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Effects of Glucose Concentration on *in vitro* Fertilization in BALB/c Mice

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Contents

BALB/c mice are widely used in genetic, tumour and immunological studies. However, the mice demonstrate a lower reproduction rate, low fertility and small litters, because of their highly genetic homozygoisty. Based on *in vitro* fertilization (IVF), a routine technique for biomedical studies, it is worth to evaluate the effects to BALB/c mice on IVF efficiency. In order to test the genetic factor affecting the IVF efficiency of BALB/c, four reciprocal IVF tests of BALB/cByJ and FVB/NCrl mice were performed. The results showed that the average fertility of IVF sponsored by FVB/NCrl spermatozoa was 69.6%, but only 12.1% was obtained from BALB/cByJ strain. Effect of glucose contained in the culture medium to the IVF efficiency of BALB/cByJ was also evaluated. The results showed that the fertility of BALB/cByJ spermatozoa incubated with 0, 2.7, 5.5, 11.1 and 22.2 mM of glucose in the TYH medium were 6.8, 9.9, 13.9, 32.7 and 22.2%, respectively. It is showed that IVF efficiency of BALB/cByJ spermatozoa could be improved depending on the concentration of glucose in the IVF medium. According to the results, it is beleived that lower IVF of BALB/cByJ mice might be due to the genetic defect in spermatozoa and increasing glucose in the IVF medium which significantly affect the IVF efficiency of BALB/cByl via activating the spermatozoa.

Introduction

BALB/c mice were first inbred in 1913 by Bagg (Staats 1981), and have been widely used in biology and medical research for many decades. BALB/c is suitable for genetic, tumour and immunological investigations with a clear genetic background. BALB/c was inbred for more than 150 generations, showing more than 99% of genetic homozygosity. The BALB/c therefore demonstrated a depressed reproduction, manifest in low fertility and small litters. Large amounts of structurally abnormal spermatozoa are produced in BALB/c (Kishikawa et al. 1999). Some inbred strains of mouse, for example C57BL/Kw and PL/J2-azh/azh, KE, also have a high occurrence of poor-quality spermatozoa (Krzanowska et al. 1995). Interestingly, the breeding performance of BALB/c in natural mating is similar to those of the other inbred mice, for example C57BL/6 and FVB/NCrl; however, *in vitro* fertilization (IVF) was less efficient.

FVB/N mice are useful for the production of transgenic mice on a fully inbred genetic background. They have a vigorous reproductive performance with large litters. Fertilized eggs of FVB/N contain large and prominent pronuclei which facilitate the microinjection of DNA, the survival rate of microinjected eggs is much higher than that of pure-line C57BL/6 (Taketo et al. 1991). The reproduction performance superior than other inbred strains for FVB/Ns. FVB/NCrl was chosen as a control group to clarify the genetic effects on the IVF efficiency with BALB/cByJ in this study.

Carbohydrate metabolism significantly contributes to embryo development of mouse besides genetic factors. Although not essential in the early perimplantation stages of mouse, glucose becomes the predominant energy substrate following the 8-cell stage embryo development (Urner and Sakkas 1996). Reports have shown that fertilization in various species requires glucose, such as in mouse (Hoppe 1976), rats (Niwa and Iritani 1978) and humans (Mahadevan et al. 1997). Sperm employ glucose as a main energy source to attain optimal capacitation and to maintain hyperactivated motility (Cooper 1984). Moreover, glucose metabolism happens during sperm fusion and decondensation into oocyte (Urner and Sakkas 1999). The present study experimentally addresses the effects of glucose concentration in IVF medium on fertility *in vitro* to rectify the efficiency of BABL/c IVF in further practical operations.

Materials and Methods

Animals

BALB/cByJ (60 female, eight male) and FVB/NCrl (48 female, eight male) mice were obtained from the National Laboratory Animal Center (NLAC), Taiwan. All animals were maintained, handled and treated following NRC (1996) guidelines.

