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INVESTIGATIONS ON IN VITRO FERTILIZATION OF RABBIT OVA*

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Tavşan Ovum'larının in vitro Fertilizasyonu Üzerine Çalışmalar.

Özet: *Bu çalışmada tavşan ovumlarının in vitro fertilizasyonu üzerinde çalışıldı. Bu amaçla* 42 *dişi ve* 6 *erkek ergin Yeni Zelanda tavşanı kullanıldı. Oniki gruba ayrılan deneme hayvanlarına* 150-250 *IU PMSG ve* 75-100 *IU HCG, süperfollikülasyon ve süperovulasyon amacıyla enjekte edildi. Toplam olarak* 62 *follikül ve* 616 *ovulasyon yeri enjeksiyonu izleyen değişik zamanlarda sayıldı.* 251 *adet* (% 37.02) *ovum in vitro fertilizasyon vasatı ile oviduct veya folliküllerin yıkanmasıyla toplandı. Toplanan ovumlar stereo mikroskop altında kontrol edildiler. Bu ovumların* 145 (% 57.76), 102 (% 40.63), *ve* 4(% 1.59)'ü *sırasıyla zona pellucida, corona radiata ve cumulus oophorus'lu olarak bulundu. Toplanan* 251 *adet ovumun* 236 *adedi in vitro fertilizasyon amacıyla kullanıldı. Bu normal ovumlar dölleme yeteneğini kazanmış spermatozoitler ile hava içinde* % 5 CO_2 *veya* % 5 CO_2 - % 95 N_2 *ile dengelenmiş parafin yağı altındaki in vitro fertilizasyon vasatı içinde inkübe edildiler.*

Inkubasyondan 24-28 *saat sonra ovumlar stereo mikroskop altında kontrol edildiler ve döllenmiş ve döllenmemiş diye sınıflandırıldılar.*

Bulgulara göre kullanılan 236 *adet ovumun* 47 (% 19.91)*'sinde in vitro fertilizasyon gözlemlendi.* 34 *adet ovum normal, geriye kalanlar ise* (13 *adet) döllenmiş olmasına rağmen anormal olarak saptandı.* 189 *adet ovum ise* (% 80.09) *döllenmemiş ya da döllenmemiş dejenere olarak bulundu.*

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Summary: *In this study, in vitro fertilization of rabbit ova was investigated. For this purpose,* 42 *females and* 6 *male rabbits (New Zealand) were used during the research. The experimental animals were divided into* 12 *groups.* PMSG (150–250 IU) *and HCG* (75–100 IU) *were injected for superfolliculation and superovulation. Totally* 62 *follicles and* 616 *ovulation places (corpus haemorrhagicum) were counted in different times following administration of gonadotrophins. Two hundred fiftyone* (37.02 %) *ova were collected by flushing oviducts and ruptured follicles with the defined medium. Collected ova were identified under stereo microscope and estimated as normal and degenerated. One hundred fourty five* (57.76 %), 102 (40.63 %), *and* 4(1.59 %) *of ova were covered with zona pellucida, corona radiata and cumulus oophorus, respectively. Totally* 236 *normal ova were used for in vitro fertilization. These normal ova were incubated with capacitated spermatozoa in the in vitro fertilization medium under paraffin oil which had been equilibrated with* 5 % CO_2 + 95 % N_2 *at* 37–38 °C *in a incubator. After* 24–28 *hours incubation, the ova were controlled under stereo microscope and were classified as fertilized or unfertilized*

According to the results, the in vitro fertilization was observed in 47 (19.91 %) *out of* 236 *ova and* 34 *of fertilized ova were normal and the rest* (13 *ova) were abnormal. One hundred eightynine ova were found as unfertilized and degenerated.*

Introduction

The fertilization of mammalian eggs outside the body, that is the penetration of the egg by a spermatozoon in some form of cell culture system, has long been the subject matter of scientists on related disciplines. In the context of a technology that might one day be applied to the field of animal breeding, in vitro fertilization has also been suggested as a possible sequel to in vitro maturation of large number of oocytes and as a means of fertilizing eggs obtained from immature animals or after excessive responses to superovulatory treatments. In the case of human medicine a technique of in vitro fertilization has already been used to overcome problems of infertility due to blocked fallopian tubes (2,7,8,10,12,19). The history of attempted in vitro fertilization in mammals is a lengthy one, dating back at least to the work of Shenk (1878) with rabbit and guinea-pig eggs (12,19).

