Zygote **13** (May), pp. 145–153. © 2005 Cambridge University Press
doi:10.1017/S0967199405003205 Printed in the United Kingdom

Medium effects on capacitation and sperm penetration through the zona pellucida in inbred BALB/c spermatozoa

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Date submitted: 23.02.05 Date accepted: 13.03.05

Summary

Inbred BALB/c mice are one of the most difficult inbred strains to fertilize *in vitro*. In this study we examined the abilities of various media used for mouse *in vitro* fertilization (IVF) to support capacitation and sperm penetration through the zona pellucida (ZP) of inbred BALB/c spermatozoa. Media examined were TYH, M16, CZB, mWhitten medium, T6, modified Tyrode's solution (mTyrode's), mKSOM, MEM and TCM199. Modified human tubal fluid (mHTF) was used as a control medium. When sperm were capacitated and inseminated in the same medium, mHTF showed the best fertilization (approximately 80%) scored by male pronuclear formation (<26%) at 5 h post-insemination (PI). When sperm were capacitated in various media and inseminated in mHTF, sperm capacitated in TYH solution (93%) but no other media (<45%) showed a significantly higher level of sperm nuclear decondensation (SND) than mHTF at 2 h PI (approximately 65%). When sperm were capacitated in mHTF and inseminated in various media, only mTyrode's (52%) was not significantly lower than mHTF (66%) in terms of SND at 2 h PI (<49%). Sperm capacitation also was examined by chlortetracycline (CTC) staining. Sperm capacitated in TYH solution showed a significantly higher percentage of capacitation (46%) than those treated in HTF (28%) and other media (<24%). These results indicate that the best approach for IVF in the BALB/c strain is capacitation in TYH and insemination in mHTF. Poor fertilization of BALB/c may result from suboptimal conditions of sperm capacitation and insemination, and overall IVF success may differ depending on strains used.

Keywords: BALB/c inbred mice, Capacitation, *In vitro* fertilization, Spermatozoa, Zona penetration

Introduction

Extensive studies have been conducted to elucidate optimal conditions for *in vitro* manipulation of gametes to improve developmental competence. Knowledge obtained from such studies leads to increased production in domestic and laboratory animals, preservation of endangered animal species, with applications in clinical treatments of human infertility. *In vitro* fertilization (IVF) is an essential part of gamete and embryo manipulation *in vitro*, and elucidation of optimal IVF conditions is an important issue for understanding developmental control of phenomena occurring during fertilization such as capacitation, gamete interaction and egg activation.

In some laboratory mice, IVF has been successfully applied to obtain embryos for cryopreservation, for the production of gene-modified animals, and for transport of animals as embryos instead of whole animals. However, mouse IVF is limited to some outbred and inbred strains and is not always successful. The least fertilizable strains using *in vitro* techniques include the C3H, BALB/c and 129 strains (Choi *et al*., 2000; Sztein *et al*., 2000; Thornton *et al*., 1999). Because these strains are the most widely used strains in biological research, it is essential to establish the IVF conditions that achieve a good fertilization level. In our previous

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Table 1 Composition (mM) of media

		Modification of KRB solution					Modification of Tyrode's solution	
	mHTF[a]	TYH[b]	M16[c]	CZB[d]	mWhitten[e]	mKSOM[f]	T6[g]	mTyrode's[h]
NaCl	101.61	119.30	94.70	82.00	88.100	95.00	99.40	99.23
KCl	4.69	4.70	4.78	4.86	4.78	2.50	1.42	2.68
$CaCl_2$	5.14	1.71	1.71	1.71	–	1.71	1.78	1.80
Ca lactate	–	–	–	–	1.71	–	–	–
KH_2PO_4	0.40	1.20	1.19	1.17	1.19	0.35	–	–
$MgSO_4$	0.20	1.20	1.19	1.18	1.19	0.20	–	–
$MgCl_2$	–	–	–	–	–	–	0.47	0.49
NaH_2PO_4	–	–	–	–	–	–	0.36	0.36
$NaHCO_3$	25.00	25.10	25.00	25.00	22.62	25.00	25.00	25.00
Glucose	2.78	5.56	5.56	5.56	5.56	5.56	5.56	5.56
Na lactate	18.36	–	23.30	30.10	22.11	10.00	24.90	25.00
EDTA	–	–	–	0.10	–	0.01	–	–
Na pyruvate	0.34	1.00	0.33	0.26	0.23	0.20	0.37	0.50
Glutamine	–	–	–	1.00	–	1.00		

All media contain 4 mg/ml bovine serum albumin (BSA), 0.05 mg/ml streptomycin sulfate and 100 U/ml penicillin G (potassium salt).
[a] Kito *et al.* (2004); [b] Toyoda *et al.* (1971); [c] Whittingham (1971); [d] Chatot *et al.* (1989); [e] Hoppe & Pitts (1973); [f] Summers *et al.* (1995); [g] Quinn *et al.* (1982); [h] Fraser (1993).

work (Kito *et al*., 2004), a comparison of fertilization competence between two media, modified human tubal fluid (mHTF) and modified Krebs–Ringer's bicarbonate solution (TYH), showed that the medium used for capacitation and fertilization is an important factor for successful IVF. This suggests that poor fertilization of a few strains possibly derives from suboptimal conditions for capacitation and sperm penetration through the zona pellucida (ZP).

