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Research Article

The Effect of Genetic Background on the In Vitro Development of Mouse Embryos in Potassium Simplex Optimized Medium Supplemented with Amino Acids (KSOM^{AA})

Haydar BAĞIŞ, Hande ODAMAN MERCAN, Hakan SAĞIRKAYA, Gazi TURGUT
TÜBİTAK, Research Institute for Genetic Engineering and Biotechnology (RIGEB),
P.O. Box 41, 41470 Gebze, Kocaeli - TURKEY

András DINNYÉS
Research Group for Animal Breeding, Hungarian Academy of Sciences and Szt István University,
Pater K. u. 1., Gödöllö, H-2103 - HUNGARY

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Abstract: In vitro culture systems are required for many biotechnological and assisted reproductive technologies. In order to perfect the conditions, the effect of genetic background was evaluated in the in vitro development of embryos from CD-1 and CB6F1 mice in potassium simplex optimized medium supplemented with amino acids (KSOM^{AA}). Presumptive zygotes were recovered from superovulated and mated females (19-20 h post-hCG injection). Two-cell stage block, morula (M), expanded blastocyst (EB), and completely hatched blastocyst (CHB) rates and total cell count were compared in the two mouse strains. The development of CD-1 vs. CB6F1 embryos to 2-cell (98% vs. 96%) and to 3- to 8-cell (89% vs. 93%, respectively) were not significantly different ($p > 0.05$). However, significant differences were detected in the proportions of CD-1 vs. CB6F1 embryos reaching later stages (79% vs. 91% for M ($p < 0.01$), 65% vs. 77% for EB ($p < 0.05$), and 34% vs. 49% for CHB stages ($p < 0.01$), respectively). The results showed that the genetic background of mouse embryos has a significant effect on in vitro developmental potential to late pre-implantation stages. Nevertheless, zygotes obtained both from CB6F1and CD-1 mouse strains have been cultured successfully in KSOM^{AA} in 5% CO_2 in air at 37 °C.

Key Words: mouse strains, zygote, 2-cell block, in vitro culture, amino acids

Fare Embriyolarının, Amino Asit İlave Edilen "Potasyum Simpleks Optimize Edilmiş Medyum (KSOM^{AA})"İçindeki İn Vitro Gelişimleri Üzerine Genetik Farklılığın Etkisi

Özet: Bu çalışmada, CD-1 ve CB6F1 ırkı fare embriyolarının, amino asit ilave edilmiş KSOM medyumundaki (KSOM^{AA}) in vitro gelişim oranları ve toplam hücre sayıları değerlendirilmiştir. Olası zigotlar, süperovulasyondan sonra çiftleştirilmiş dişilerden (hCG enjeksiyonundan 19-20 saat sonra) elde edilmiştir. İki farklı fare ırkı arasında 2-hücre bloğu, morula, genişlemiş blastosist, sarkmış blastosist ve toplam hücre sayısı açısından değerlendirmeler yapılmıştır. KSOM^{AA} içinde 2-hücreli safhaya gelişen embriyoların yüzdesi CD-1 ve CB6F1 için sırasıyla % 98 ve % 96 ; 3-8-hücreli safhaya gelişenlerin yüzdesi % 89 ve % 93 bulunmuştur. İki ırk arasında gerek 2-hücreli, gerekse 3-8-hücreli safhaya gelişim açısından istatistiksel bir farka rastlanmamıştır. Bununla beraber, CD-1 ve CB6F1 ırkları arasında morula (M), genişlemiş blastosist (EB) ve sarkmış blastosist (CHB) safhalarına gelişim yüzdeleri M için % 79 ve % 91 ($p < 0,01$); EB için % 66 ve % 77 ($p < 0,05$); CHB için % 34 ve % 49 ($p < 0,01$) oranlarıyla istatistiksel bir fark ortaya koymaktadır. Sonuçlar göstermektedir ki; CB6F1 ve CD-1fare ırklarından elde edilen muhtemel zigotların kültürleri KSOM^{AA} içinde ve % 5 CO_2, % 95 hava ve 37 °C'lik ortamda etkin şekilde yapılabilmektedir.

