitro development of mouse zygotes

To examine the influence of oil (paraffin vs. mineral) on embryo development, embryos were cultured in CZB medium covered with either 1 mL of paraffin or 1 mL of mineral oil.

During 96 h and 120 h of in vitro culture, the proportion of embryos developing to blastocysts was significantly higher using paraffin oil than when using mineral oil ($P < 0.001$) (Tab. IX).

3.3. The effects of the number of observations on the in vitro development of mouse zygotes

The effect of observation times on embryo development was also determined. After 96 h and 120 h of in vitro culture, the proportion of embryos developing to blastocysts was similar whether the embryos were observed only at these times or at 24, 48 and 72 h as well (Tab. X).

4. DISCUSSION

The proportion of embryos developing to blastocysts (96 h) and blastocysts plus hatched blastocysts during 120 h in culture (Tabs. II–V) was similar to those reported by Chatot et al. [3] using CZB, but lower

Table IX. The effects of the types of oil on the development of zygotes in CZB medium.

	Paraffin oil	Mineral oil	T- test	*P*	Time of examination[b] (h)
No. zygotes	24.0 ± 1.00	20.0 ± 0.00			
M	2.5 ± 0.50	8.0 ± 1.00			
B	**18.5 ± 1.50**	**3.5 ± 0.50**	**4.3**	***P* < 0.025**	96
Others	3.0 ± 1.00	8.5 ± 1.50			
B	**20.5 ± 2.50**	**7.0 ± 1.00**	**3.64**	***P* < 0.05**	120
Others	3.5 ± 1.50	13.0 ± 1.00			

[a,b] M, B, and time of examination are the same as in Table II; others including 2 to 4 cell, abnormal and dead embryos.
The values given are mean ± SEM.

Table X. The effect of observing frequency on the development of zygotes.

Time of examination[b] (h)	Developmental stage	No. zygotes		X^2	*P*
		54.0 ± 1.15	38.3 ± 0.67		
24	2-C[a]	36.0 ± 0.58			
	4-C	2.3 ± 0.33			
48	8-C	0.7 ± 0.33			
	M	34.3 ± 1.20			
72	M	25.3 ± 0.88			
	EB	11.7 ± 1.45			
96	M	3.0 ± 0.58	0.7 ± 0.67		
	B	**43.3 ± 0.88**	**31.3 ± 2.33**	**0.2370**	**NS**
120	M	0.7 ± 0.67	0.7 ± 0.67		
	B	**45.7 ± 0.67**	**33.3 ± 0.88**	**0.0624**	**NS**

[a,b] M, B and time or examination are the same as in Table II C and EB are respectively referred to as cell and early blastocysts. The values given are mean ± SEM.

than those reported by Erbach et al. [9] using KSOM and by Quinn et al. [4] using HCO_3HTF. These differences could be related to different compositions of the three media and the mouse stains used.

It is known that the main differences among these media are in the concentrations of NaCl and glucose and the addition of glutamine and ethylenediaminetetraacetic acid (EDTA) (Tab. I). High concentrations (125 mM) of NaCl in culture media has been reported to decrease the rates of protein synthesis, change the patterns of protein synthesis and reduce the rates of mRNA synthesis and its stability, and the relative amount of mRNA for IGF-I,

IGF-II and their cognate receptors [21]. These changes of protein and mRNA synthesis subsequently inhibit the development of mouse zygotes to blastocysts [6–7] since the development and differentiation of preimplantation embryos require protein synthesis. In contrast, the media containing a low sodium concentration (85 mM) may overcome these problems and support embryo development [22, 23]. The CZB medium found to be the most appropriate in the present study contains only 82 mM of NaCl. In contrast, the KSOM medium contains 95 mM of NaCl which is the lower than 125 mM of NaCl, but higher than 82 mM of NaCl in CZB. This could contribute to lower the developmental rates of embryos in KSOM than in CZB.

In the mouse, zygotes can utilize only pyruvate (or oxaloacetate) as energy sources, but lactate can support the development from the 2-cell stage onwards and together with glutamine helps support in vitro development. Many studies have indicated an increase in glucose uptake between the 1-cell and 2-cell stage, but not between the 2- and 8-cell stage, and that glucose can be used as the sole substrate from the 8-cell stage onwards [15, 24]. In this experiment, glucose concentrations in CZB, KSOM and HCO_3HTF were 5.6, 0.2 and 2.78 mM, respectively (Tab. I) and the high concentrations of glucose in CZB might better support the development of mouse zygotes onward because there was a higher proportion of 2-cell embryos in KSOM and HCO_3HTF than CZB, during 96 h and 120 h of in vitro culture.

