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THE IN VITRO CULTIVATION OF MOUSE OVA FROM ONE CELL TO BLASTOCYST

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Fare ovumlarının tek hücreliden blastosist aşamasına kadar in vitro kültürü

Özet: *Preimplantasyon aşamasındaki fare embriyoları yaşamları için gerekli ortamı sağlayan vasatlar içersinde gelişme gösterebilirler. Ancak embriyolar vasatlarda oluşabilecek değişikliklere diğer doku ve hücrelerden daha hassastırlar.*

Bu çalışmada PMSG ve HCG enjekte edilmiş dişi farelerin erkek farelerle çiftleştirildikten sonra oviductlarının yıkanması sonu elde edilen tek hücreli döllenmiş ovumlarının blastosist oluncaya kadar in vitro kültüre edilmeleri inlenmiştir.

Bunun için servikal dislokasyonla öldürülen dişi farelerin oviductları 50–70µl. PBI + 4 *mg/ml. BSA* + 0.3 *mg./ml. hyaluronidase* içeren *petri kutularına konmuş, oviductun ampullasının bir iğne yardımıyla yırtılmamasıyla bu vasat içine dökülen tek hücreli döllenmiş yumurtalar bir pipet yardımıyla toplanarak parafinle örtülü* 50 – 100µ l. *M* 16 + *BSA vasatında,* 37 *C derecede,* % 5 *lik* CO_2*li ortam içersinde tutulmuşlar ve gelişmeleri periodik olarak izlenmiştir.*

Summary: *The mouse embryo can develop normally in vitro in a simple, chemically defined medium which fully meets the specific nutritional requirements of the embryos during pre-implantation development. However the embryos are far more sensitive to variations in the culture medium than other cells and tissues.*

Introduction

There has been an increasing interest in the in vitro culture of fertilized mammalian embryos over the past 50 years, with major ardvances in cell and tissue culture techniques satisfyign the requirements of embryos at their different cleavage stages.

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Mark and Long (10) first reported studying the mouse embryo in vitro but they only observed the ova for 12 hours and did not see any cleavage division. Lewis and Wright (9) used drops of plasma and embryo extract to observe and to photograph the early stages of the mouse embryo, but they also did not see any cleavage division. Hammond (8) was the first investigator to successfully culture mouse embryos through several stages of division in vitro. As a culture medium, he employed a simple, physiological salt solution contaning sodium chloride, potassium chloride and magnesium chloride with a glucose concentration of 1 mg. per ml. supplemented with about 5 % egg white. Using this medium, he observed hatching of the blastocyst from the zona pellucida.

A very important factor for the in vitro cultivation of all mammalian cells is the composition of the culture media. The most commonly used chemically defined media for culturing mouse embryos are based upon the Krebs-Ringer bicarbonate solution (7,17). Whitten (13,14) cultured 8-cell mouse embryos to blactocysts in a modified Krebs Ringer solution supplemented with lactate, glucose, crystalline bovine serum albumin and antibiotics. Later Brinster (1,2) cultured 2-cell mouse eggs to blastocysts and after Whittingham (16) achieved fertilization of mouse eggs in vitro, Whitten and Biggers (15) and Whittingham (16) successfully cultured mouse embryos through the entire pre-implantation period of development.

The aim of this study is to make well-designed studies which will yield quantitative information. It sounds more logical to make the largest number and most detailed studies on the embryos of laboratory animals and then to attempt to extrapolate this information to the embryos of larger species and of course if a good foundation of knowledge is available about the embryos of laboratory animals it will be possible to desing more meaningful experiments in which the eggs of larger animals are used. In this way the most efficient use can be made of embryos from the costly large domestic animals.

Materials and Methods

Eight weeks old 11 F1 hybrid females from C57Bl/6J♀♀ x CBA/Ca♂♂ were superovulated with 5 I.U. Pregnant Mare's Serum Gonadotrophin (PMSG, Folligon Intervet) by intra peritoneal followed 48 hours later by 5 I.U. Human Chorionic Gonadotrophin (HCG, Chorulan Intervet). The injected females were placed with

males (CFLP) at the time of HCG and checked for vaginal plugs the following morning (= Day 1). A total of 325 fertlized 1-cell eggs were recovered from donor females with vaginal plugs autopsied between 9.30-10.30 Day 1. The donors were killed by cervical dislocation and the oviducts were placed in a petri dish containing 50-70µl. PBI+4 mg/ml. BSA (Bovine Serum Albumin) + 0.3 mg/ml. Hyaluronidase (Bovine Testis). Each oviduct was checked for a transparent swelling. The 1-cell embryos were located within the ampullary portion of the oviduct, embedded in cumulus and usually stuck together as a single large mass of ova and cumulus cells. To remove this cumulus masses from the ampulla the expanded ampulla was torn and the mass expelled in the hyaluronidase solution. Liberation of the intact cumulus mass was effected using a pair of watchmaker forceps and a disposable 25. gau. needle. The eggs were released from the cumulus cells after 2-2 1/2 minutes at 37°C when an average of 30 eggs were recovered (11) The denuded eggs were then washed through 6 drops of M 16 + 4 mg/ml. BSA to complete cumulus dispersal and remove the PBI + hyaluronidase (11).