Anahtar Sözcükler: fare ırkları, zigot, 2-hücre bloğu, in vitro kültür, amino asitler

Introduction

Emerging embryo technologies are offering new opportunities in reproductive medicine and animal biotechnology. A key element for success, the in vitro culture (IVC) of mammalian embryos has been widely investigated in efforts to approximate the efficiency and quality provided in vivo. The success of IVC is influenced by various factors, among them basic media composition, culture atmosphere and temperature, oxygen tension, osmotic pressure, composition of nutrients, free radical scavengers, volume of culture drops, and embryo manipulation (1,2). In many mammalian embryos, in

vitro development might be arrested at certain stages. This phenomenon in mice has been known as the two-cell block, which can be strongly influenced by the genetic background of the embryos (3,4). Development of pronuclear-stage embryos to blastocyst was limited in several strains, and it is more efficient in some inbred and hybrid strains (4,5). The biological reason behind this arrest is the maternal-zygotic transition of gene activities, which results in a drastic change in embryo protein synthesis. The success of the early stages of embryonic development depends on the proteins and mRNA stored within the mature oocytes. Once they are depleted, and the IVC conditions are not appropriate, embryonic development is either arrested, or the resulting embryos' cell number is reduced because they cannot complete the developmental stages at the right time (6). Attempts to overcome this "block" as well as to elucidate the mechanisms causing this retardation resulted in culture media recreating an environment closer to that in the oviduct and the uterus, including a variety of chemically defined media (7-9). To overcome the developmental block, Lawitts and Biggers (10,11) developed the sequential potassium simplex optimization medium (KSOM), which successfully supported the development of pronuclear-stage embryos from many outbred and inbred strains to the blastocyst stage (12-14). The addition of essential and non-essential amino acids to KSOM, in similar concentrations to that used for the culture of some human cell lines (15), further improved the development to blastocyst stage (16), the rate of hatching and the total cell counts of blastocysts in mice (13). Pre-implantation development of one-cell F1 hybrid mouse embryos was increased when a mixture of 20 amino acids was added to mouse tubal fluid (MTF) medium (17). The aim of this study was to examine the effect of the genetic background on IVC success in two mouse strains relevant for our studies. The ratios of in vitro development to various stages and the total cell counts in KSOM supplemented with amino acids in CD-1 and CB6F1 mice was examined.

Materials and Methods

Animals, superovulation and embryo collection

All animal care and use procedures were in accordance with the International Guide for the Care and Use of Laboratory Animals and were approved by TÜBİTAK's Research Institute of Genetic Engineering and Biotechnology (RIGEB) Animal Care and Use committee. Four- to five-week old outbred CD-1 and hybrid (C57BL/6J x BALB/c) CB6F1 mice were used. The animals were housed in 14:10 h light/dark cycle (lights on at 05:00 h) at 21 ± 0.5 °C and humidity 50-60%. Females were superovulated by intra-peritoneal (i.p.) injections of 5 IU pregnant mare's serum gonadotropin (PMSG, Sigma, G-4877) at 13:00 h, followed by an i.p. injection of 5 IU human chorionic gonadotropin (hCG, Pregnyl, Organon) 48 h later (18,19) and then placed individually with stud males. Successfully mated females were identified by vaginal plugs. Approximately 19-20 h post-hCG oviducts were excised and cumulus oocyte complexes (COCs) were released from oviductal ampullae and then transferred into drops of M2 medium containing hyaluronidase (80 IU/ml) for 3 min (18). Finally, all embryos were washed three times in M2 medium, and presumptive zygotes were selected for IVC studies.

Preparation of culture media

Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated. Embryos were cultured in KSOM^{AA} (see formulation in Tables 1A and B) (13,20). KSOM^{AA} was prepared freshly for each replicate from five stock solutions, with non-essential (MEM, Sigma M7145) and essential (BME, Sigma B6766) amino acids added (Table 2), diluted with embryo-tested water (Sigma W1503) and then filter-sterilized (0.22 µm Millipore filters). Osmolarity measured by freeze-point depression osmometer (Osmomat 030, Gonotec GmbH) was 265 ± 10 mOsm (14).

Embryo Culture

Culture plates (60-mm tissue culture plate; Nunc150288 cat. no.) were equilibrated with 5% CO_2 in air in a submarine incubator (21) overnight. Zygotes were transferred from M2 medium into culture drops following three washes (22) and cultured in groups of 10 per 10 µl droplet of medium overlaid with embryo-tested mineral oil (Sigma, M 8410) in an incubator with 5% CO_2 in air at 37 °C for 6 days (144 h post-hCG), without replacement with fresh medium.