Medium preparation

All reagents and chemicals are cell culture grade, and purchased from Sigma (St Louis, MO, USA). Krebs–Ringer bicarbonate medium (Table 1) was employed for IVF treatment by named of TYH (Toyoda et al. 1971); the osmolality of the medium is 290 ± 10 mOsm. Standard TYH medium for IVF is usually supplemented with 5.5 mM glucose during pre-incubation and insemination (Tsuchiya et al. 2001). Thus, the TYH medium contains 5.5 mM glucose as the control concentration in the present study and the medium with 0 and 2.7 mM of glucose were prepared as the low glucose concentration and the medium with 11.1 and 22.2 mM of glucose as high glucose concentration.

Modified-Whitten's medium (MWM) (Whitten 1971) was used for the culture of fertilized eggs. The MWM contains 5.5 mM glucose, 0.05 mM EDTA and 3 mg/ml bovine serum albumin (BSA), the osmolality is 270 ± 10 mOsm.

Table 1. The composition of the TYH medium[a]

Component	Concentration	
	mg/100 ml	mM
NaCl	697.6	119.4
KCl	35.6	4.8
$CaCl_2$	19.0	1.7
$MgSO_4 \cdot 7H_2O$	29.3	1.2
KH_2PO_4	16.2	1.2
$NaHCO_3$	210.6	25.1
Glucose	100.0	5.5[b]
Sodium pyruvate	11.0	1.0
Potassium penicillin G	7.5	
Streptomycin sulphate	5.0	
BSA	400.0	

[a]Krebs–Ringer bicarbonate medium with 5.5 mM glucose was employed for *in vitro* fertilization (IVF) treatment by named of TYH (Toyoda et al. 1971), the osmolality of the medium is 290 ± 10 mOsm.
[b]Glucose concentration in the medium as standard was 5.5 mM. It was modified to 0, 2.7, 5.5, 11.1 and 22.2 mM for studying effect of glucose concentration in the medium on the IVF efficiency of BALB/cByJ and FVB/NCrl mice.

Collection and pre-incubation of spermatozoa

Sexually mature (10–12 weeks old) male mice were killed by cervical dislocation. The epididymides were excised and washed in TYH to remove traces of blood and fat (Wakayama et al. 1996). After the cauda epididymis were cut with a pair of sharp iridectomy scissors, one drop (3–5 μl) of the dense sperm mass was placed in 0.3 ml of pre-warmed TYH and then covered with mineral oil. The suspended sperm capacitated by incubation in 5% CO_2 at 37°C for 1.5 h. The concentration of motile sperm at the end of capacitation was $2–4 \times 10^7$ cells/ml.

Oocytes collection

Oocytes were obtained from 4 to 5 weeks old female mice which were injected with 5 IU PMSG (gonadotropin); 48 h later by injection with 5 IU hCG (human chorionic gonadotropin) for superovulation treatment. The treated mice were killed 13–14 h following hCG injection, and the ampullae of oviducts were removed. Eggs with cumulus cells were dissected out from the ampullae and deposited in the TYH medium under oil in preparation for IVF.

In vitro fertilization

Following pre-incubation, the sperm suspensions were placed into the dish that contained eggs in the TYH medium. The final sperm concentration for insemination was 1×10^6 cells/ml. IVF was sustained in 5% CO_2 incubation at 37°C for 6 h. The eggs were washed three times with the MWM medium to elute residual cumulus cells and sperm, and then were transferred to a new dish containing MWM for another 16 h incubation. The fertility was determined by observing eggs development. The presence of a 2-cell stage embryo indicated successful fertility, implying that the sperm were successfully capacitated, underwent acrosome reaction, penetrated the egg, fused with nucleus and developed into 2-cell stage embryo.

Experiment 1. Genetic effect of BALB/cByJ and FVB/NCrl

Four groups of reciprocal fertilization tests were performed in the IVF experiments. They were BALB/cByJ oocytes × BALB/cByJ sperm, BALB/cByJ oocytes × FVB/NCrl sperm, FVB/NCrl oocytes × BALB/cByJ sperm and FVB/NCrl oocytes × FVB/NCrl sperm. The experiments aimed to evaluate the difference between the IVF efficiency of the two strains, FVB/NCrl and BALB/cByJ. In this experiment, the standard TYH medium with 5.5 mM of glucose was used.