In this study, 10 media used for mouse IVF and embryo culture were compared as to their abilities to support IVF of an inbred BALB/c strain. This strain was selected because IVF of the BALB/c strain is highly sensitive to culture milieu. We tested four media modified from Krebs–Ringer's bicarbonate solutions (KRB)–TYH (Toyoda *et al*., 1971), M16 (Whittingham, 1971), CZB (Chatot *et al*., 1989) and mWhitten (Hoppe & Pitts 1973); two media based on Tyrode's solution – T6 (Quinn *et al*., 1982) and modified Tyrode's solution (mTyrode's) (Fraser, 1993); one medium named mKSOM newly developed by a procedure called simplex optimization (Summers *et al*., 1995); and two complex tissue culture media–Eagle's Minimum Essential Medium (MEM) (Ho *et al*., 1995) and Medium 199 (TCM199) (Bleil, 1993). All media were examined as to their ability to support both capacitation and fertilization relative to mHTF, an important concern in that our previous study (Kito *et al*., 2004) showed mHTF supported both capacitation and zona penetration of the BALB/c strain. Capacitation in all media was also examined by observing the staining pattern by chlortetracycline (CTC) (Ward & Storey, 1984).

Materials and methods

Animals

Inbred BALB/cA males and B6D2F1 (C57BL/6N♀ × DBA2N♂) females were purchased from CLEA Japan (Tokyo, Japan). Animals were maintained at 22 ± 2 °C under a lighting regimen of 12 L: 12 D (lights on from 07:00 to 19:00 hours). All animals were treated according to the Recommendations for Handling of Laboratory Animals for Biomedical Research, compiled by the Safety and Ethical Handling Regulations Committee for Laboratory Animal Experiments, the National Institute of Radiological Sciences, Japan.

Medium preparation

All salts were purchased from Nacalai Tesque (Kyoto, Japan) unless indicated otherwise. Glutamine (G-5763), sodium pyruvate (P-4562), sodium lactate (L-7900) and chlortetracycline (C-4881) were obtained from Sigma Chemical (St Louis, MO), and EDTA (disodium salt) (no. 343-01861) from Wako Pure Chemical Industries (Osaka, Japan). Medium compositions except for MEM and TCM199 are indicated in Table 1. The MEM with Earle's balanced salt solution (no. 61100-087) and TCM199 (no. 31100-035) were obtained from Invitrogen (GIBCO, Carlsbad, CA) and supplemented with 25 mM $NaHCO_3$ and 0.27 mM pyruvate (Bleil, 1993; Ho *et al*., 1995). The essential and non-essential amino acids were added to MEM from 100× and 50× concentrates (GIBCO), respectively. Media were stored without

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bovine serum albumin (BSA), pyruvate or glutamine at 4 °C for no more than 1 week. All media contained 4 mg BSA (Nacalai Tesque, 012-02, Kyoto, Japan), 0.05 mg/ml streptomycin sulfate (S-1277 Sigma) and 100 U/ml penicillin G (P-4687, Sigma). Media were equilibrated at 37 °C overnight under 5% CO_2 with saturated humidity.

Oocyte collection and sperm preparation

The procedure used for oocyte collection and sperm capacitation was as described previously (Kito *et al.*, 2004). Briefly, mature 8- to 10-week-old female B6D2F1 mice were injected intraperitoneally with 5 IU of equine chorionic gonadotropin (Serotropin; Teikoku Hormone, Tokyo, Japan) and 5 IU human chorionic gonadotropin (hCG, Gonatropin; Teikoku Hormone) 46–48 h apart. The cumulus–oocyte complexes were collected from the oviduct 15–16 h after hCG administration. Cumulus cells were removed with 1 mg/ml bovine testis hyaluronidase (type I-S, H-3506, Sigma) containing 0.01 mg/ml soybean trypsin inhibitor (202–09221, Wako Pure Chemical) in a flushing-holding medium (FHM) (Lawitts & Biggers, 1993) and rinsed three times prior to further treatment. Cumulus-free oocytes from individual animals were distributed in equal numbers in each treatment. Sperm were collected both from the distal cauda epididymides and vas deferentia under mineral oil and capacitated at a concentration of $1-2 \times 10^7$ cells/ml for 1.5–2 h under 5% CO_2 at 37 °C. Sperm viability was subjectively scored under a dark-field dissecting microscope, and only males with >60% of sperm viability were used in the study.

