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Effect of CZB Medium on the Two Cell Block of Preimplantation Mouse Emryos

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Abstract: This experiment was designed to determine the effects of CZB medium on in vitro culture of murine embryos obtained from FVB/N, CD-1, and CB6F1 strains. Pronuclear stage embryos were recovered from superovulated and mated females 17 h post-hCG. Two-cell stage block, morula and blastocyst stages were used as the end point of the experiments. There is no statistical difference among strains and media for the development to the two-cell stage. However, more FVB/N embryos (88.3 and 82.4%) than CD-1 (57.4 and 57.1%) and CB6F1 (69.8 and 28.9%) reached the 3-8 cell stage in CZB and M16 media, respectively. CB6F1 embryos developed better (69.8%) in CZB than in M16 (28.9%). Morula development for FVB/N embryos was superior (75.0%) in CZB when compared to M16. Collectively, these data suggest that there are strain differences for in vitro embryo development and the in vitro culture medium has a profound effect on in vitro development.

Key Words: Mouse strains, embryo, 2-cell block, in vitro culture

CZB Medyumunun İmplantasyon Öncesi Fare Embriyolarında Görülen İki Hücre Bloğu Üzerine Etkisi

Özet: Bu çalışma, FVB/N, CD-1 ve CB6F1 ırklarından elde edilen embriyoların in vitro kültürü üzerine CZB medyumunun etkilerini belirlemek için dizayn edilmiştir. Pronükleer dönemdeki embriyolar süperovulasyona tabi tutulmuş ve çiftleştirilmiş dişilerden hCG enjeksiyonundan 17 saat sonra elde edilmiştir. İki hücre aşamasındaki blok, morula ve blastosist aşamaları deneylerin son noktası olarak kullanılmıştır. İki hücre aşamasına gelişimde ırklar ve medyumlar arasında istatistiksel bir fark bulunmamıştır. Ancak, FVB/N embriyoları (%88.3 ve 82.4) CD-1 (%57.4 ve 57.1) ve CB6F1 (%69.8 ve 28.9) embriyolarından daha çok oranda 3-8 hücre aşamasına sırasıyla CZB ve M16 medyumları içerisinde ulaşmıştır. CB6F1 embriyoları CZB içerisinde (%69.8) M16 içerisindekilerden (%28.9) daha iyi gelişmiştir. FVB/N embriyolarının morula gelişimi M16 ile karşılaştırıldığında CZB içerisinde daha yüksektir (%75.0). Sonuç olarak, bu veriler in vitro embriyo gelişimi için ırk farkının olduğunu ve in vitro kültür medyumunun in vitro gelişim üzerinde önemli bir etkisinin olduğunu göstermektedir.

Anahtar Sözcükler: Fare ırkları, embryo, 2-hücre bloğu, in vitro kültür

Introduction

During the early stages of embryo development, fundamental changes take place that are crucial for subsequent normal development. These changes include combination of the parental genomes, activation of the embryonic genome, alterations of energy-generating pathways, and, in at least some species, activation of intracellular homeostatic mechanisms and reorganization of the cytoplasm and organelles (1,2). The significance of the cytoplasmic reorganization is unknown but these events occur in vivo as well as in vitro and correlate strongly with the developmental ability of embryos. When

regulation of ion homeostasis is disturbed in cultured embryos, development is severely compromised or blocked completely (3).

In the mouse, several strains exhibit a block at the two-cell stage. The "block" is also present during in vitro culture of embryos from other species, and it occurs at stages of development characteristic for those particular species. Early cleavage is dependent on proteins and mRNA stored within the oocyte. The block consists of a drastic change in embryo protein synthesis, which is the inner signal that ends maternal control of cleavage and begins embryonic control of growth. Once this block has been initiated, the embryo cannot be salvaged and dies (4). Various somatic cells used in embryo co-cultures (5) or in the preparation of conditioned medium (6) have enabled such arrested development to be overcome. The compositions of initially used culture media were mostly based on serum with pyruvate, lactate, glucose, and antibiotics to retard bacterial growth (4). These in vitro culture media formulations led to inconsistent results due to the vast biochemical control within a developing embryo. Early cleavage stage embryos induce metabolic changes within the embryo, which could probably be detrimental during in vitro development. Therefore, the complementary impact of somatic cell cultures on mammalian embryonic development has been attributed to the transfer of embryonic factors or the metabolism and/or inactivation of embryotoxic factors (5,6). Interest in production of mammalian embryos in defined in vitro conditions has led to the use of media such as synthetic oviductal fluid (SOF) (7,8) and simplex optimization medium with high potassium (KSOM) (9) for embryo culture. With the advancement of culture conditions, mammalian embryos can be produced in vitro in semi-or completely defined conditions (8,10). To understand the requirements for development of immature oocytes through maturation, fertilization and onward to uterine stage embryos, unknowns should be eliminated from supporting chemical and physical conditions, so that the mammalian embryos can be utilized in research and industry without any concern. In designing culture media for preimplantation embryos, we should endeavor to assist embryos through the critical early development period in ways that mimic the help normally provided by the oviduct. Therefore, the objective of this study was to evaluate the effects of the CZB medium in its ability to overcome the two-cell block on different mouse strains by comparing it with M16 medium.