Embryo evaluation

During IVC embryo development was evaluated every 24 h, starting with day 2 (48 h post-hCG). Two-cell, 3- to 8-cell, morula, expanded and completely hatched blastocyst stages (Fig. 2) were distinguished. At the

Table 1A. Preparation of concentrated stock solutions for KSOM.

Stocks	Component	g/100ml	Final medium mM
Stock A (10X)*	NaCl	5.550	95.0
	KCl	0.186	2.50
	KH_2PO_4	0.048	0.35
	$MgSO_4.7H_2O$	0.049	0.20
	Na lactate (60%)	1.869	10.0
	Glucose	0.036	0.20
	EDTA	0.004	0.01
	Penicillin	0.060	97.5 µ/ml
	Streptomycin	0.050	37.5 µ/ml
Stock B (10X)*		g/100ml	
	$NaHCO_3$	2.101	25.00
	Phenol red	0.010	0.001 g or 1 ml of 1% solution
Stock C (100X)*		g/10ml	
	Na Pyruvate	0.022	0.20
Stock D (100X)*		g/10ml	
	$CaCl_2.2H_2O$	0.252	1.71
Stock E (100X)*		g/10ml	
	L-glutamine	0.146	1.00

* Storage: Stocks A, D and E can be kept for up to 3 months at 4 °C, but it is important to change stocks B and C every other week. Stocks at –20 °C can be kept for longer periods.

Table 1B. Preparation of KSOM medium from concentrated stock solutions.

Stocks	ml/50 ml KSOM[AA]
A (10x)	5.00
B (10x)	5.00
C (100x)	0.50
D (100x)	0.50
E (100x)	0.50
H2O	38.5
BSA	50 mg
MEM	0.25
BME	0.50

Osmolarity range is 252 to 260 mOsm. Filter sterilized and stored at 4 °C for 1 week.

termination of culture (day 6), cell number per blastocyst (Fig. 3) was counted in four replicates. First, blastocysts were fixed in 3% formaldehyde for 15 min at 37 °C and then nuclei were stained with 1 µg/ml bisbenzimide (Hoechst 33342, Sigma B2261) in M2 for 15 min at room temperature. Blastocysts were mounted in a drop of mounting medium (50% glycerol, 50% PBS, 5 mg/ml sodium azide and 1 µg/ml bisbenzimide) on glass microscope slides. A glass coverslip was gently flattened over the mounting medium and sealed in place with clear nail polish and stored at room temperature overnight (23). Nuclei were counted at a magnification x40 using an inverted Zeiss epifluorescence microscope (Axiovert 35M) with a 365 nm band pass excitation filter and a 420 nm long pass barrier filter.

Table 2. Concentrations of amino acids added to KSOM.

Amino Acid	BME [50x] g/l	MEM [100x] g/l
L-Alanine.HCl	-	0.89
L-Arginine.HCL	1.05	-
L-Asparagine.H_2O	-	1.5
L-Aspartic Acid	-	1.33
L-Cystine.2HCl	0.6	-
L-Glutamic Acid	-	1.47
Glycine	-	0.75
L-Histidine (free base)	0.4	-
L-Isoleucine	1.3	-
L-Leucine	1.3	-
L-Lysine.HCL	1.849	-
L-Methionine	0.375	-
L-Phenylalanine	0.825	-
L-Proline	-	1.15
L-Serine	-	1.05
L-Threonine	1.2	-
L-Tryptophan	0.2	-
L-Tyrosine	0.9	-
L-Valine	1.175	-

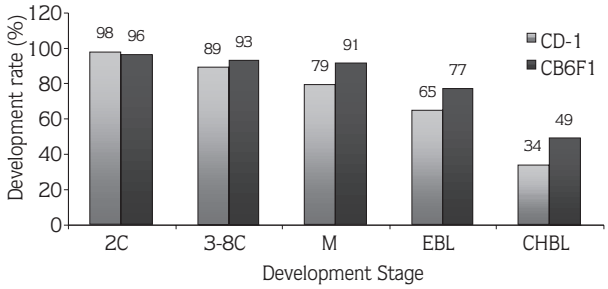

Figure 1. Developmental rates of presumptive zygotes from CD-1 and CB6F1 strains.