It has been shown in several mammals that some physiological changes (Capacitation) occur in sperm of the female reproductive tract that enables the sperm to fertilize the egg (1,8,9,10,14,16,17,18). It is generally believed, however, that before the requirement for capacitation of spermatozoa was appreciated, the supposed fertilization of eggs in vitro with samples of ejaculated spermatozoa or those prepared from the male tract was either a chance occurence or, more probably, an incorrect diagnosis (10). The development and repeatable techniques for fertilization of rabbit ova in vitro rest largely upon the discovery significance of sperm capacitation in mammalian fertilization (1).

Capacitation is the enzymatic removal of a substance or coating, probably a polysaccharide, on the sperm cell head that is acquired in the seminal plasma (14).

This capacitation is appearent and studied most easily in the rabbit. Samples of capacitated rabbit sperm are usually obtained by flushing the uterine horns 10–12 hours after natural mating, but recent data (1) suggest that samples for use in vitro fertilization experiments are better taken at a later time.

Ova recovered from the superovulated donors are placed in synthetic medium + rabbit serum until the semination, when they are transferred to sperm samples flushed from the uterus with the synthetic medium + serum. The gametes are brought together at 37 °C in a self sealing culture dish. The ova are transferred to a culture medium.

Cytological examination of the ova after semination will allow the most accurate assesment of normality fertilization (1,3,4,5,6,13, 15,19).

At this time the male and female pronucleus and first and second polar bodies should be clearly visible, and the fertilizing sperm tail can often be located (1).

The purpose of this study is to assess the fertilization of rabbit occytes under various of in vitro conditions.

Material and Methods

In vitro fertilization of rabbit ova was investigated in 42 females and 6 males New Zealand white rabbits used as donors of ova and

spermatozoa. All does were 5–9 month–old virgins that were individually caged at least 21 days before being used, to avoid pseudopregnancy, the males proven fertility.

The materials were divided into 12 groups each containing two donors and one capacitator female.

Each ovum donor was treated with an intramuscular injection of 150 IU PMSG (Gestyl ORGANON, Folligon INTERVET, Suigonan VEMIE) followed 72–88 hours later with 75–100, IU HCG (Pregnyl ORGANON, Chorulon INTERVET) for superovulation. Ova of the firtst group were recovered from large follicles 10 hours after the HCG injection. Ovaries were excised, submerged in a saline and carried into a 38 °C culture room. Extraneous tissue was removed. Ova were liberated by puncturing each follicle with a sharp probe. Ova recovered four ovaries in the first experiment were collected and pooled in this way before the recovery of spermatozoa.

In total eleven experiments, ova were collected by flushing oviducts 16 1 / 2–21 1 / 2 hours after injection of HCG. Collected ova were then examined by phase contrast microscopy for the presence of a first polar body within the perivitelline space.

Capacitated spermatozoa were recovered from capacitators mated to two–six bucks 18–24 hours previously. Capacitators were anesthesied and their female reproductive tracts were immediately exposed. Each uterine horn was flushed 3–4 ml volume of medium. Each sperm suspension was taken into the warm room, where it was put into a small tissue culture dish and covered with paraffin oil equilibrated with 5 % CO_2 in air or 5 % CO_2– 95 % N_2.

In vitro insemination was carried out by adding the ova to the sperm suspansion. Incubation was carried out at 38 °C in a most air atmosphere. These surgical procedures were carried out under aseptic conditions and under pentobarbital anesthesia.