Materials and Methods

Animals, housing, and maintenance:

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. Male and female inbred FVB/N, outbred CD-1 and hybrid CB6F1 mice, 6-8 weeks old were used in this work. Males were housed individually and females were grouped five per cage in solid bottom polycarbonate cages with stainless steel wire lids. The mice were fed ad libitum with autoclaved food and water. The animals were housed in 14:10-h light/dark cycle at 21 ± 0.5ºC and humidity 50-60%.

Superovulation and Embryo collection:

Six- to eight-week-old mice of all the strains were superovulated by intra-peritoneal (IP) injections of 5 IU Pregnant Mare Serum Gonadotropin (PMSG; Sigma) and 5 IU Human Chorionic Gonadotropin (hCG) (Pregnyl; Organon) 48 h apart (10). Then the females were placed in cages individually with a stud male mouse. All donor females were checked for successful mating by examination for the presence of a vaginal plug the next morning. Mated females were killed (approximately 17 h post-HCG injection) by cervical dislocation and the oviducts were excised and placed in tissue culture dish with M2 medium (11,12). Cumulus oocyte complexes (COCs) were released from oviductal ampullae and transferred into drops of M2 medium containing hyaluronidase (80 IU/ml). COCs were incubated for 3 min to separate cells from the oocytes. Then all embryos were washed three times in hyaluronidase-free M2 medium. At this stage, only those embryos exhibiting two pronuclei were included in the experiments.

Culture media:

Embryos were cultured in CZB medium (13). The formulation of this medium is shown in Table 1. Media were filter-sterilized through 0.22 µm Millipore filters and gassed with 5% CO_2 and stored at 4ºC. Fresh media were prepared every two weeks. The osmolarity of media was tested by freeze-point depression, and its range was 285 ± 10 mOsm. Fifty microliters of culture drops were prepared in 35-mm petri dishes (Falcon 3002) and covered with embryo-tested light mineral oil (Sigma; M8410). The culture drops were equilibrated with 5%

Table 1. Compositions of CZB and M16 media.

Components	Concentrations (mM)	
	CZB	M16
NaCl	81.62	94.62
KCl	4.83	4.83
KH_2PO_4	1.18	1.18
$MgSO_4.7H_2O$	1.18	1.18
$NaHCO_3$	25.12	25.00
$CaCl_2.2H_2O$	1.70	1.70
D-Glucose	-	5.55
Sodium lactate	31.30	22.00
Sodium pyruvate	0.27	0.55
EDTA (disodium salt)	0.11	-
Glutamine	1.00	-
Sodium penicillin G (U/ml)	100.00	100.00
Streptomycin (mg/ml)	0.70	0.50
BSA (mg/ml)	5.00	4.00

CO_2 in an incubator at 37ºC for 4-5 h before embryos (12 per drop) were added. One-cell embryos were cultured in 5% CO_2, and 95% air at 37ºC.

Embryo evaluation:

Embryos were observed periodically at 200x on an inverted Zeiss DIC microscope and graded for stage of development, including compaction, blastocoel formation and hatching at 24 h (day 2, after hCG), 48 h (day 3), 72 h (day 4) and 96 h (day 5). Fifty percent of the culture volume was replaced with fresh medium at 24 h intervals. Embryos that did not progress to the next cleavage stage were separated at the time of each medium change.

Statistical Analysis:

The proportions of embryos reaching 2-cell, 3-8-cell, morula, and blastocyst stages were recorded. Each reported data point represents an observation involving at least 3 replicates. The developmental effects of CZB and M16 (13) on the three different strains were analyzed by Student's t-test. Differences of $p<0.05$ were considered significant.

Results

In this study, it was tested whether the effect of CZB medium could overcome the two-cell block in three different mouse strains, outbred CD-1, inbred FVB/N and hybrid CB6F1. M16 was used as a control medium. The results of this comparative study are shown in Table 2. The percentages of embryos that developed to the 3-8 cell stage were 88.3, 59.5, and 67.9 % in CZB; 55.4, 17.9,and 27.3 % in M16 for FVB/N, CD-1 and CB6F1 mice, respectively. There was no statistically difference between the two media tested in their ability to sustain the development of embryos from the mice strain used until the 3-8 cell stage for FVB/N and CD-1. However, a significantly higher ($p<0.05$) percentage of CB6F1 embryos cultured on CZB medium (69.8%) reached the 3-8C stage than those cultured on M16 medium (28.9%). Nonetheless, more embryos reached the morula stage in CZB medium than in M16 for all three strains used (75.0 vs. 55.4% for FVB/N; 40.6 vs. 23.8% for CD-1; and 52.5 vs. 11.1% for CB6F1). Interestingly, none of the 86 embryos from CD-1 reached the blastocyst stage when cultured in CZB medium, while 14.5% of embryos of the same strain cultured in M16

Table 2. The numbers and percentages of embryos from three different mouse strains cultured in CZB and M16.