In vitro fertilization (IVF)

Capacitated sperm suspensions were diluted 1:10 and sperm concentration was counted under a haemocytometer and further diluted to a final concentration of $1-2 \times 10^5$ sperm/ml (total of approximately 1:50–100 dilution of capacitated sperm suspension). Sperm and egg were co-incubated in a 100 μl drop covered with mineral oil (M-8410, Sigma) in a 60 mm Petri dish (no. 1007, Becton Dickinson, Franklin Lakes, NJ). In the IVF experiment, media were divided to two groups: one group included modified KRB solutions (TYH, M16, CZB and mWhitten), the other included Tyrode's solutions (T6 and mTyrode's), mKSOM and complex tissue culture media (MEM and TCM199). A series of three experiments was performed. In the first experiment, capacitation and insemination were performed in the same medium, and eggs were fixed at 5 h post-insemination (PI) in 2% formaldehyde and 2% glutaraldehyde (Kito & Bavister, 1996). In the second experiment, sperm were capacitated in various media and were inseminated in mHTF. In the third experiment, sperm were capacitated in mHTF and inseminated in various media. In the latter two experiments, eggs were fixed for examination of fertilization at 2 h PI. Fixed ova were mounted on glass slides and overlaid with coverslips supported by 3:1 paraffin wax–Vaseline mixture. Ova were stained with aceto-orcein and examined for zona penetration and male pronuclear formation by Nomarski interference microscopy (Nikon, Tokyo, Japan). Zona penetration was defined as ova with at least one sperm head within the ZP. Ova were scored for male pronuclear (MPN) formation in the first experiment and for sperm nuclear decondensation (SND) in the other two experiments. Ova with no sperm heads that had resumed second meiosis or ova that had only one pronucleus were scored as parthenogenotes and were excluded from the study.

Chlortetracycline (CTC) staining of sperm

In CTC staining experiments, media were divided into three groups: the first group included modified KRB solutions (TYH solution, M16, mWhitten and CZB); the second group included mKSOM, T6 and mTyrode's; and the third group included complex tissue culture media (MEM and TCM199). As a control medium, mHTF was always included. Sperm were capacitated for 1.5 h at $1–2 \times 10^7$ cells/ml and viable sperm were collected by separation with Percoll (Pharmacia, Piscataway, NJ) gradient (Kito *et al.*, 2004), which makes it possible to recover >90% motile sperm in a higher concentration than separation by Sephadex (S. Kito, personal observation). Control uncapacitated sperm were dispersed in phosphate-buffered saline with 4 mg/ml BSA for 5 min at 37 °C before Percoll separation. Percoll concentration was adjusted by mixing with water and 10× concentrated medium without $NaHCO_3$, which was added from 1 M solutions in the final stage. Media with Percoll were equilibrated at 5% CO_2 for 2 h. Sperm were gently placed on top of the 1 ml of 30% and 80% Percoll layers in a conical tube (no. 2095, Becton Dickinson). The suspension was centrifuged at room temperature at 750 *g* for 10 min and the sperm pellet was transferred to 1 ml medium. After gentle mixing with medium, sperm were washed by centrifugation 5 min at 750 *g*. Sperm pellets were immediately transferred for staining with CTC as described elsewhere (Fraser, 1993; Ward & Storey, 1984). Sperm capacitation was assessed by fluorescence microscopy (Olympus, Tokyo, Japan) with a 405 nm band pass filter and a DM 455 nm dichroic mirror. A minimum of 200 sperm with a normal or quasi-normal head (Fraser & Herod, 1990) was scored for each treatment. Sperm were classified as uncapacitated (F pattern), capacitated (B pattern) or acrosome-reacted