Brackett's (1) medium for rabbit in vitro fertilization supplemented by the addition of 20 % heated rabbit serum and 10 % serum solution was used.

Brackett's medium for in vitro fertilization of rabbit ova

Component	g / l	mM
NaCl	6.550	112.0
KCl	0.300	4.02
$CaCl_2 2H_2O$	0.330	2.25
$NaH_2PO_4H_2O$	0.113	0.83
$MgCl_2 6H_2O$	0.106	0.52
$NaHCO_3$	3.104	37.0
Glucose	2.500	13.90
Crystalline bovine serum albumin	3.000	
Penicillin-G-Sodium	0.031	
Distilled water to 1000 ml		

The 10 % serum solution was composed of acidic saline with 0.25 % glucose, and 10 % heated rabbit serum.

Results

The number of counted follicles and ovulation places (points), recovered and used ova, fertilized and unfertilized ova are shown in Table 1. According to Table 1, totally 678 follicles and ovulation places (corpus haemorrhagicum) were counted. Totally 251 (37.02 %) were collected by flushing oviducts and ruptured follicles. Two hundred thirtysix normal ova were used for in vitro fertilization. As far as the results are concerned, in vitro fertilization was observed in 47 (19.91 %) out of 236 ova, 34 of fertilized ova were normal and the rest (13 ova, 5.50 %) were abnormal, 189 (80.09 %) ova were found as unfertilized and degenerated.

Morphological pecuilarities of recovered ova are shown in Table 1. One hundred fourtyfive (57.76 %), 102 (40.63 %), and 4 (1.59 %) of ova were covered with zone pellucida, corona radiata and cumulus oophorus.

In experiment 7–12, normal fertilized ova were observed in 34 (25.95 %) out of 131 used ova. 22 (64. 70 %), 10 (29.41 %), and 2 (5.88 %) ova were found two–cell stage, four-cell stage and eight-cell stage respectively, but there was no pronuclear stage of ova.

Discussion

Capacitation is appearent and is studied most easily in the rabbit. Samples of capacitated rabbit sperm are usually obtained by flushing the uterine horns 10–12 hours after natural mating. But recent data

Table 1. Follicles and ovulation places, number of recovered and used ova and results of fertilized and unfertilized ova.

Exp. No.	Follicles and ovulation places	No. of used ova	No. of recovered ova	Fertilized ova		Unfertilized ova	
				Normal	Abnormal	Normal	Degenerated
1	62 Fol.	4	4	—	—	4	—
2	83 Ov.Pl.	12	10	—	6	3	1
3	78 " "	26	20	—	2	7	11
4	85 " "	35	31	—	3	28	—
5	12 " "	4	4	—	—	4	—
6	51 " "	36	36	—	—	36	—
7	98 " "	33	33	16	—	15	2
8	86 " "	34	34	4	1	9	20
9	39 " "	19	19	7	—	9	3
10	29 " "	13	13	1	—	—	12
11	40 " "	25	22	5	1	12	4
12	15 " "	10	10	1	—	9	—
Total	678	251	236	34 14.40%	13 5.50%	136 57.72%	53 22.45%
				47 (19.91%) Total fertilized ova		189 (80.09%) Total unfertilized ova	

Table 2. Morphological pecuilarities of collected ova.

Exp. No.	No. of recovered ova	Morphological pecuilarities of recovered ova		
		With Zona Pellucida	With Corona Radiata	With Cumulus ophorus
1	4	—	—	4
2	12	12	—	—
3	26	26	—	—
4	35	—	35	—
5	4	4	—	—
6	36	36	—	—
7	33	—	33	—
8	34	—	34	—
9	19	19	—	—
10	13	13	—	—
11	25	25	—	—
12	10	10	—	—
Total	251	145 (57.76%)	102 (40.63%)	4(1.59%)

suggest that samples for use in vitro fertilization experiments are better taken at a later time (1,10). According to Seitz et al (15), the best in vitro fertilization rates were achieved when spermatozoa had resided in the uterus for 16–18 hours. Hahn (8), reported that optimum capacitation time for sperms was 13 hours.

