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Research Article

Effect of Culture Medium Volume on the Development and Viability of Microinjected or Noninjected One-Cell Hybrid and CD-1 Strain Mouse Embryos

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Abstract: We investigated the effects of drop volume on the development of mouse presumptive zygotes to the completely hatched blastocyst stage. For this aim, we established 3 experimental groups. In the first group, we cultured hybrid presumptive zygotes to the hatched blastocyst stage in 10 presumptive zygotes per 5, 10, 20, 40 and 50 µl drops. In the second group, we used microinjected hybrid zygotes for culture. In the last group, we cultured CD-1 mouse presumptive zygotes to the hatched blastocyst stage. There was no statistical difference in the first group. In contrast, the data demonstrated that there were significant differences in the second and third groups. We also determined the cell number of blastocyst stage embryos in each group and evaluated the results statistically. In conclusion, although the development rate of hybrid mouse zygotes to the hatched blastocyst stage was the highest in 5 µl culture drops, the highest blastocyst cell number was achieved in 40 µl. The highest rates for the development to the hatched blastocyst stage and the blastocyst cell number were also obtained in 40 µl culture drops for microinjected hybrid zygotes. In the CD-1 strain, the highest cell number was obtained in 10 µl. It is clear from the present study that it is important to consider the culture medium volume as a crucial factor affecting the in vitro development of pre-implantation embryos. The genetic background and the manipulation process such as pronuclear microinjection of the zygotes could influence their in vitro developmental potential in terms of medium volume.

Key Words: Medium volume, mice, microinjection, hybrid, zygote, culture

Farelerde Kültür Vasat Hacminin, Mikroenjeksiyon Geçirmiş ve Geçirmemiş Tek Hücreli Hibrid Embriyolar ile CD-1 Irkı Embriyoların Gelişimleri ve Canlılıkları Üzerine Etkileri

Özet: Bu çalışmada fare zigotlarının kültürlerinin yapıldığı vasat damlası hacminin, embriyoların tam olarak zonalarını terk ettikleri safhaya dek gelişimleri üzerine etkileri araştırılmıştır. Bu amaçla üç deney gurubu oluşturulmuştur. İlk gurupta, 5, 10, 20, 40 ve 50 µl vasat damlaları içinde 10'ar hibrid fare zigotunun kültürü yapılmıştır. İkinci gurupta ise farklı olarak, mikroenjeksiyon işlemi geçirmiş olan hibrid fare zigotları kullanılmıştır. Son gurupta ise, CD-1 ırkı fare zigotlarının kültürü yapılmıştır. İlk gurubun gelişim değerleri arasında herhangi bir istatistiksel farka rastlanmamıştır. Tersine, ikinci ve üçüncü deney gurupları içinde istatistiksel olarak önemli farklılıklar olduğu görülmüştür. Ek olarak, her bir deney gurubuna ait blastosist safhasına erişen embriyoların hücre sayıları da saptanmış ve bulguların istatistiksel değerlendirmeleri yapılmıştır. Sonuç olarak, normal hibrid fare embriyoları için gelişimsel oran açısından en yüksek değer 5 µl hacmindeki kültür damlasında gözlenirken; en yüksek hücre sayısı ise 40 µl hacmindeki damlada elde edilmiştir. Mikroenjeksiyon geçirmiş hibrid fare zigotları için ise hem gelişim oranları hem de blastosist safhasındaki hücre sayıları açısından en yüksek değerler 40 µl hacmindeki kültür damlaları içinde elde edilmiştir. CD-1 ırkında ise en yüksek hücre sayısına 10 µl hacmindeki kültür vasatı damlasında ulaşılmıştır. Sonuç olarak, implantasyon öncesi safhalardaki fare embriyolarının in vitro gelişimleri üzerinde kültür vasatı hacminin önemli etkilere sahip bir faktör olduğu açıkça görülmüştür. Ek olarak, kültüre alınan embriyoların ait olduğu ırkların genetik yapıları ve pronükleer DNA mikroenjeksiyon gibi manipulasyonların da embriyoların in vitro gelişimsel potansiyellerini etkilediği düşünülmektedir.