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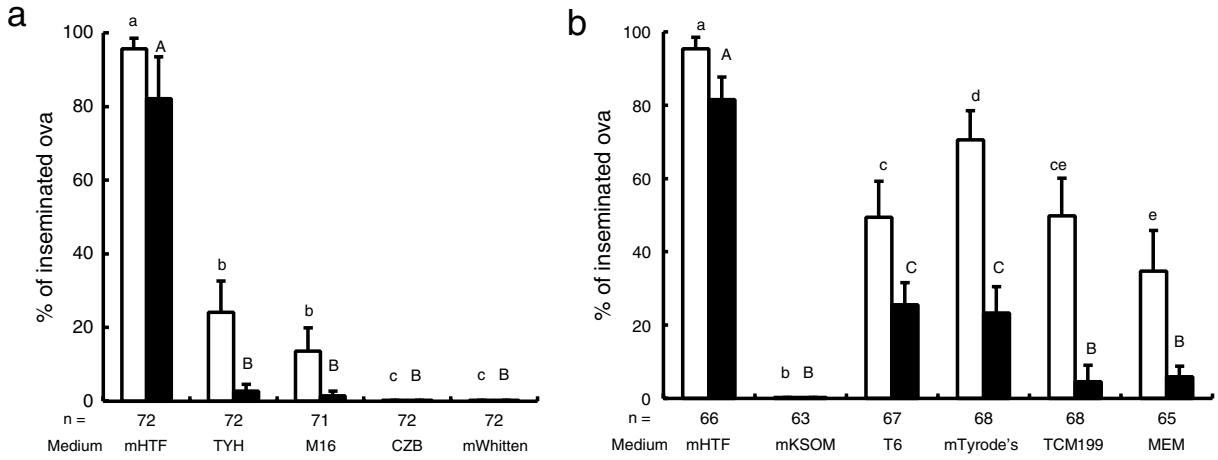

Figure 1 Penetration through the zona pellucida and male pronuclear (MPN) formation of BALB/c sperm capacitated and inseminated in the same medium at 5 h post-insemination (PI). Eggs were fixed and stained for observation under Nomarski interference microscopy. Open bars indicate percentages of ova whose zona pellucida was penetrated by one or more sperm. Filled bars indicate percentages of ova with one or more male pronuclei (MPN). (*a*) Comparison among mHTF and media of modified KRB group. (*b*) Comparison among mHTF, mKSOM, media in the modified Tyrode's group and complex tissue culture media. Bars indicate mean standard error. *n*, total numbers of ova inseminated in six replicate experiments. Percentage data were arcsin transformed and analysed by analysis of variance, and multiple comparisons were made using the Least Significant Difference test. Within each fertilization process (noted with lowercase letters for 'sperm penetration through the zona pellucida' and uppercase letters for 'MPN formation'), data that are significantly different ($p < 0.05$) are labelled with different letters.

(AR pattern), depending on staining pattern of the sperm head region as described by Fraser (1993).

Experimental design and statistical analysis

Each series of IVF experiments was replicated six times and CTC staining was replicated four times. The IVF data were recorded as percentages of total oocytes inseminated and data of CTC staining pattern as a percentage of total sperm counted. All percentage data were transformed using arcsin to control for unequal variance (Tukey–Freeman transformation) (Zar, 1996). Data were analysed by analysis of variance (ANOVA) with a random block design using the SAS program with each male assigned as a block. Multiple comparisons were made using the Least Significant Difference (LSD) test. A probability of $p < 0.05$ was considered to be statistically significant.

Results

In the first series of experiments, IVF was performed in the same medium throughout capacitation and insemination, and MPN formation was examined at 5 h PI (Fig. 1). Although mTyrode's had significantly higher zona penetration (70.6 ± 8.0%) than others (<50%), no medium showed zona penetration and MPN formation rates as high as those in mHTF (95.5 ± 3.1% zona penetration and 81.5 ± 6.2% MPN formation, $p < 0.05$; Fig. 1a, b).

In the second series of experiments, sperm capacitated in various media were inseminated in mHTF and oocytes were examined at 2 h PI (Fig. 2). Zona penetration rates by sperm capacitated in M16 (61 ± 6.3%) and TCM199 (59.3 ± 12.8%) were not significantly different from that of sperm capacitated in mHTF (77.6 ± 5.8%), and sperm capacitated in TYH (95.2 ± 3.4%) penetrated significantly better than those capacitated in mHTF ($p < 0.05$; Fig. 2a, b). The SND showed a similar tendency to zona penetration among media: sperm capacitated in various media other than TYH showed a significantly lower SND rate (<45%) than those capacitated in mHTF (66.2 ± 7.4 or 64.2 ± 6.9%, $p < 0.05$; Fig. 2a, b). Sperm capacitated in TYH (93.4 ± 4.0%) had a significantly higher SND than those capacitated in mHTF (66.2 ± 7.4%, $p < 0.05$; Fig. 2a). In addition, extrusion of the second polar body was higher in oocytes inseminated by sperm capacitated in TYH solution (63.0 ± 11.0%) than in those capacitated in mHTF (10.1 ± 3.7%, $p < 0.05$; data not shown).