The washed eggs were then quickly transferred to the final culture microdrop 50-100µl.M 16+BSA equilibrated earlier with 5 % CO_2in air under paraffin oil (0.83-0.87 S.G.) in a sterile petri dish (Fig.1). The composition of the M 16 culture medium used to culture 1-cell eggs to blastocysts is given in Table 1.

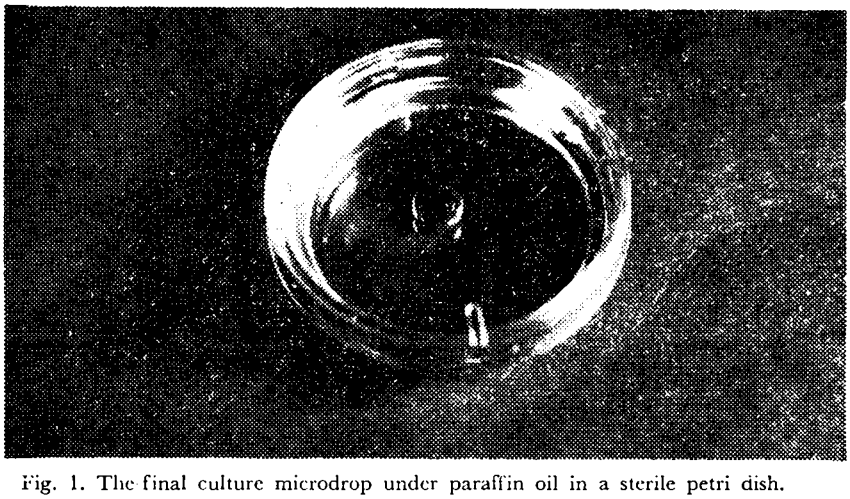

Fig. 1. The final culture microdrop under paraffin oil in a sterile petri dish.
Şekil 1. Kültür damlasının, steril petri kutusu içersinde, parafın altında görünümü.

Table 1. The composition of M. 16 culture medium. (for 100 mls.)

NaCl	0.472 g.
$CaCl_2 2H_2O$	0.026 g.
KCl	0.011 g.
$NaH_2PO_2 2H_2O$	0.006 g.
Na pyruvate	0.003 g.
Glucose	0.1 g.
Penicilline	0.006 ml. 100.000 I.U./ml.
Streptomyin	0.005 ml. 50 mg/ml.
$MgCl_2 6H_2O$	0.01 g.
$NaHCO_2$	0.21 g.
Na lactate	0.305 g.
Phenol red	Trace

To this must be added 4 mg/ml. BSA before use.

The paraffin oil prevents evaporation of the culture drop and helps maintain sterility of the culture medium. To maintain the pH of the medium between 7.2 and 7.4 embryos were cultured throughout at 37°C in an atmosphere of 5 % CO_2in air. The petri dishes were incubated in a sealed anaerobic culture jar containing the required gas mixture. This system gave the opportunity to examine and photograph the embryos as development proceeded.

Table 2. The timing and location of embryos during pre-implantation development. in vivo (11).

Copulation age	Ovulation age	No cell	Location
0–24	22–29	1	Ampulla
24–38	24–57	2	Upper oviduct
38–50	44–59	3–4	Midlower oviduct
50–64	50.59	5.8	Lower Oviduct-uterus
60–80	77.80	Morulae	Uterus
74.82	77.82	Blastocyst	Uterus

Results

90 % of the total 1 cell embryos developed into blastocysts following culture in vitro. The rate of development was similar that in vivo. The hatching of blastocysts from their zona pellucida started late on the fourth day and proceeded through the fifth day of cultivation. The rate of 1-cell eggs cultured in vitro is presented in Table 3.

Table 3. In vitro culture of 1-cell mouse eggs.

