In *Vitro* Fertilization of Mous Ova Obtained By Three Ways and Their Cleavage in Commercially and Chemically Defined Media (*)

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Abstract: In this study ova were collected from the regularly mated, pseudopregnant, and superovulated females Balb/c or C57/J6 mice. The ova were *in vitro* fertilized by sperms collected from the vas deferens and epididymis of male mice in fertilization medium (FM). The fertilized ova were cultured in four types of media (M16, M2, MCM, KSOM). The results have shown that in *in vitro* fertilization and early embryonic development depend on the way the ova were collected and the culture media used. The ova collected from regular mating were superior to those collected from pseudopregnant females or superovulated ones as regards to their ability for *in vitro* development (35.9%, 8%, 12.8%) respectively. The different culture media used in this study had different abilities for the maintenance of development of ova (MCM=63.8%, M2=53.1%, M16=9.4, and KSOM=16.5%) obtained by the three methods of collection. Superovulated females produce larger number of normal and abnormal ova which had lower capabilities for *in vitro* embryonic development compared to those obtained from pseudopregnant or normally mated females.

Key Word: *in vitro* fertilization, cleavage, culture media.

Introduction

For proper *in vitro* fertilization and early embryonic development, the embryo culture medium should satisfy certain requirement such as optimum pH value and osmolarity. pH value of the medium affects the fertilization rate in the harmster (Bavister 1969), and fetilization of mouse eggs (Iwamatsu & Chang, 1971). A culture medium having an osmolarity just lower than the blood serum is favourable for development of mammalian eggs *in vitro* (Miyamoto and Chang, 1972). The normal osmolarity of the tissue culture media and blood serum is approximately 308 m. osmol

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(Brinster, 1965). However, a culture medium with a lower osmolarity (276 m.osmol) was found to be more favourable for the development of two-cell stage mouse embryo into a blastocyts (Brinster, 1965a). A modification of the simplex optimization Medium (SOM), designated KSOM, contain a high concentration of K^+ (2.5 mM). It supports growth beyond the two cell block; provides a larger yield of blastocysts (88%) (Erbach *et al.*, 1994). Glucose plays an inhibitory role during the early stages embryonic development (Chatot *et al.*, 1989). Sakkas and his co worker, (1993), studied the effects of glucose and fructose on fertilization, cleavage and viability of mouse embryos *in vitro* using three types of media a) the stander M 16, b) M16 without glucose (M16-G), and c) M16 with fructose and without glucose (M16F-G), they found that the M16 support fetilization. In 65% of OFI strain embryos cultured in M16 support fertilization. In 65% of OFI strain embryos cultured in M16 developed was blocked at the 2-cell stage, whereas in M16-G and M16. F-G embryos, only 22% and 23% respectively were blocked. The objective of this study is to compare two commercially produced culture medium (M2 and M16 from Sigma, U.S.A.) with (KSOM) (Erbach, G. *et al.*, 1993) and the modified culture medium (MCM) to be prepared at our Lab. The comparison aimed to assess the effects of the different culture media on fertilization and early embryonic development of mouse embryo *in vitro.*

Material and Methods

(1) ***Ova Collections***: The Ova were collected from females (6-8 week old) either (Balb/c) or (C57/J6) strain of mice, (all animals were kept in a room with 25 °C 10hr. light, and 14hr. dark), according to the following:

1. ***Regular mating***: Groups of females were placed with male (4 female with one male) for mating. In the following morning, the mating was confirmed by the presence of vaginal plug. Then the ova were collected from mated females and the cumulus cells were removed using hyaluronidase (300 IU/ml, Sigma, U.S.A.) in M2 with HEPES media (for 1-2 min). Then washed two times with fertilization media (FM) and *in vitro* fertilized.

2. ***Pseudopregnancy***: A number of females were placed with vasectomised males in a ratio of 4:1 and checked daily for the presence of vaginal plug. Then the mated females were killed and the ova were collected in M2 with HEPES medium. Then the cumulus cells were removed as above and the ova were fertilized *in vitro.*

3. ***Superovulation***: Groups of females (3-6) were superovulated by intraperitioneal injection of 10 IU PMSG (Folligon) from (Intervet, Holland). This was followed after 48 hours by an injection of LH, (Chorulen), from (intervet, Holland). After 16 hours

Table 1. Composition of the different media used for the *in vitro* fertilization (IVF) and culture of mouse embryo.