In the third series of experiments, sperm capacitated in mHTF were inseminated in various media and oocytes were examined at 2 h PI (Fig. 3). Insemination in T6 (56.2 ± 4.0%) and mTyrode's (55.7 ± 5.1%) resulted in moderate levels of penetration through the ZP (Fig. 2b), although still significantly lower than with

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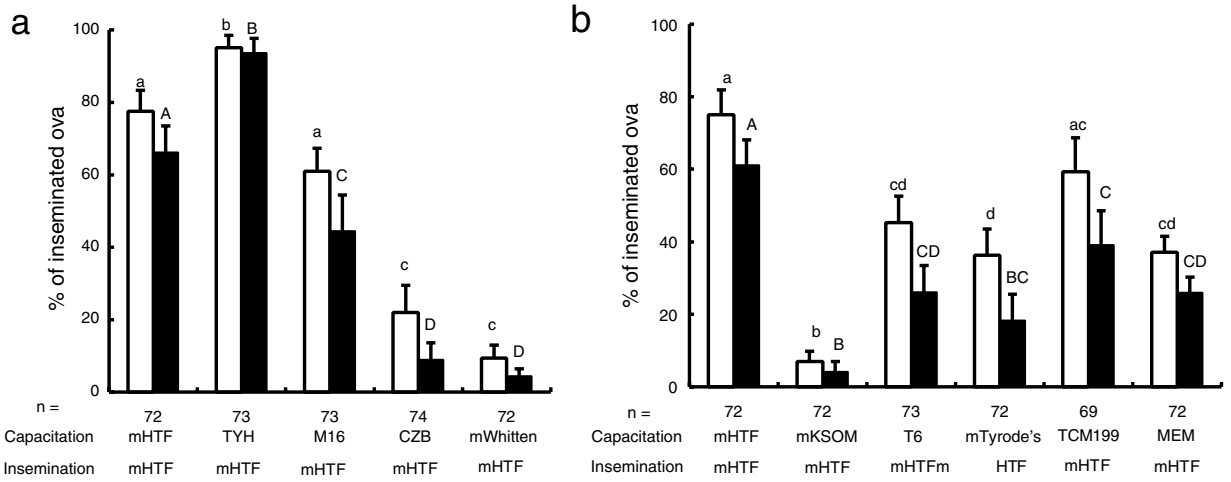

Figure 2 Sperm penetration through the zona pellucida and sperm nuclear decondensation (SND) of BALB/c sperm capacitated in various media and inseminated in mHTF at 2 h PI. Eggs were fixed and stained for observation under Nomarski interference microscopy. Open bars indicate percentages of ova whose zona pellucida was penetrated by one or more sperm. Filled bar indicates percentages of ova with one or more decondensed sperm nuclei (SND). (*a*) Comparison among mHTF and media of modified KRB group. (*b*) Comparison among mHTF, mKSOM, media of modified Tyrode's group and complex tissue culture media. Bars indicate mean standard error. *n*, total numbers of ova inseminated in six replicate experiments. Percentage data were arcsin transformed and analysed by analysis of variance, and multiple comparisons were made using the Least Significant Difference test. Within each fertilization process (noted with lowercase letters for 'sperm penetration through the zona pellucida' and uppercase letters for 'SND'), data that are significantly different ($p < 0.05$) are labelled with different letters.