Experiment 2. Effect of glucose concentration in IVF medium

Standard TYH medium for IVF was supplemented with 5.5 mM glucose during pre-incubation and insemination. In this experiment, the glucose concentration was varied between low (0, 2.7 mM) and high (11.1, 22.2 mM) containing groups to elucidate the effect of glucose concentration on BALB/cByJ and FVB/NCrl IVF efficiency.

Statistical analysis

All quantitative data were expressed as mean ± standard deviation (SD). Differences between groups were evaluated by a Student's *t*-test. A p-value of < 0.05 was considered to be statistically significant.

Results

Genetic effects in mice fertilization *in vitro*

FVB/NCr1 mice were used as a reference strain to explore the genetic factor of BALB/cByJ mice on low IVF efficiency. The sperm and oocytes from both FVB/NCr1 and BALB/cByJ were reciprocally fertilized *in vitro* to evaluate the IVF efficiency (Table 2). At least three independent experiments were performed by four groups of different IVF tests as described in Materials and Methods. The fertility efficiencies of BALB/cByJ oocytes × BALB/cByJ sperm, BALB/cByJ oocytes × FVB/NCrl sperm, FVB/NCrl oocytes × BALB/cByJ sperm and FVB/NCrl oocytes × FVB/NCrl sperm were 7.0, 17.1, 65.9 and 73.3%, respectively. In conclusion, the average fertility sponsored by FVB/NCrl sperm was 69.6%. Whereas, the average fertility sponsored by BALB/cByJ sperm was 12.1% only (Table 2).

Effect of glucose concentration in medium on IVF

This experiment investigated the effect of glucose concentration in the TYH medium on IVF efficiency, and to determine the best condition for IVF in BALB/cByJ mice. Five glucose concentrations, 0, 2.7, 5.5, 11.1 and 22.2 mM in the medium, were evaluated in BALB/cByJ and FVB/NCrl IVF.

A total of 306, 307, 213, 365 and 276 oocytes of BALB/cByJ were used in the IVF tests in which the TYH media contain 0, 2.7, 5.5, 11.1 and 22.2 mM of glucose, respectively. In the parallel experiments, there were 229, 179, 120, 135 and 147 oocytes of FVB/NCr1

Table 2. The fertility of BALB/cByJ and FVB/NCrl mice by *in vitro* fertilization IVF[a]

	FVB/NCrl oocytes × BALB/cByJ sperm	BALB/cByJ oocytes × BALB/cByJ sperm	BALB/cByJ oocytes × FVB/NCrl sperm	FVB/NCrl oocytes × FVB/NCrl sperm
No. of donor mice (female/male)	18/3	30/3	30/3	15/3
No. of oocytes	213	175	217	165
No. of 2-cell stage embryos[b]	15	30	143	121
Fertility (%)	7.0	17.1	65.9	73.3

[a]Four groups of reciprocal fertilization tests were performed in the IVF experiments. IVF was performed in TYH and cultivated at 37°C for 6 h. Eggs were transferred to a new dish containing modified-Whitten's medium for another 16-h incubation.
[b]The presence of a 2-cell stage embryo indicated successful fertility.

applied in the IVF tests using the THY media with 0, 2.7, 5.5, 11.1 and 22.2 mM of glucose, respectively. The number of oocytes in each test was the sum over four independent experiments.

The experimental results showed that the BALB/cByJ fertility of the control group (5.5 mM) was 13.9 ± 7.7%, and those of the 0 and 2.7 mM groups were 6.8 ± 2.9% and 9.9 ± 3.2%, respectively (Fig. 1a). The fertility of both low-concentration groups was less than that of the control group, but did not reach statistical significance