Component (g/L)	Fertilization media (FM)	Culture media (MCM)	KSOM	M16 (Sigma)	M2 (Sigma)
NaCl	5.99	5.97	5.51	5.53	5.5
KCl	0.20	0.35	0.18	0.36	0.35
KH_2PO_4	-	0.16	0.047	0.16	0.16
$NaH_2PO_4.2H_2O$	0.058	-	-	-	-
Ca Lactate. H_2O	-	0.45	-	-	-
Mg_2SO_4	-	0.14	0.024	0.143	0.14
$NaHCO_3$	2.1	1.9	2.22	2,1	0.35
Glucose	1.0	1.0	0.036	1.0	1.0
Na Pyruvate	0.055	0.03	0.022	0.036	0.03
Na Lactate 60% (syrap)	3 ml	-	3 ml	4.349	4.34
Penicilin G	0.075	0.075	0.075	0.075	0.075
Streptomycin SO_4	0.05	0.05	0.05	0.05	0.05
Phenol Red	2 ml	2 ml	2 ml	0.01	0.01
$CaCl_2.H_2O$	-	-	0.18	0.25	0.25
Glutamine (20 mM)	-	-	0.5 ml	-	-
EDTA (Na)	-	0.0029	0.0029	-	-
BSA *(Fraction V)	1.0	3.0	1.0	4.0	4.0
Fructose	1.0	-	-	-	-
HEPES *(Free acid)	-	-	-	-	4.96

(FM) = Fertilization media prepared at our lab from (Dodds, and Seidel, 1984).
(MCM) = Modified culture media prepared at our lab.
(KSOM) = Culture media prepared at our lab from (Erbach *et al.*, 1994).
(M16 & M2) = Commercially pre-made culture media from (Sigma, U.S.A.).
(*) BSA=Bovin serum albumin powder. HEPES=N-2-Hydroxyethyle piperazine-2-ethanesol sulfonic acid.

later (in the next morning), the females were killed and the ova were collected and released from the cumulus cells as described above.

(2) ***In Vitro Fertilizationa (IVF)***: Sperms were collected from adult (Balb/c or C57/J6) males mice, by killing the males to extract and squees the vas deferens and cauda epididymis into fertilization media (FM) (Dodds and Sideal, 1984) (Table 1). About 10 µl of sperm suspention were added from above to 0.5 ml of fertilization medium to containing the collected ova and kept in the incubator (37 °C with 5% CO_2 in air) for about 1-2 hours (Dodds and Sideal 1984). Later on the ova were distributed and washed for two times changed into the one of the selected culture media (M2, M16, KSOM or MCM). The embryos were culture for 3 days and the data of fertilized and the developed embryos were recorded daily.

(3) ***Culture Media:*** Four types of culture media were used, two commercially pre made culture media, namely M2 with HEPES and M16 media from (Sigma, U.S.A.) and two other culture media were prepared at our laboratories, namely KSOM (Erbach G. *et al.*,, 1994), and MCM (Table 1).

Table 2. The numbers and means (±SE) of the normal and abnormal ova collected by three ways of collection.

Way of ova collected	No. of females used	Total no. of ova collected	Mean no. of ova/female	No. of abnormal ova
Regular mating	67	644	9.61 (±0.26)	10 (1.5%)
Pseudopregnant	19	215	11.31 (±0.88)	$7^{(a)}$ (3.25%)
Superovulation	71	1361	$19.1^{(c)}$ (±1.17)	$65^{(b)}$ (4.77%)

(a) and (b): Significantly different at ($P<0.01$) compared to regularly mated.
(c): Significantly different at ($P<0.05$) compared to the others.

Statistical analysis: Chi-square analysis (X^2) (2x2 contingency table) were used to compare between each two means of each treatment. The ranks of transformation method were used to compare the development of different way of collected ova in the four different culture media (Prof. I. Ahmed personal communication)..