Media	Strain	Developmental Stages (X ± SD %)				
		PN	2C	3-8C	M	BL
CZB	FVB/N	120	110 (91.6±21.3)	106 (88.3±9.7)a	90 (75.0±17.3) d	9 (7.5±5.0) g
	CD-1	212	156 (73.6±15.7)	122 (57.5±8.6) b	86 (40.6±9.3) c	0 (0) h
	CB6F1	282	234 (83.0±8.6)	197 (69.8±25.5) b	148 (52.5±18.9) c	3 (1.1±2.9) i
M16	FVB/N	74	73 (98.6±6.4)	61 (82.4±6.4) a	41 (55.4±30.6) c	2 (2.7±7.7) i
	CD-1	84	68 (80.9±1.8)	48 (57.1±4.1) b	20 (23.8±9.1) e	12 (14.3±9.2) g
	CB6F1	45	36 (80.0±9.0)	13 (28.9±19.8) c	5 (11.1±14.9) f	1 (2.2±2.0) i

PN: Pronuclear; 2C: 2 cell; 3-8C: 3-8 cell; M: morula; BL: blastocyst. Different superscripts in the same column denote statistical differences at $p<0.05$.

medium developed into the blastocyst stage. In contrast, the percentage of FVB/N embryos reaching the blastocyst stage was significantly higher in CZB medium than in M16 (7.5 vs. 2.7%). These results indicated that the two-cell block observed for each medium was dependent on the mouse strain used.

Discussion

It is expected that in vitro culture medium should provide presumptive zygotes with an environment in which they can overcome the developmental block and develop to the blastocyst stage. Our results show that the best results were obtained from FVB/N embryos cultured in CZB. However, FVB/N embryos when cultured in M16 also showed a good developmental potential until the morula stage. The lower rate for the morula stage was obtained from CB6F1 embryos cultured in M16. Surprisingly, CD-1 had the highest blastocyst development in M16.

The CZB medium used in our study additionally contained glutamine and EDTA and no glucose when compared with M16. While NaCl and sodium pyruvate concentrations of CZB were lower than than those of M16, sodium lactate, BSA and streptomycin concentrations of CZB were higher (Table 1). It is known that the main energy sources of media used to culture embryos are pyruvate, lactate and glucose (14,15). In mice, preimplantation embryos consume pyruvate preferentially during the early developmental stages, before glucose becomes the most important energy substrate in the blastocyst (16). It has also been demonstrated that glutamine is beneficial to embryo development during the first 48 hours of culture and during this time glucose is detrimental to embryo development (4). However, as mentioned above, on day 3 glucose is necessary for higher developmental ratios (13). It has further been reported that optimum development was obtained with CZB medium containing 1 mM-glutamine and no glucose for the first 48 hours of culture (13,17). Glutamine on the other hand had no effect on the proportion of embryos developing to the blastocyst stage; however, it significantly reduced cell numbers at the blastocyst stage (18). In addition, it has been stated that there is a possibility that blocked mouse embryos have the ability to metabolize lactate and pyruvate, but do not produce sufficient energy in this way to develop in vitro, and the addition of glutamine and the removal of glucose might allow increased glutamine metabolism to provide the additional energy substrates necessary for development of 1-cell mouse embryos beyond the 2-cell stage (13). Metabolic studies on embryos of several species indicate that mitochondria are less important in the earliest stages of embryo development, since oxidative metabolism becomes much more pronounced at the morula and blastocyst stages.

From these results, one can conclude that CZB is a more effective medium than M16 in terms of the culture of inbred strain (FVB/N) pronuclear embryos until the morula stage; however, this beneficial effect of CZB diminishes when embryos reach the blastocyst stage. This could be related to the absence of satisfactory energy source and/or antioxidants in the culture medium to allow them to reach the blastocyst stage.

In conclusion, a single medium may not be sufficient to meet all changing requirements within an embryo despite the fact that a medium could be optimal for a cell through the 4-cell stage. Therefore, it is important to optimize an embryo culture medium that will support early embryonic development outside the uterus. Apparently, improvements in media used for in vitro culture of embryos have been made, but still a medium that will give more consistent results for embryos from a range of diverse mouse genotypes may be required. The development of such media will be partly dependent on further studies aimed at gaining a better understanding of the effects of each media component on the two-cell block.

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