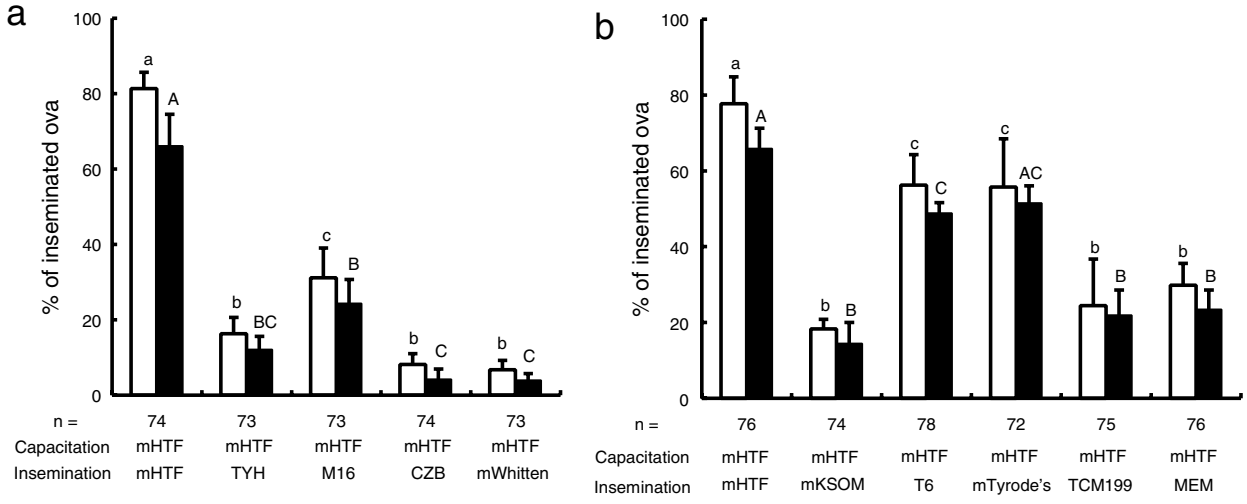

Figure 3 Sperm penetration through the zona pellucida and sperm nuclear decondensation (SND) of BALB/c sperm capacitated in mHTF and inseminated in various media at 2 h PI. Eggs were fixed and stained for observation under Nomarski interference microscopy. Open bars indicate percentages of ova whose zona pellucida was penetrated by one or more sperm. Filled bars indicate percentages of ova with one or more decondensed sperm nuclei (SND). (*a*) Comparison among mHTF and media of modified KRB group. (*b*) Comparison among mHTF, mKSOM, media in modified Tyrode's group and complex tissue culture media. Bars indicate mean standard error. *n*, total numbers of ova inseminated in six replicate experiments. Percentage data were arcsin transformed and analysed by analysis of variance, and multiple comparisons were made using the Least Significant Difference test. Within each fertilization process (noted with lowercase letters for 'sperm penetration through the zona pellucida' and uppercase letters for 'SND'), data that are significantly different ($p < 0.05$) are labelled with different letters.

mHTF ($77.7 \pm 4.6\%$, $p < 0.05$; Fig. 3b). Sperm nuclear decondensation in mTyrode's ($51.5 \pm 4.5\%$) was not significantly different from that in mHTF ($65.9 \pm 5.4\%$; Fig. 3b).

Sperm capacitation was examined by CTC staining pattern at 1.5 h of sperm capacitation (Fig. 4). The B (capacitated) pattern sperm was not significantly lower in M16 ($23.9 \pm 1.1\%$) than in mHTF ($28.2 \pm 2.7\%$;

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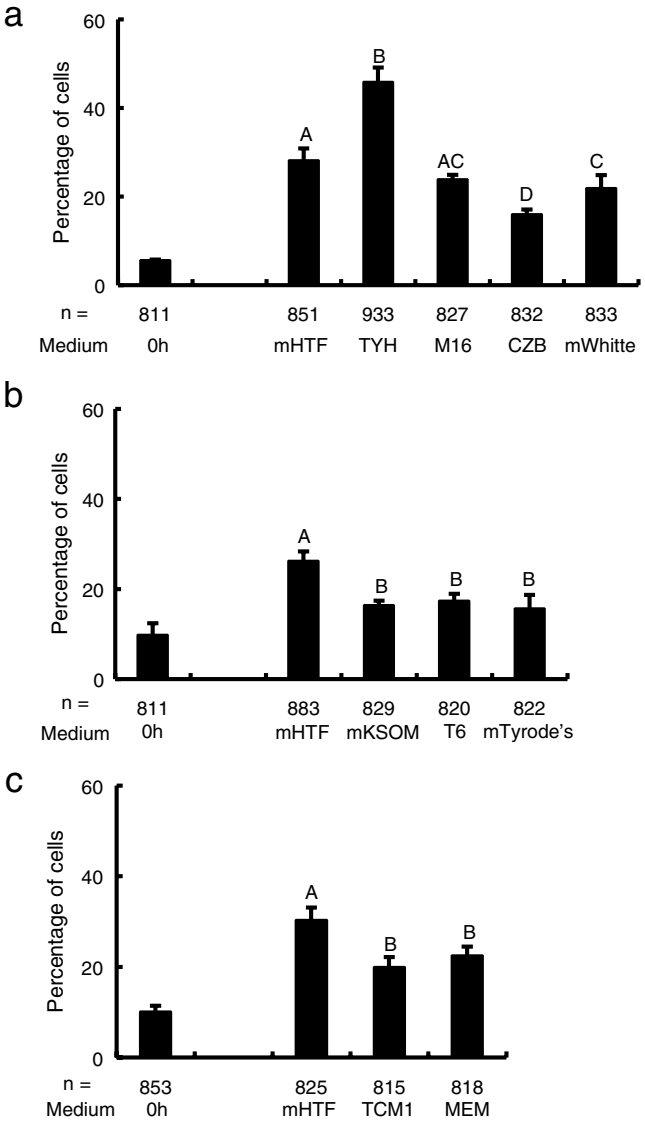

Figure 4 CTC fluorescence patterns of BALB/c sperm capacitated for 1.5 h in various media. Sperm were capacitated at the concentration of $1–2 \times 10^7$ cells/ml for 1.5 h. After centrifugation in Percoll gradient and washing, sperm were stained by CTC and fixed for counting staining patterns under fluorescent microscope. Fresh sperm (0 h) were incubated in phosphate-buffered saline supplemented with 4 mg/ml bovine serum albumin and 0.3 mM pyruvate for 5 min at 37 °C followed by Percoll separation and CTC staining. (*a*) Comparison among sperm capacitated in mHTF and media of the modified KRB group. (*b*) Comparison among sperm capacitated in mHTF, mKSOM and media of modified Tyrode's group. (*c*) Comparison among sperm capacitated in mHTF and complex tissue culture media. Bars indicate mean standard error. *n*, total numbers of sperm counted in four replicate experiments. Percentage data were arcsin transformed and analysed by analysis of variance, and multiple comparisons were made among incubated sperm using the Least Significant Difference test. Data that are significantly different ($p < 0.05$) are labelled with different uppercase letters.