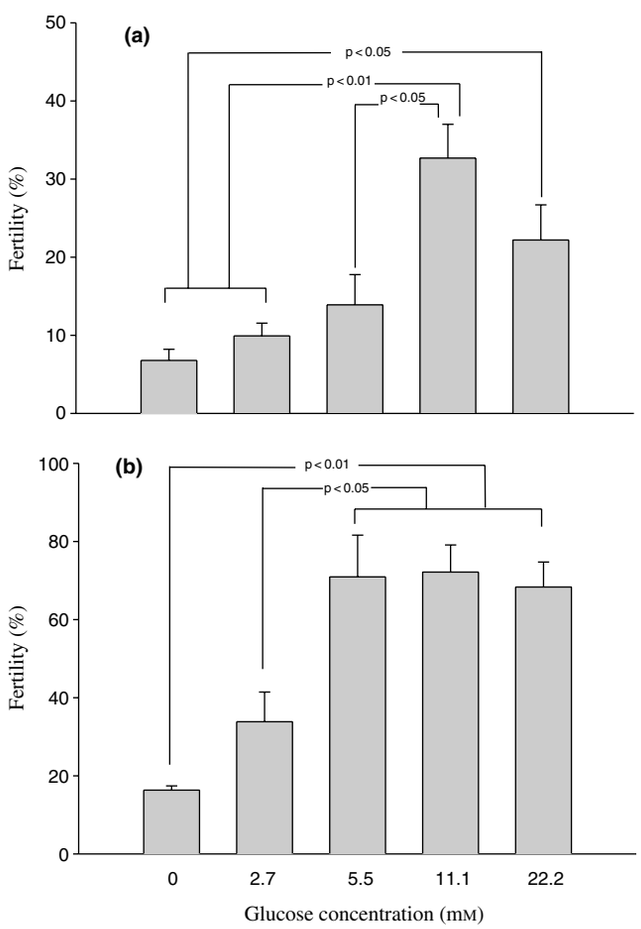

Fig. 1. Effect of glucose concentration in the TYH medium on the *in vitro* fertilization (IVF) of BALB/cByJ (a) and FVB/NCrl (b) mice. Significant difference ($p < 0.05$ and $p < 0.01$) was marked between each related group of different glucose concentrations. Each datum was collected from four independent experiments and showed as mean ± SD in each bar

($p = 0.13$ for 0 mM vs 5.5 mM; $p = 0.38$ for 2.7 mM vs 5.5 mM). High fertility was observed in the high glucose concentration groups, 11.1 mM (32.7 ± 8.6%) and 22.2 mM (22.2 ± 9.0%), which showed significant differences compared with the control and the low glucose concentration groups ($p < 0.05$, Fig. 1a).

The fertility of FVB/NCrl in the low glucose concentration groups, 0 and 2.7 mM, were 16.3 ± 2.2% and 33.8 ± 15.2%, respectively (Fig. 1b). The fertility of the control group 5.5 mM (70.9 ± 21.4%) and the high-concentration groups, 11.1 mM (72.2 ± 13.9%) and 22.2 mM (68.3 ± 12.8%), had high IVF efficiency and showed significant differences compared with those of the low glucose concentration groups ($p < 0.01$, 0 mM vs 5.5, 11.1 and 22.2 mM; $p < 0.05$, 2.7 mM vs 5.5, 11.1 and 22.2 mM). But there were no differences between the control group and the high-concentration groups.

Discussion

The present study showed the IVF efficiency of FVB/NCrl reached 73.3%, but only 17.1% in BALB/cByJ (Table 2). Such low IVF efficiency specially occurred when sperm were derived from BALB/cByJ males. The IVF efficiency reached 69.6% when FVB/NCrl donated sperm, regardless of whether the oocytes came from BALB/cByJ or FVB/NCrl. Yet, the efficiency of IVF declined to 12.1% when BALB/cByJ donated sperm. The influence on IVF efficacy from the female mice was lower than that from the males. The assumption can be supported by the evidence that IVF fertility reach 65.9% in the mating combination of BALB/cByJ oocytes with FVB/NCrl sperm.

Successful fertilization requires spermatozoa to penetrate cumulus cells, recognize, and bind to the zona pellucida, then fuse to form a zygote. These steps include species-specific cellular recognition, intracellular and intercellular membrane fusion and enzyme-catalyzed modifications of cellular investments (Wassarman 1987). The vitality and health of spermatozoa promote fertilization. Styrna et al. (1991) demonstrated that BALB/c males produced up to 44% morphologically abnormal spermatozoa. Morphologically abnormal spermatozoa are less able to pass through the cervix, uterotubal junction and oocyte vestments (Kishikawa et al. 1999). Furthermore, sperm head shape and normality is highly correlated with IVF rate (Liu and Baker 1992). Evidence indicated that Y chromosome plays an importantly participate in determining the total proportion of sperm

head abnormalities. The mutant gene Y^{del} for example, was caused by a partial deletion of Y chromosome (Styrna et al. 1991). The proximity of chromosome 17 to the H-2 locus and the mid-region of chromosome 4 were also suggested relating to the normality of sperm head during spermatogenesis (Styrna 1991).