Results

A total of 2220 ova collected from 157 female's mice (14 ova/female) for this study, 3.7% were abnormal ova (broken-down cytoplasm). The superovulated females has produced more ova than the regular mated and pseudopregrant ones; their mean number of ova per female (19.16) was significantly higher ($P<0.05$) than those of regularly mated (9.61) and pseudopregnant (11.31) ones. Nevertheless the percentage of abnormal ova in superovulated females (4.77%) was significantly higher ($P<0.01$) than in pseudopregnant (3.25%) and regularly mated (1.5%) ones (Table 2).

In Vitro fertilization and embryonic development: Examination of ova incubated first with sperms in the fertilization medium followed by incubation in the culture medium revealed that they were of three types: (a) Single cell uncleaved ova which were considered undeveloped, (b) cleaved ova at the 2-, 4-, or more cell stage which were considered as developing embryo, (c) degenerating or fragmented ova which were considered as abnormal d. The total number of ova were obtained from regularly mated, pseudopregnant and superovulated females, and the effects of different culture media on their subsequent development are shown in (Table, 3) and (Fig. 1). The percentage of the total number of developing embryos in all culture media was higher in ova obtained from regular mated females (35.9%) than in those obtained from pseudpregnant (8%) and superovulated ova (12%). Comparison of embryo development in the different types of culture media regardless of the way it was obtained, have shown that the highest percentage of developing embryo were found in MCM (30.7%) and M2 culture media with HEPES (27.7) followed by M16 (10.6%) and KSOM (9.6%). It is apparent then; that embryonic development depends on the way the ova are collected and the

Table 3. The total number and the percent of development (%) of the *in vitro* fertilized ova obtained by three different ways and cultured in four types of media.

Way of ova obtained	Total no. of ova development & %[1]	Type of culture media			
		CM	KSOM	M16	M2
Regular mating	481	105	103	128	145
	a) 227 (47.2%) b) 173 (35.9%) c) 81 *(16.9%)	a) 30 (28.5%) b) 67 (63.8%)(*) c) 8 (7.7%)	a) 60 (58.3%) b) 17 (16.5%) c) 26 (25.2%)	a) 71 (55.4%) b) 12 (9.4%) c) 45 (55.2%)	a) 66 (45.5%) b) 77 (53.1%)* c) 2 (1.4%)
Pseudopregnancy	163 a) 132(81%) b) 13 (8%) c) 18 (11%)	56 a) 49 (87.5%) b) 6(10.7%) c) 1(1.8%)	35 a) 32(91.4%) b) -2(5.7%) c) (2.9%)	26 a) 23(88.5%) b) 1 (3.8%) c) 2 (7.7%)	46 a) 28(60.9%) b) 4(8.7%) c) 14(30.4%)
Superovulation	640 a) 321(50.1%) b) 82(12.8%) c) 237(37.1%)	171 a) 120(70.1%) b) 29(17%) c) 22(12.9%)	110 a) 25(22.7%) b) 5(4.5%) c) 80(72.8%)	146 a) 44(30.1%) b) 19(13.1%) c) 83(56.8%)	213 a) 132(62%) b) 29(13.6%) c) 52(24.4%)
Total no. of ova/in all culture media	1284 a) 680(52.9%) b) 268(20.8%) c) 336(26.1%)	332 a) 199(59.9%) b) 102(30.7%) c) 31(9.4%)	248 a) 117(47.2%) b) 24(9.7%) c) 107(43.1%)	300 a) 138(46%) b) 32(107%) c) 130(43.3%)	404 a) 226(55.9%) b) 110(27.3%) c) 68(16.8%)

(1) a) undeveloped ova (one cell). b) developed embryo (2 cell and more) .c) abnormal development (degenerated and fragmented. Ova) (*) highly significant at ($P<0.01$) compared to the other developed ova).

type of media used. Of the ova obtained from the regular mating, more than 50% development into the two-cell stage when cultured in M2 or MCM. This was significantly higher than the percentage of ova development into that stage in the other media (M16 and KSOM). On the other hand, ova obtained from the superovulation females did not show significant variations as regards to their developmental capabilities in the different media except the KSOM where only (4.5%) showed sign of development (Table 3).