	First day controls		Second day controls		Third day controls		Fourth day controls	
	10.30	17.30	10.30	17.30	10.30	17.30	10.30	17.30
1- cell	40	27						
2- cells		13	18	2				
4- cells			21	24				
8- cells				13	29			
16-32 cells					10	7		
Early morulae						15	9	
Late morulae						17	19	3
Blastocyst							7	13
Hatched blastocyst							4	20
Degenerated			1	1	1	1	1	4

Discussion and Conclusion

The results of this study supported the observations (1,2,3,4,16, 17) that a simple, chemically defined medium such as M 16 can support full development of pre-implantation mouse embryos in vitro.

The relationship between time and development stage of mouse ova in vivo (10,12) as given in Table 2 was very similar to that observed in the present study during the culture in vitro of fertilized 1-cell mouse embryos (Fig.2-7).

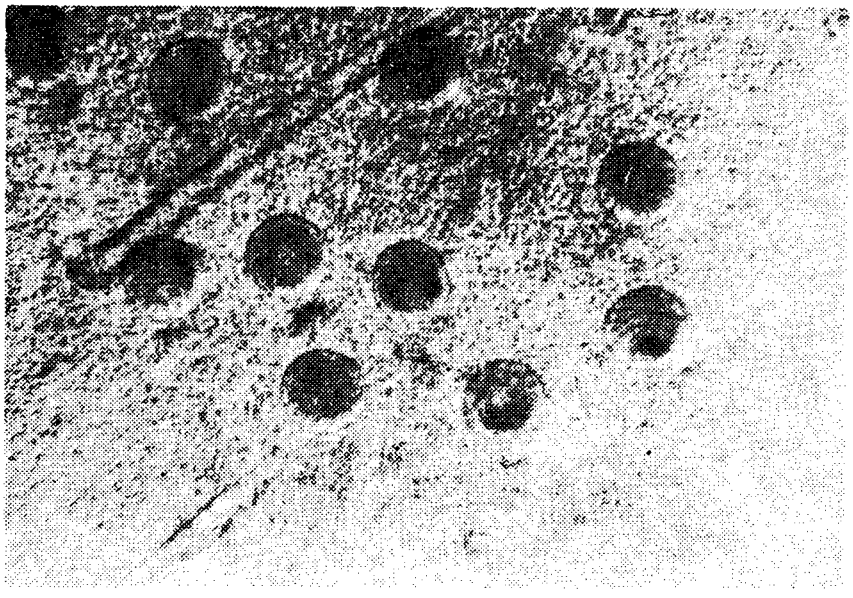

Fig. 2. One-cell embryos and cumulus cells.
Şekil 2. Tek hücreli embriyolar ve cumulus hücreleri.

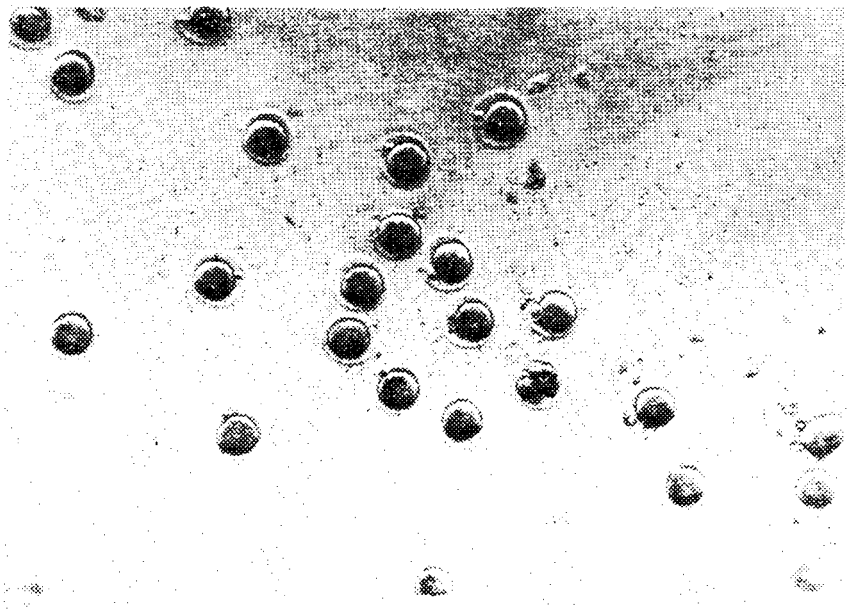

Fig. 3. The denuded one-cell embryos. 180x
Şekil 3. Cumulus hücrelerinden arındırılmış tek hücreli embriyolar.