Statistical Analysis

Each experiment was repeated at least four times. Developmental effects of KSOM^{AA} on two different strains were compared with Chi-square analysis. Differences of $P < 0.05$ and $P < 0.01$ were considered significant. Data analysis was carried out by using Graphpad Software Program (Version 2.02, Dr. Granger, LSU Medical Center).

Results

There were no statistical differences in the proportions of CD-1 and CB6F1 mice embryos developing in KSOM^{AA} to the 2-cell stage (98% vs. 96%, respectively) and to the 3- to 8-cell stages (89% vs. 93%, respectively) (Fig. 1). However, for the proportions of embryos reaching morula (M), expanded blastocyst (EB) and completely hatched blastocyst (CHB) there were statistical differences in CD-1 vs. CB6F1 mice (79% vs. 91% for M ($P < 0.01$); 65% vs. 77% for EB ($P < 0.05$); and 34% vs. 49% for CHB stages ($P < 0.01$), respectively) (Fig. 1). The percentages of presumptive zygotes that developed to the expanded and hatched blastocyst stages by 120 h and 144 h, respectively, are summarized in Table 3. In blastocysts of CD-1 and CB6F1

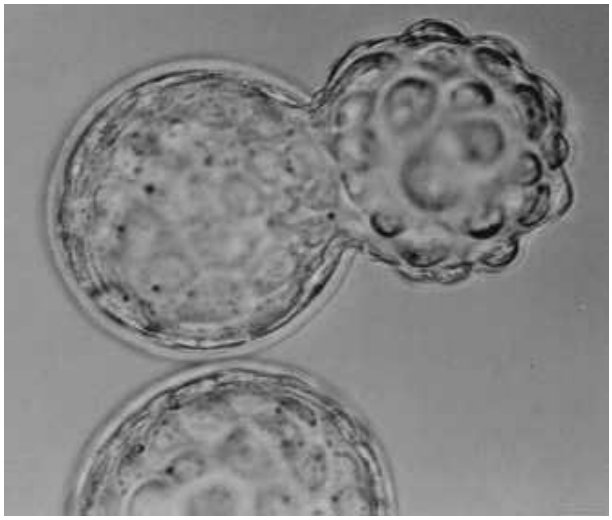

Figure 2. Partially hatched blastocyst stage embryos (Day 5).

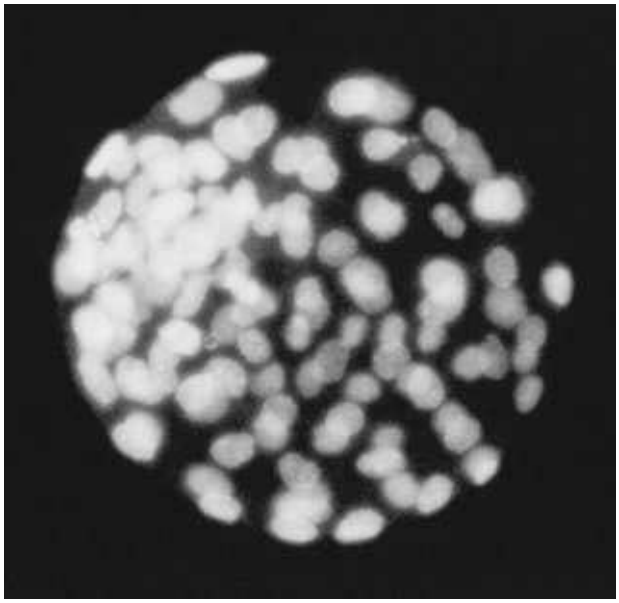

Figure 3. Day-6 blastocyst-stage embryos after fluorescent staining of nuclei.

Table 3. Development of embryos from two mouse strains in KSOM^{AA}.

	Developmental Stages					
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Strain	PZ	2C	3-8C	M	EB	CHB
CD-1	168	164 (98%)	149 (89%)	132 (79%)**	110 (65%)*	57 (34%)**
CB6F1	160	153 (96%)	149 (93%)	145 (91%)**	123 (77%)*	78 (49%)**

PZ: presumptive zygotes; 2C: 2-cells; 3-8C: 3 to 8-cells; M: morula; EB: expanded blastocyst; CHB: completely hatched blastocyst.
* superscripts in the same column indicate statistically significant differences (*: $p < 0.05$, **: $p < 0.01$).

origin, the average cell counts were not statistically different (72 ± 7.58, and 73 ± 8.0, respectively).