Anahtar Sözcükler: Vasat hacmi, fare, mikroenjeksiyon, hibrit, zigot, kültür

Introduction

The success of in vitro culture is influenced by various factors, such as basic media composition, culture atmosphere and temperature, oxygen tension, osmotic pressure, composition of nutrients, free radical scavengers, volume of culture drops, embryo manipulation and single or group culture (1,2). In the past decade, most research has focused on optimizing the

culture media, the embryo/medium volume rates and the medium exchange during the culture. More research still needs to be performed on the ideal composition of the culture conditions necessary to obtain higher rates of blastocyst formation. More recently, published data have shown that small cultures (3,4), as well as the communal growth of embryos (5), may lead not only to improved embryo development but ultimately to higher pregnancy rates. Recent studies also show that embryos produce their own growth factors and receptors, thus creating an autocrine loop, which stimulates development (6,7). The regulation of the growth and survival of the cells of the early embryo is, however, poorly understood. Mammalian preimplantation embryos develop in vitro with simple medium requirements and have no absolute requirement for exogenous hormones, vitamins, or growth factors. The rate of embryo development in vitro is density-dependent. Embryos growing in relatively small volumes develop more successfully than those growing in large volumes (3,6). The synthesis of some exogenous growth factors is performed by preimplantation of embryos of a number of growth factor ligands and their receptors to enhance the embryo metabolism in vitro and to compensate for the adverse effects of culture in large volumes (6,8,9). Growth of preimplantation embryos is influenced by autocrine trophic factors, which act after the 2-cell stage (7). In many mammalian embryos, in vitro development might be arrested in certain stages. This phenomenon in the mouse is known as the 2-cell block, which can be strongly influenced by the genetic background of the embryos and culture conditions (9-12). Transgenic animals are important for scientific, pharmaceutical and agricultural purposes. Microinjection has been used for the production of transgenic animals; however, the efficiency is very low, limiting the application (13-15). Therefore, microinjected embryos are very precious. Embryo density can affect murine preimplantation embryo development in vitro (5,16). Paria and Dey (6) demonstrated that the ratio of the number of embryos per microliter of medium affected preimplantation embryo development. Autocrine or paracrine growth or survival factors released by the embryo might support itself and other embryos in their development (6,7). A smaller incubation volume could prevent a dilution of these specific embryo-derived factors. Our objective was to evaluate the optimum volume of medium per drop for in vitro development of hybrid, microinjected and CD-1 1-cell mouse embryos to the hatched blastocyst stage.

Materials and Methods

Animals, Superovulation and Embryo Collection

All animal care and use procedures were in accordance with the Institutional Guide for the Care and Use of Laboratory Animals and were approved by the TÜBİTAK, Research Institute of Genetic Engineering and Biotechnology (RIGEB) Animal Care and Use Ethis Committee. Four to five week-old hybrid (C57BL/6J x BALB/c) CB6F1 and CD-1 mice were used. The animals were housed in a 14:10 hr 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 of pregnant mare's serum gonadotropin (PMSG; Sigma; G-4877) at 13:00 h, followed by an i.p. injection of 5 IU of human chorionic gonadotropin (hCG; Pregnyl, Organon) 48 h later (13-15). They were then placed individually with stud males. Successfully mated females were identified by vaginal plugs (day 0). 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 (13,15). Finally, all embryos were washed 3 times in M2 medium, and presumptive zygotes were selected for culture. One-cell embryos were collected 20 to 21 h after hCG.

Preparation of Culture Media

Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO,USA) unless otherwise indicated. Embryos were cultured in KSOM (12). KSOM was prepared freshly for each replicate from stock solutions, supplemented with non-essential (MEM, Sigma M7145) and essential (BME, Sigma B6766) amino acid solution, diluted with embryo-tested water (Sigma W1503) and then filter-sterilized (0.22 µm Millipore filters). Osmolarity as measured by freeze-point depression osmometer (Osmomat 030, Gonotec GmbH) was 265 ± 10 mOsm (17).

Pronuclear Injection

CB6F1 embryos with 2 pronuclei were selected under a stereo microscope and transferred into a 20 µl microdrop of M2 medium (14) under mineral oil (M-3516; Sigma). The embryos selected for manipulation were transferred to the injection chamber. The injection

chamber included an inverted microscope equipped with Differential Interference Contrast (DIC) optics (Axiovert 35M, Zeiss), 2 Leitz micromanipulators (Leitz), and automatic microinjectors (Eppendorf 5242). Holding pipettes and injection needles were made as described previously (13-15). Approximately 1 to 2 pl of DNA solution (Green Flourescent Protein, Gfp, gene) (3 ng/µl) was microinjected into the male pronuclei (18). All microinjected embryos (n = 480) were pooled and randomly divided into 5 groups.