Fig. 4a). Sperm capacitated in TYH solution showed a significantly higher B pattern ($45.9 \pm 3.2\%$) than in mHTF ($p < 0.05$; Fig. 4a).

Discussion

It is well documented that the choice of medium is important for manipulation of gametes and embryos *in vitro* (Bavister, 1995; Van de Sandt *et al*., 1990: Rose & Bavister, 1991). We have previously showed that IVF results for inbred mice differ depending on the media used, and that deficiencies in sperm capacitation and sperm penetration through the zona are primary reasons of poor results of IVF in the BALB/c strain (Kito *et al*., 2004). In this study, we further examined BALB/c mouse IVF by using 10 media invented or modified for embryo and gamete manipulation of various inbred and outbred strains of mice.

The first report of successful IVF was documented by using a modified KRB solution called TYH (Toyota *et al*., 1971). Modified KRBs have often been used as basic medium for mouse IVF; such media include M16 (Sakkas *et al*., 1993; Sato & Ishikawa, 2004), mWhitten (Hoppe & Pitts, 1973) and CZB (Chatot *et al*., 1989; Sato & Ishikawa, 2004). The other most frequently used mouse IVF media are Tyrode's solution with some modification (Fraser, 1993; Quinn *et al*., 1982; Szczygiel *et al*., 2002). Complex tissue culture media with Earle's salt, namely TCM199 (Bleil, 1993) and MEM (Ho *et al*., 1995), have also been used for mouse IVF. Another medium used for IVF is mHTF, based on the composition of the human oviducts and modified with increased calcium by Nakagata (1996). Finally, mKSOM, developed for mouse embryo culture by a computer-assisted simplex program, was reported to support IVF of outbred and inbred mice by increasing glucose concentration (Lawitts & Biggers, 1993; Summers *et al*., 1995). However, all these media were mostly developed for outbred or F1 hybrid mice; only a few inbred strains, if any, have been examined for their ability to support successful IVF, and poor fertilizability *in vitro* is often encountered in various inbred strains.

Poor *in vitro* fertilization of inbred mice is a problem in practical applications of IVF such as colony maintenance, cryopreservation or production of transgenic animals. Inbred mouse strains with poor fertilizability *in vitro* include C3H, 129 and BALB/c (Choi *et al*., 2000; Glenister & Thornton 2000; Sztein *et al*., 2000; Thornton *et al*., 1999). BALB/c is regarded as one of the least fertilizable strains even *in vivo* (Roudebush & Duralia, 1996) and it has been known to have 60–80% deformed sperm heads (Burruel *et al*., 1996). Experiments using intracytoplasmic sperm injection in which normal fertile males with a normal

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spermatozoal head shape were produced by injection of deformed sperm suggest that this abnormality is derived from local dysfunction of the seminiferous tubules or of the Sertoli cells rather than being a genetic disorder (Burruel *et al*., 1996). While it may be thought that a high abnormality of sperm shape results in low fertilization *in vitro*, our results describe the culture conditions as being the most important factors for successful fertilization in this strain. This and previous studies (Kito *et al*., 2004) show that mHTF can support a high level of capacitation and sperm penetration through the ZP when a single medium is used for both capacitation and insemination (Fig. 1). Media from the modified KRB group and mKSOM have a poor ability to support IVF of BALB/c strains, and the media in Tyrode's group and complex tissue culture media group can support only a limited level of fertilization.

To further examine the effects of media on capacitation and sperm penetration through the zona, each medium was tested for its ability to support capacitation or sperm penetration through the zona. To avoid the possibility that sperm could be capacitated in mHTF during 5 h sperm:egg co-incubation period and thus penetrate the ZP, a sperm:egg co-incubation period of 2 h was utilized.