The inherited defect of sperm in BALB/cByJ may be a limitation of fertilizing by IVF. Our result suggests that the low fertility of IVF on BALB/cByJ males is attributable to the heredity of poor quality spermatozoa, by following excessively inbreeding of BALB/c for over 100 generations. Interestingly, five to six pups per litter can be bred from natural mating of BALB/cByJ. Many factors affect the fertility of female reproductive tract *in vivo*, such as biochemical factors (hormones, cholesterol, ions and fatty acids) and physiological factors (temperature, CO_2/O_2 content and pH value; Choi and Toyoda 1998). Sperm may efficiently be capacitated under certain conditions and therefore fertilize. These effects wait to be confirmed by further study.

The present study showed that increasing glucose concentration from 5.5 mM to 11.1 and 22.2 mM in the TYH medium can improve IVF efficiency of BALB/cByJ (Fig. 1a). The result showed that without changing other components in TYH, suitable glucose addition in the medium (e.g. 11.1 mM) can increase the IVF rate of BALB/cByJ. However, there were no difference among 5.5, 11.1 and 22.2 mM glucose concentration in the TYH medium at FVB/NCrl IVF (Fig. 1b). The fertility kept in the range from 68.3 to 72.2%. We proposed that modified to high glucose concentration (11.1 and 22.2 mM) in IVF medium did not make effort in FVB/N's IVF because of the inherent effect of its spermatozoa.

Both the fertility of BALB/cByJ and FVB/NCrl tended to decline when the glucose concentration in TYH decreased (2.7 and 0 mM). A small proportion of fertilized eggs were observed in the group at 0 mM glucose concentration (6.8% for BALB/cByJ; 16.3% for FVB/NCrl). We purposed the result to be due to the full utilization and metabolism of glucose inside the spermatozoa and the cumulus cells themselves. Urner and Sakkas (1996) claimed that an absence of glucose in the medium does not affect the fusion of spermatozoa and oocyte, but inhibited sperm to bind and penetrate into oocyte.

The carbohydrates are the major energy source of the spermatozoa; thus glucose consumption over the period of fertilization should be vast. Researches on mouse IVF were used to use medium containing 5.5 mM glucose (Urner and Sakkas 1996; Leppens-Luisier and Sakkas 1997; Choi and Toyoda 1998; Redkar and Olds-Clarke 1999; Urner and Sakkas 1999). In our study, we increased the glucose concentrations two to four times (11.1 and 22.2 mM) in the TYH medium. The experimental results showed adequate supply of glucose significantly increase IVF efficiency of BALB/cByJ. In our observation, the motility of BALB/cByJ spermatozoa surpassed that of FVB/NCrl (data not shown). Accordingly, increasing the glucose concentration in the TYH medium can provide more energy and improve the IVF of BALB/cByJ.

The final transition cannot occur in the absence of an appropriate exogenous glycolysable substrate in medium, although the sperm are essentially capacitated (Fraser and Herod 1990). Many researchers had tried to change types of carbohydrate in medium to compare the effects on IVF (Hoppe 1976; Fraser and Herod 1990; Urner and Sakkas 1996; Redkar and Olds-Clarke 1999). For example, fructose, 2-deoxyglucose, 3-*o*-methylglucose, pyruvate and lactate in TYH. However, the ratio of the capacitation to the acrosome reaction was lower than glucose added, because glycolytic pathway during sperm capacitation mainly oxidizes by glucose (Hoppe 1976; Fraser and Herod 1990).

The effects of strains of mouse, sperm quality, capacitation, acrosome reaction, cultural environment and culture medium composition, all are critical in mouse IVF. Results in this study demonstrated that low fertility *in vitro* of BALB/cByJ mice might be due to the hereditary defect in spermatozoa. Appropriately increasing the glucose concentration in IVF medium could improve the rate of fertilization *in vitro*.

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