Embryo Culture

Culture plates (60-mm tissue culture plate; Nunc; 150288) were equilibrated in a culture chamber overnight. Embryos were transferred to medium into culture drops and cultured in groups of 10 presumptive zygotes per 5, 10, 20, 40, or 50 µl drops of medium in a petri dish overlaid with embryo-tested mineral oil (Sigma, M 8410). Embryo culture was conducted in a KSOM medium plus amino acids at 37 °C in an incubator with atmosphere containing 5% CO_2 and 95 %N_2 for 5 days (120 h post-hCG), without replacement with fresh medium the whole culture period (12). All embryos (n = 900 (hybrid) and n = 560 (CD-1)) were pooled and randomly divided into 5 groups. Embryos were evaluated every 24 h for their development. Finally 2-cell, 3- to 8-cell, morula, expanded and completely hatched blastocyst stages were distinguished.

Total Cell Number Determination

Total cell numbers per blastocyt were counted at the termination of culture (120 h post-hCG, day 5). In 3 replicates, a total of 30 blastocysts were fixed in 3% formaldehyde for 15 min at 37 °C and then nuclei were stained with 1 µg/ml of 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 of sodium azide and 1 µg/ml of 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 (19). Nuclei were counted at a magnification of 40x using an inverted Zeiss epifluorescence microscope (Axiovert 35M) with a 365 nm band pass excitation filter and a 420 nm long pass barrier filter.

Statistical Analysis

Each experiment was repeated 3 to 4 times. One-way ANOVA was performed using the General Linear Models procedures (SPSS Version 10.0). Groups were compared by means of 1-way ANOVA using least square differences (LSD) as a post-hoc test. A Tukey post-hoc test followed by 1-way ANOVA was used to compare the cell numbers of blastocysts. Differences of $P < 0.05$ were considered significant.

Results

In the present study, we studied the possible effects of drop volume on the development of mouse presumptive zygotes to the completely hatched blastocyst stage in 3 experimental groups. In the first group, we cultured hybrid presumptive zygotes to the hatched blastocyst stage in 5, 10, 20, 40 and 50 µl drops (Table 1). There was no significant difference between the drop volumes. In the second group, we used microinjected hybrid zygotes for culture and found that there were statistical differences between 10 (83.75 ± 4.78) and both 40 (92.00 ± 2.44) and 50 µl drops (94.00 ± 4.24) in the 2-cell stage ($P < 0.05$); between 40- (90.00 ± 0.00) and all of 5 (78.00 ± 2.44), 10 (72.00 ± 12.88) and 20 µl drops (77.25 ± 2.06) in the 3 to 8-cell stage; between 5 (78.00 ± 2.44) and 10 µl (68.25 ± 10.43) in the morula stage; between 10 (45.75 ± 2.98) and 40 µl drops (56.25 ± 9.46) in the blastocyst stage; between 10 (25.00 ± 7.07) and all of 5 (11.25 ± 2.50), 20 (8.50 ± 1.73), 50 µl (10.50 ± 3.31); between 20 and 40 µl (22.00 ± 2.44); and between 40 and 50 µl in the hatched blastocyst stage (Table 2). In the third group, we cultured CD-1 mouse presumptive zygotes to the hatched blastocyst stage and showed that there were statistical differences between 5 and all of 20, 40, 50 µl drop volumes (Table 3). Total cell numbers of hybrid embryos (mean ± standard deviation) cultured in groups per 5, 10, 20, 40 or 50 µl drops were 75.28 ± 7.22, 73.63 ± 7.43, 70.92 ± 8.48, 76.50 ± 5.39 and 73.42 ± 7.04, respectively (n = 30 in each group). Significant differences were determined between 20 and 40 µl (Table 4, $P < 0.05$). Total cell numbers of microinjected hybrid embryos per 5, 10, 20, 40 or 50 µl drops were 75.00 ± 6.83, 76.36 ± 5.76, 68.00 ± 6.78, 77.62 ± 3.29, and 75.16 ± 1.72, respectively (n = 30 in each group). We found statistical differences between 20 µl and all the other drop sizes. In the CD-1 strain, total cell

Table 1. Development of CB6F1 presumptive mouse zygotes to the hatched blastocyst stage in KSOM.