Initially, sperm were capacitated in the various media and inseminated in mHTF. When fertilization was examined at 5 h PI in a previous study (Kito *et al*., 2004), sperm capacitated in TYH solution produced the same level of fertilization as sperm capacitated in mHTF. Thus, this experiment yielded unexpected results – sperm nuclear decondensation was higher when sperm were capacitated in TYH solution than in mHTF at 2 h PI (Fig. 2a), indicating that sperm of the BALB/c strain capacitated better in TYH than the other media. This was further supported by CTC staining experiment in which a significantly higher percentage of B-pattern sperm was observed when sperm were capacitated in TYH than in the other media (Fig. 4a). In the next series of experiments, medium effects on sperm insemination were examined by capacitation in mHTF and insemination in various media. The results indicate that a few media (i.e. Tyrode's group media) can support moderate levels (approximately 50%) of SND, but that none showed better SND than mHTF (Fig. 3). Overall these results indicate that the requirements for sperm capacitation and penetration through the ZP are likely to be different in the BALB/c strain, and capacitation in TYH solution and insemination in mHTF are the preferred conditions for this strain. Because our data indicate that penetration through the ZP and sperm–egg fusion does not necessarily reflect the capacitation state of sperm, IVF studies have to be interpreted carefully when using sperm:egg interaction as the indicator of sperm capacitation *in vitro*.

The major differences in composition between mHTF, TYH and the rest of the media are their concentrations of lactate, pyruvate, glutamine and calcium. Among these, glutamine, which is included in CZB, mKSOM and complex culture media in the concentration range 1–2 mM, is unlikely to be the factor affecting capacitation and penetration through the ZP. The effect of pyruvate on capacitation and sperm penetration through the ZP is controversial. Fraser & Herod (1990) and Hoppe (1976) indicated that glucose, but not pyruvate and lactate, is essential for capacitation of outbred or hybrid mice and that pyruvate and lactate are required for oocytes to accomplish SND and MPN during fertilization *in vitro*. On the other hand, others (Miyamoto & Chang 1973; Tsunoda & Chang, 1975) indicated that pyruvate is beneficial for IVF in mice and rats. Although all media tested in this study included pyruvate at concentrations varying from 0.2 mM to 1 mM, pyruvate concentration does not seems to affect fertilization outcome. Lactate is another energy substrate reported to have a negative affect on sperm capacitation. Neill & Olds-Clarke (1988) demonstrated that better fertilization occurred with sperm capacitated in lactate-free medium than in lactate-containing medium and that the inhibitory action of lactate results from its chelation of free calcium below the level required for fertilization. Our results are consistent with theirs, as capacitation in the lactate-free medium (TYH) resulted in the highest and fastest fertilization among the media containing lactate at concentrations of 10–30 mM with the exception of complex tissue culture media (Fig. 2a). Poor fertilization levels of sperm capacitated in complex tissue culture media, which do not include any lactate, can be explained by the detrimental effects of various components such as vitamins and coenzymes. Based on these studies, lactate should be considered a major factor that slows or inhibits sperm capacitation, although lactate sensitivity may vary depending on the strains tested.

Our results suggest the importance of calcium in sperm penetration through the ZP in the BALB/c strain, because only mHTF, which contains 3 times the concentration of calcium (5.14 mM) as the other media (1.7–1.8 mM), allows a high percentage of sperm penetration. It is well documented that calcium is one of the most important factors for early embryo development including fertilization and sperm capacitation (Yanagimachi, 1994). Fraser (1987) reported that in outbred TO mice, a high concentration of calcium (3.6 mM and 7.2 mM) during insemination significantly reduces fertilization. In contrast, Itagaki & Toyoda (1992) reported that high calcium concentration (5.14 mM) resulted in high levels of fertilization in outbred ICR mice. In the present study, a high level of male pronuclear formation in mHTF at the same

calcium concentration as used by Itagaki & Toyoda (1992) was achieved when this medium was used for both capacitation and insemination. In mHTF enough calcium may still be available for sperm capacitation after chelation by lactate, and a high concentration of calcium may be required for sperm penetration through the ZP in the BALB/c strain. We are currently elucidating factors involving in capacitation and fertilization to improve the IVF medium based on KSOM, because use of a single medium for *in vitro* maturation, fertilization and embryo culture is a less stressful culture environment than conditions that change according to the developmental stage.

Given our result of different requirements for capacitation and fertilization, how does the oviduct adjust the environment for capacitation and fertilization *in vivo*? Around the period of fertilization, ionic compositions in the oviduct change dramatically by influx of follicular and bursal fluid (Borland *et al*., 1977). In addition, cumulus–oocyte complexes can maintain secretions from follicles for considerable periods of time. As follicular fluid has stimulatory effects on sperm capacitation and fertilization (Edwards, 1974), the influx of follicular fluid into the oviduct may alter the environment so that capacitation and fertilization can occur under ideal conditions, or some other factors in follicular fluid such as amino acids or macromolecules may play an important role for controlling fertilization *in vivo*.

Acknowledgement

We thank the Laboratory Animal Development and Management Section, National Institute of Radiological Science, Chiba, Japan, for taking care of the animals used in this research. We are grateful to Mr Craig Steger and Dr Diane Cookfair for critically reviewing this manuscript.

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