In this study, samples of capacitated rabbit sperm were usually obtained by flushing the uterine horns 18 ½ – 24 hours after natural mating and the best in vitro fertilization rates were achieved when spermatozoa had resided in the uterus for 18 1 / 2 hours. These results are similar to those reported by Seitz et al (15) and are different from those reported by others (8).

Bedford and Chang (1) concluded that several characteristics of spermatozoa including quality, mass activity and motolity were important in fertilization. Hahn et al (8), claimed that at least 10^5 spermatozoa must be found in the fertilization medium for in vitro fertilization. In these experiments, the best results were obtained in experiment 7, reported here in (Table 3).

Kılıçoğlu and Tekeli (11), Brackett and Server (5) determined the recovery rates of ova 52–75 % and 48.4 % respectively. The recovery rate of ova in this experiment was found to be lower than the rates obtained by Kılıçoğlu and Tekeli (11) and Brackett and Server (5).

In literatures, there ara some reports related with the rates of fertilized ovum; Bedford and Chang (1), Chang and Bedford (7), Mills et al (13), Hahn et al (8) have determined the following rates 30–94 %, 12–90 %, 43.5–73.5 %, 44–90 %. In this study the rate of fertilized ovum was observed to be 19,91 %. These results are found to be less than those of the others (1,7,8,13).

Collected ova were placed into a spermatozoit suspension and covered with paraffin oil which had been equilibrated with 5 % CO_2 in air. Then the dish was wrapped with foil to avoid light and was incubated under a moist 5 % CO_2 in air atmosphere Five hours after the insemination the ova were transferred to 10 % serum solution and incubated under an air atmosphere until the time of examination, but in this study, although the incubation of gametes under a moist 5 % CO_2 in air atmosphere were not applied, in vitro fertilization of rabbit ova was achieved. Incubation of gametes under a moist 5 % CO_2 in air atmosphere reported by Brackett and Williams was found to be not necassary. In this study, in vitro fertilization was achieved and observed in 47 (19.91 %) out of ova.

Table 3. Characteristic pecuilarities of spermatozoa and results of in vitro fertilization.

Exp. No.	Pecuilarities of used spermatozoa			Results of invitro fertilization		
	No. of sperma-tozoa	Mass* activity	% Motility**	No. of incubated ova	No. of fertilized ova	%
1	$3x10^3$	+-+	60	4	—	—
2	$4x10^4$	++	30	10	—	—
3	$6x10^4$	++	30	20	—	—
4	$5x10^4$	+-+	30	31	—	—
5	$3.\ 2x10^3$	0	10	4	—	—
6	$2.\ 4x10^3$	0	10	36	—	—
7	$1.25x10^5$	++	80	33	16	48.48
8	$1.\ 3x10^4$	++	65	34	4	11.76
9	$1.\ 9x10^4$	+-+	65	19	7	36.84
10	$6x10^4$	++	45	13	1	7.69
11	$4.\ 8x10^4$	+-+	60	22	5	22.72
12	$2x10^4$	++	45	10	1	10.00

x. (0) No mass activity
(+) Slow wave motion
(++) Rapid wave motion with formation of eddies at the end of waves
(+++) Eddies

xx. 80–100 % Very good
60–80 % Good
40–60 % Normal
20–40 % Slow
0–20 % Very slow

Table 4. Stages of in vitro fertilized rabbit ova.