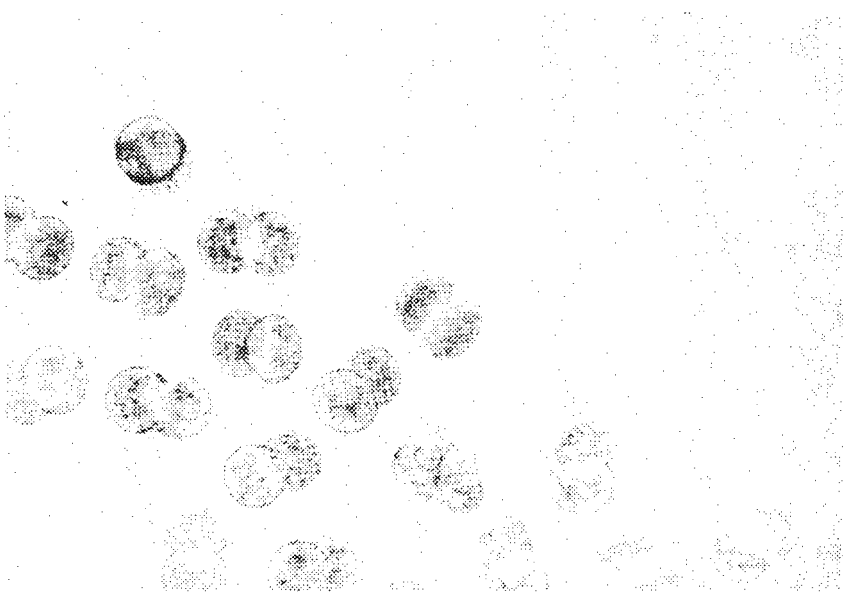

Fig. 4. Two-cell embryos in M 16 culture medium.
Şekil 4. M 16 kültüründeki iki hücreli embriyolar.

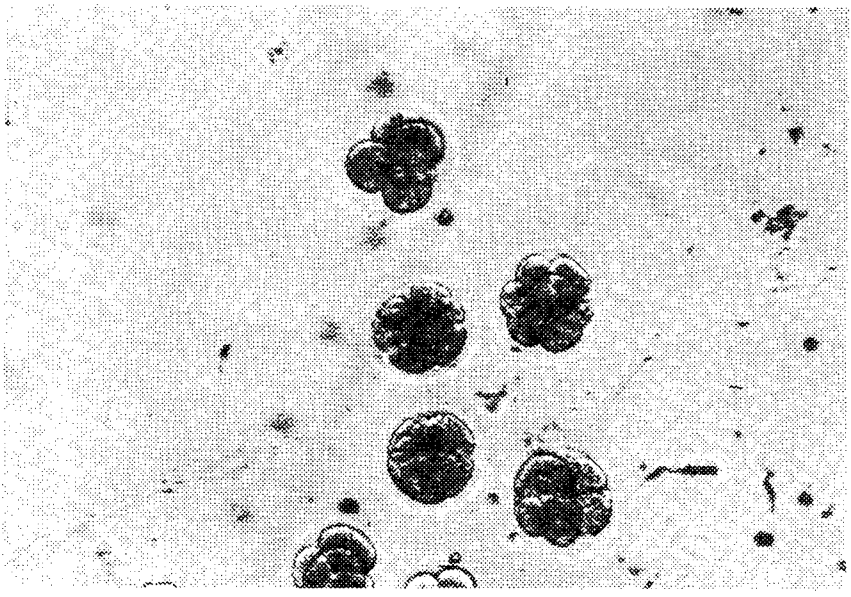

Fig. 5. 4 and 8-cell embryos in M 16 culture medium. 180x
Şekil 5. M 16 kültüründeki 4 ve 8 hücreli embriyolar.