Discussion

In this study, the effect of genetic background on the rate of 2-cell block and further development in vitro was investigated by comparing zygotes of CD-1 and CB6F1 mouse strains cultured in KSOM supplemented with amino acids. The results demonstrated that although no difference was found in the early developmental stages, later in vitro development was significantly affected by the genetic origin of the embryos. Embryos obtained from outbred mice exhibit a "block" in development when cultured in sub-optimal conditions (24,25). The results show that in the KSOM^{AA} system no "block" was observed in CD-1 and CB6F1 embryos and they equally developed well to 3- to 8-cell stages. However, later development from CB6F1 embryos was significantly higher than that of CD-1 embryos. The results of this study are similar to previous research, demonstrating that the supplementation of amino acids to KSOM and other media has some advantages on the pre-implantation development of mouse zygotes (26,27), especially on blastocysts development and total cell count (12,13). It was reported that non-essential amino acids and glutamine accelerate the time of the first three cleavage divisions and increase the time of compaction in the mouse when added to modified MTF (26). It has been speculated that the oviduct secretes some essential growth factors and/or amino acids that are needed by pronuclear-stage ova and 2-cell embryos for the activation of their embryonic genome and to the subsequent development into healthy blastocysts and fetuses (28). The presence of amino acids in the culture medium resulted in a higher rate of partial hatching (up to 80%), increased blastocyst cell numbers, particularly that of the ICM, which was more organized, than in blastocysts produced without amino acids (13). The effect of various compounds in a medium is a result of numerous interactions among them (10). The effects of glucose and KH_2PO_4 on the 2-cell block of outbred mouse zygotes have been demonstrated (14). Although the detrimental effect of glucose by inducing 2-cell block has been reported in early studies (8), it is now known that it has no inhibitory effect when used in KSOM (14). In contrast, the addition of both glucose and KH_2PO_4 in similar concentrations have been inhibitory in other media (14), indicating that the interactive effects of glucose and KH_2PO_4 may depend on the composition of the basic media. Optimum concentrations of glucose and KH_2PO_4 to minimize the 2-cell block were found to be 0.20 mmol/l glucose and 0.35 mmol/l KH_2PO_4 (14). The best results were obtained with KSOM, which was formulated using the sequential simplex optimization technique to overcome the 2-cell block (10,12-14). In this study, the concentrations of glucose and KH_2PO_4 were determined by the above experiments, which resulted in a more efficient IVC system compared to the previous results obtained in CZB (Chatot, Ziomek, Bavister) medium used without glucose, and in a later study the percentages of CD-1 vs. CB6F1 embryos developed to the 2-cell stage were 73.6% vs. 83.0%, respectively (29). Other studies have raised the possibility that mouse strains might differ in their sensitivity towards KH_2PO_4 (14,25). In a previous study, 1.18 mmol/l KH_2PO_4 was used in CZB medium (29). In the present study, 0.35 mmol/l KH_2PO_4 in KSOM^{AA} was used. We demonstrated that the concentration of this component in culture media may be crucial to overcome the 2-cell block, regardless of the genetic origin of the embryos used. The expanded blastocyst rates obtained in this study are comparable

with a previous one for development from hybrid zygotes and are similar for CD-1 zygotes (13). The hatched blastocyst rates are also similar to other results (12,13), demonstrating that KSOM media prepared in the laboratory can be similar in efficiency to commercially available forms, and that partial or complete changes of media drops during IVC are not necessary. However, expanded and completely hatched blastocysts produced in $KSOM^{AA}$ had lower cell numbers than reported previously (12), indicating that the system is not optimized yet.

In conclusion, we demonstrated that the presumptive zygotes obtained from hybrid and outbred mouse strains can be cultured efficiently with $KSOM^{AA}$ in 5% CO_2 in air at 37 °C without changing the culture medium during IVC. The genetic background of the zygotes influenced their in vitro developmental potential into late pre-implantation stages. Further studies are needed to demonstrate the interactions among various genotypes and the culture systems, and to optimize IVC conditions to suit all mouse strains.

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