Exp. No.	In Vitro Fertilization					
	No. of used ova	Normal fertilized ova	Pronuclear stage	Two-cell	Four-cell	Eight-cell
7	33	16	–	8	7	1
8	34	4	–	4	–	–
9	19	7	–	4	2	1
10	13	1	–	1	–	–
11	22	5	–	4	1	–
12	10	1	–	1	–	–
Total	131	34 (25.95%)	–	22 (64.70%)	10 (29.41%)	2 (5.88%)

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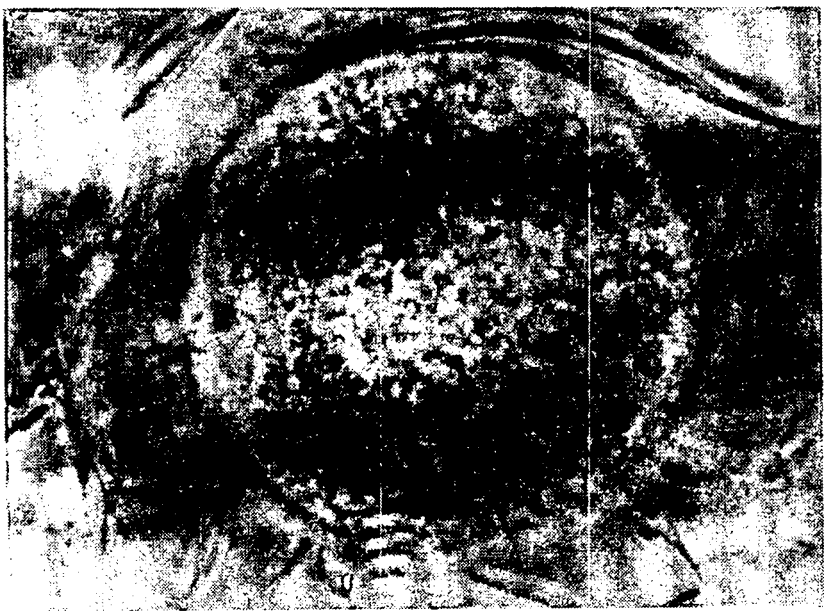

Figure 1. Rabbit ovum penetrated by capacitated spermatozoa, (x300).
Şekil 1. Besiyerinde birarada inkübasyona bırakılan ovum ve spermatozoitler.

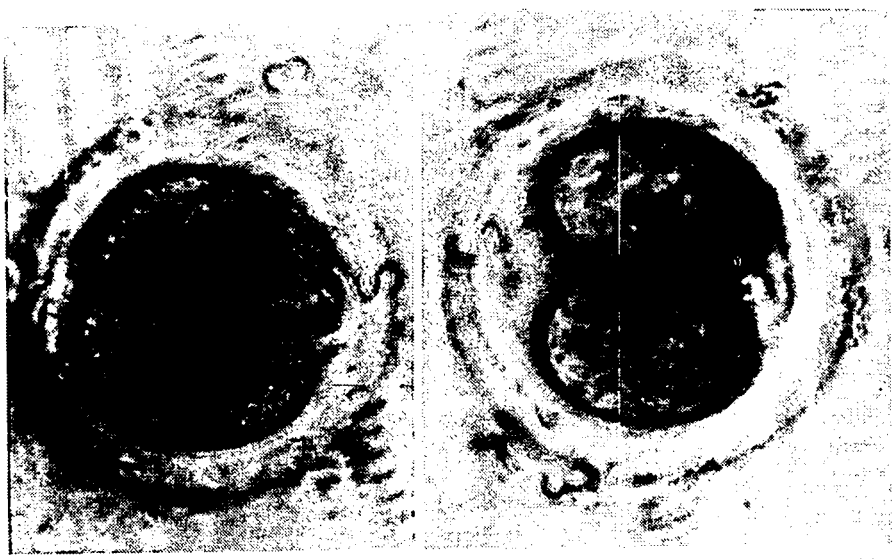

Figure 2. a-Two-cell stage rabbit ovum fertilized in vitro, (x150). b-Four-cell stage rabbit embryo fertilized in vitro, (x150).

Şekil 2. In vitro olarak döllenmiş iki hücreli (blastomerli) embriyo, (x150). b-In vitro olarak döllenmiş dört hücreli (blastomerli) embriyo, (x150).