Drop Size	Zyote	2-Cell	3-8-Cell	Morula	Blastocyst	Hatched
5 µl	190	90.40 ± 5.89[a]	88.50 ± 6.55[a]	84.20 ± 6.14[a]	64.10 ± 9.83[a]	37.22 ± 10.40[a]
10 µl	210	90.26 ± 6.29[a]	87.46 ± 8.84[a]	84.76 ± 7.19[a]	69.10 ± 13.61[a]	36.90 ± 15.46[a]
20 µl	170	88.66 ± 13.08[a]	83.48 ± 17.69[a]	78.32 ± 14.21[a]	67.50 ± 16.95[a]	30.16 ± 10.21[a]
40 µl	160	87.50 ± 7.90[a]	81.16 ± 5.46[a]	78.50 ± 10.83[a]	72.00 ± 18.99[a]	31.00 ± 8.94[a]
50 µl	170	89.56 ± 7.37[a]	87.22 ± 4.55[a]	77.22 ± 12.65[a]	71.32 ± 13.84[a]	25.34 ± 9.83[a]

[a-d] Different superscripts in the same column demonstrate statistical difference at $P < 0.05$

Table 2. Development of CB6F1 microinjected mouse zygote to the hatched blastocyst stage in KSOM.

Drop Size	Zyote	2-Cell	3-8-Cell	Morula	Blastocyst	Hatched
5 µl	90	90.75 ± 4.34[ab]	78.00 ± 2.44[b]	78.00 ± 2.44[a]	47.00 ± 9.79[ab]	11.25 ± 2.50[b]
10 µl	90	83.75 ± 4.78[b]	72.00 ± 12.88[b]	68.25 ± 10.43[b]	45.75 ± 2.98[b]	25.00 ± 7.07[a]
20 µl	100	90.75 ± 6.99[ab]	77.25 ± 2.06[b]	70.50 ± 3.3[ab]	46.25 ± 5.37[ab]	8.50 ± 1.73[b]
40 µl	100	92.00 ± 2.44[a]	90.00 ± 0.00[a]	72.00 ± 2.44[ab]	56.25 ± 9.46[a]	22.00 ± 2.44[a]
50 µl	100	94.00 ± 4.24[a]	80.50 ± 7.59[ab]	77.00 ± 6.78[ab]	48.25 ± 3.94[ab]	10.50 ± 3.31[b]

[a-d] Different superscripts in the same column demonstrate statistical difference at $P < 0.05$

Table 3. Development of CD-1 presumptive mouse zygote to the hatched blastocyst stage in KSOM.

Drop Size	Zyote	2-Cell	3-8-Cell	Morula	Blastocyst	Hatched
5 µl	120	99.73 ± 1.250[a]	90.00 ± 0.00[ab]	90.00 ± 0.00[a]	60.00 ± 23.09[a]	42.50 ± 5.00[a]
10 µl	110	100.00 ± 0.00[a]	92.00 ± 5.41[a]	89.00 ± 9.86[a]	75.00 ± 17.32[a]	29.50 ± 13.20[ab]
20 µl	120	91.87 ± 9.86[a]	85.62 ± 5.15[ab]	85.62 ± 5.15[a]	80.00 ± 0.00[a]	16.87 ± 9.43[b]
40 µl	110	93.75 ± 4.78[a]	83.75 ± 4.78[b]	82.50 ± 5.00[a]	71.75 ± 6.23[a]	16.25 ± 11.08[b]
50 µl	100	82.50 ± 5.00[b]	85.00 ± 5.77[ab]	77.50 ± 15.00[a]	77.50 ± 15.00[a]	20.00 ± 0.00[b]

[a-b] Different superscripts in the same column demonstrate statistical difference at $P < 0.05$

Table 4. Total cell numbers of blastocysts (mean ± standard deviation) after post-hCG 120 h.