Glutamine may improve the development of mouse zygotes from outbred strains [3]. Similar beneficial effects of glutamine on embryonic development have been reported in other species, including the hamster [18, 25], rabbit [26], pig [27] and sheep [28]. The role of glutamine in embryonic development may be to act as a source of amine groups for transamination and/or as a source of energy, and a regulator of the detrimental effects of excessively high NaCl concentrations [16, 22]. The combination of 1 mM of glutamine with 105 mM of NaCl appears to be optimal [22]. In the present study, glutamine concentrations in CZB, KSOM and HCO_3HTF were respectively 1 mM, 1 mM and 0.0 mM (Tab. I) and our results clearly showed that CZB and KSOM were much better than HCO_3HTF.

Previous studies have indicated that EDTA may exert its beneficial effect on embryo development by chelating toxic metals, or by facilitating the transport of other factors important for embryo development [3, 29–31], or by increasing protein synthesis [32], through its action on the cell surface of the embryo [33]. Furthermore, EDTA may be more important for in vitro embryo development beyond the 2-cell stage than either protein or amino acids [29] and it improves the development of postimplantation embryos [34]. The EDTA content used varies with the different studies reported. Some studies have shown that the optimum EDTA concentration is 10 μM [3, 33] while other studies have indicated that the optimum EDTA concentration is 100 μM [3, 32, 34]. Therefore, one could also assume that such discrepancies indicate that the efficient range of EDTA concentration is very large and not significant between two values (10–100 μM). However, our results showed that CZB containing 100 μM of EDTA was better at supporting embryo development in vitro than KSOM and HCO3HTF respectively containing 10 μM and 0.0 μM of EDTA (Tab. I).

It has been shown that the number of TE and ICM at the blastocyst stage could affect the ability of embryonic development beyond the blastocyst stage [35, 36]. This is supported by the present study. However, the results presented here on the number of TE, ICM and TE + ICM are not in agreement with those previously reported by Erbach et al. [9] who showed that during 96 h of in vitro culture of mouse zygotes, the number of TE, ICM and TE + ICM in the blastocysts was higher in KSOM than in CZB. This difference may be due to the differences between mouse strains, between culture

conditions and the time at which the blastocysts were sampled. The present study has indicated that the ratio of ICM/TE of blastocysts in CZB and KSOM is not different, but a significant difference in the number of ICM, and the total number of ICM plus TE cells between CZB and KSOM may decide the fate of embryo development after embryo transfer. These findings suggest that an appropriate differentiation of ICM and TE cells in the blastocysts may play an important role in subsequent healthy embryonic development.

Culture conditions in in vitro embryo development have been extensively investigated [1], but a little attention has been paid to the volume of oil used. An interesting finding in this study is that the volume of paraffin oil directly affects the subsequent embryo development, depending on the type of media used. In HCO_3HTF medium, the use of 0.5 mL paraffin oil is better than the use of 1 mL paraffin oil. However, in KSOM and CZB, the application of 1 mL paraffin oil is better than the use of 0.5 mL paraffin oil. The exact reasons for this remain elusive, but the use of 1 mL oil might provide a better environment for embryo development, compared with the use of 0.5 mL oil.

The application of mineral oil significantly reduced embryo development compared with the presence of paraffin oil although these two types of oil have been broadly used in embryo culture [2, 10, 11, 37]. A recent study indicates that the use of mineral oil significantly reduces the rate of pig embryos developing to the blastocyst stage after IVF [38]. Adverse effects of mineral oil on the embryo may be that a toxic compound might be present in the oil [19]. In addition, physical properties of the mineral oil (with a density of 0.84 $g \cdot mL^{-1}$) and the paraffin oil (with a density of 0.86 $g \cdot mL^{-1}$) are different. The difference of physical properties between these two types of oils might change the compositions of the culture media, thus influencing embryo development.

During repeated observations, embryos are exposed to light and transient temperature fluctuation as they are moved repeatedly from the incubator to the atmosphere and returned. This could result in an interference with the optimal in vitro development of mouse embryos. It has been shown that increasing the length of exposure of mouse zygotes to room temperature significantly reduces the rate of development to blastocysts since temperature reductions may slow the metabolic processes of zygotes and disrupt their enzyme systems and mitotic spindle formation [1, 39]. However, 2-cell embryos are less affected by temperature fluctuation [39]. While handling conditions in the present studies were optimized to minimize temperature fluctuation by a reduced number of observations, the results in the present study have shown that repeated observations did not subsequently reduce development, compared with those observed only once at the end of the developmental period.

In summary, the results in the present study clearly demonstrate that the CZB medium is better than the KSOM and HCO_3HTF media; the volume of paraffin oil used has the potential to affect the development of preimplantation embryos depending on the type of culture media; paraffin oil is better than mineral oil in protecting zygotes in culture and finally repeated observation does not reduce the embryo developmental potential when the zygotes of B6CBF1 mice are cultured.

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