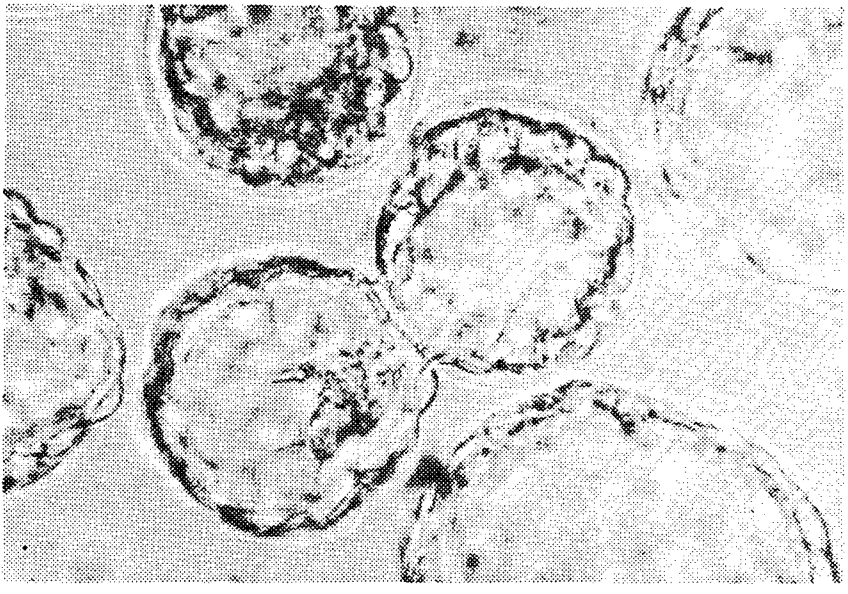

Fig. 6. Hatching blastocyst. 360x
Şekil 6. Açılım yapan blastosist.

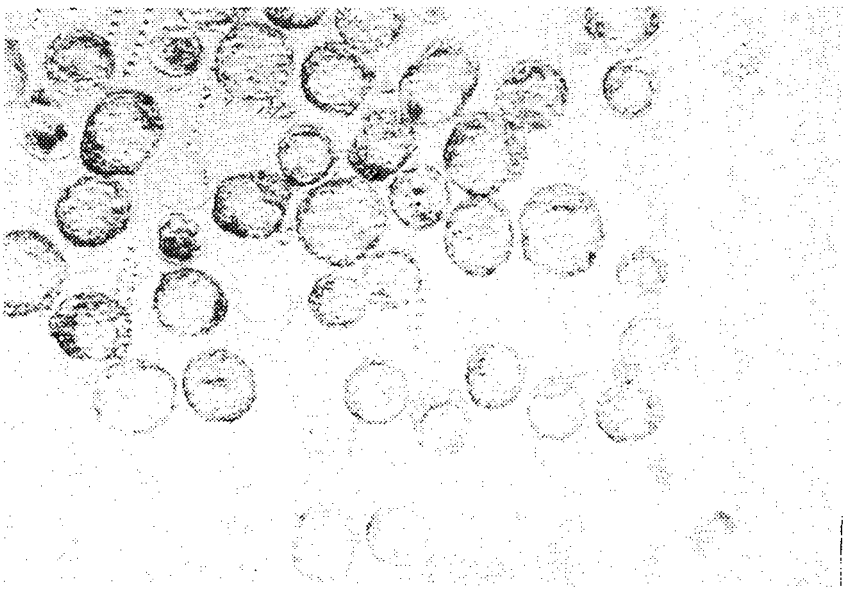

Fig. 7. Hatched blastocysts and the free zona pelucidas. 180x
Şekil 7. Serbest zona pelucidalar ve zona pelucidasız blastosistler.

The salts of all culture media closely resemble the ions in blood (6). Whitten (13) using 8-cell mouse embryos found that omission of calcium, magnesium or potasium from the medium prevented growth and that embryonic development was delayed in the absence of phosphate. Brinster (6) has also showed that the reduction in calcium concentration resulted in a significant decrease in the number of blastocysts developing from 2-cell mouse embryos.

The effect of hydrogen ion concentration on the development of mammalian embryos has been studied primarily in the mouse. Whitten (13) found that the development of 8-cell mouse embryos occured between pH 6.9 and 7.7. Brinster (2) found that the maximum development of 2-cell mouse embryos into blastocysts occured between pH 5.78 and 7.78 in an atmosphere of 5 $\%CO_2$ in air. However further studies (3) demonstrated that the optimum pH for development of the 2-cell mouse embryo was dependant on the concentration of pyruvate or lactate in the medium.

It has been shown that 8-cell mouse embryos could develop into blastocysts when cultivated in Krebs-Ringer solution containing 1 mg.

per ml. glucose and crystalline bovine serum albumin at a concentration between 0.03 and 6 % (13). Brinster (4) has studied the effects of various exogenous amino nitrogen sources on the development of 2-cell mouse embryos into blastocysts. He found that a range of concentration between 1-10 mg. per ml. of bovine serum albumin allowed maximum development. However, there is no essential requirement for the development of 2-cell mouse embryos. In 1968 Brinster (5) confirmed that when glutathione is the only fixed nitrogen source in the culture medium 2-cell mouse embryos could develop into blastocysts.

As a result, methods for the recovery and manipulation of mouse embryos have been described (1-6,10,15) but information concerning many specific aspects of development of the pre-implantation mammalian embryos are still unknown. The detailed studies which will performed on convenient economical laboratory animals especially the mouse, should provide suitable experimental research models and indicate profitable areas to exploid later using the embryos of farm animals.

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