Drop Size	CB6F1	CB6F1 microinjected	CD-1
5 µl	75.28 ± 7.22[ab]	75.00 ± 6.83[a]	67.37 ± 6.04[bc]
10 µl	73.63 ± 7.43[ab]	76.36 ± 5.76[a]	72.22 ± 7.12[ab]
20 µl	70.92 ± 8.48[b]	68.00 ± 6.78[b]	76.70 ± 5.51[a]
40 µl	76.50 ± 5.39[a]	77.62 ± 3.29[a]	72.37 ± 4.43[ab]
50 µl	73.42 ± 7.04[ab]	75.16 ± 1.72[a]	65.11 ± 8.86[c]

[a-b] Different superscripts in the same column demonstrate statistical difference at $P < 0.05$

numbers of embryos per 5, 10, 20, 40 or 50 µl drops were 67.37 ± 6.04, 72.22 ± 7.12, 76.70 ± 5.51, 72.37 ± 4.43 and 65.11 ± 8.86, respectively (n = 30 in each group). Significant differences were determined between 5 and 20 µl; and between 50 and all of 10, 20 and 40 µl (Table 4, $P < 0.05$).

Discussion

It has been reported that communal growth, as well as minimizing the incubation volume may facilitate blastocyst formation (3-5). A number of previous investigations have shown that in vitro development of mammalian embryos is facilitated by culturing them in

groups. (20). Lane and Gardner (3) did not find a significant difference in blastocyst formation in mice in relation to the incubation volume when embryos were cultured individually. By culturing embryos in groups, they found a significant increase in blastocyst formation in the 5 µl and 320 µl groups (but not in 20 µl) with increasing embryo concentration. Later, Gardner et al. (4) noted a higher rate of blastocyst formation in mice when reducing the embryo incubation volume ratio. However, as in the group with the smallest incubation volume (2 µl), 10 embryos were placed in a 20 µl droplet; one cannot make a distinction between group culture and small incubation volume. The major finding of the present investigation is that there is a cooperative interaction among preimplantation embryos in vitro. This interaction could be mediated by specific growth factors released by them such as insulin, insulin-like growth factor I and II (IGF-1 and IGF-II), platelet activating factor (PAF), leukemia inhibiting factor (LIF), and epidermal growth factor (EGF) (21-23). Rappolee et al. (24) investigated the production of growth factors by mouse embryos in vitro. In vivo, these factors are secreted by the reproductive tract or by the embryo itself and remain highly concentrated in the minuscule fluid volume surrounding the embryo (25,26). Therefore, in the present study, the culture medium was not changed during the whole culture period. In droplets with less than 10 µl of medium, development is comprised (3,16). Another problem is the possible accumulation of some toxic substances like ammonia (3) and oxygen-derived free radicals (27), which may be harmful to embryos. The size of the droplet was selected according to the calculation of Lane and Gardner (3), who determined the nutrient uptakes of individual ovine embryos, and it was calculated as 20 µl of SOF medium. This volume was suggested by Canseco et al. (16) to be 10 µl for murine embryos. Paria and Dey (6) showed that 2-cell mouse embryos cultured singly in 25 µl drops had inferior development to the blastocyst stage and lower cell numbers per blastocyst than those cultured in groups of 5 or 10. In the present study, although the development rate of hybrid mouse zygotes to the hatched blastocyst stage was highest in 5 µl culture drops, the highest blastocyst cell number was achieved in 40 µl. It is well known that the cell number is crucial for successful implantation. However, there was no statistical difference between 5 and 40 µl in the development rate to the hatched blastocyst stage. We concluded that the high development scores were obtained with 10 embryos per 40 µl culture drop for hybrid zygotes. Similarly, the highest rates for both the development to the hatched blastocyst stage and the blastocyst cell number were also obtained in 40 µl culture drops for microinjected hybrid zygotes. In the CD-1 strain, the highest cell number was obtained with 10 embryos per 10 µl drop and there was no statistical difference between 10 µl and the drop volume having the highest rate.

In conclusion, the results of this study suggest that it is important to consider the medium volume in the same number of embryos as a factor that influences pre-implantation embryos in an in vitro system. The genetic background and manipulated embryos of the zygotes influenced their in vitro developmental potential into the late pre-implantation stages in terms of medium volume. Further studies are needed to demonstrate the interactions among various genotypes, microinjected embryos and the culture systems and to optimize in vitro conditions to suit every mouse strain.

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