Discussion

In accordance with the findings of Al-Himaidi, (1988), and (1993) the results of this study have shown that the superovulation produces more normal and abnormal ova than the regular mating. The *in vitro* fertilization (IVF) and early embryonic development depend on the way the ova were obtained and on the type of culture media used. The ova collected from regular mating females were better than those collected from pseudopregnancy and

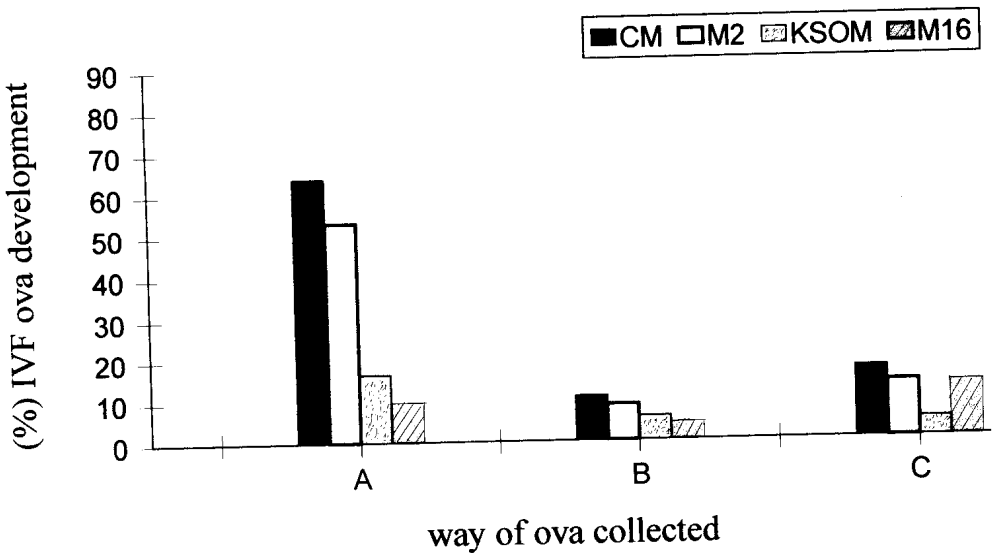

Fig 1. The development (%) (2 cell and more) of the *In Vitro* fertilized mouse ova collected by three ways of and cultured in four types of media. A=Regular mating, B=Pseudopregnancy, C=Superovulation, CM=Culture medium, M2 with HEPES culture media (from Sigma U.S.A.), KSOM=Potasium simplex optimiztion medium, M16 culture media (from Sigma U.S.A.)

superovalted females as regard to the IVF and subsequent development of the embryo. This could be attributed to the fact that ova of the regularly mated females were exposed directly to sperms and oviductal fluids before they were collected. The fertilization rates noted in the (IVF) could be attributed to the short capacitation time, which the sperms subjected to (Hashlamoun L. *et al.*, 1985). This could also be ascribed to the various metabolic challenges, which the mammalian embryo is subjected to from the time of fertilization to implantation (Sakkas *et al.* 1993). This is further argument by the continuous change in the environment that faces the embryo as it traverses the oviduct and uterus (Gardner and Leese 1986, Brown and Whittingham, 1992).

Our modified culture medium (MCM) seemed better that the other three media for this study. The difference in the development capabilities of the *in vitro* fertilized ova in the different media may be due to the different chemical composition of each media. Our modified culture media (MCM) differed from those of others (Abramczuk, *et al.*, 1977; Dodds and Seidel, 1984) in that it contain EDTA and Ca-lactate and also in that it lacked $CaCl_2H_2O$, Na Lactate, 60% (Syrup). Regardless of the way they were collected from the female, percentage of the total number of developing ova was least in those cultured in KSOM. Our result in this respect diagree with those reported by Erbach *et al.*, (1994). Discordant with the observations of

Sakkas *et al.*, (1993) and Leepens *et al.*, (1997), the result of our study has shown that the embryonic development rate was low in the M16. The low embryonic development rate noted in this study in *in vitro* fertilized ova cultured in M16 ratifies the recommendation of Sakkas, (1993) that M16 is not suitable for the IVF. It might be due to strain variation because we used Balb/c and C57/J6 mice and they use OF1 mice.

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الإخصاب الصناعي الخارجي لبويضات الفئران المعملية والتي تم الحصول عليها بثلاث طرق وتفلجها في بيئات تجارية وبينات محضرة في المعمل (*)

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الملخص :لقد تم دراسة عملية الإخصاب الصناعي الخارجي (IVF) وتفلج هذه البويضات المتحصل عليها بثلاث طـــرق من إناث الفئران المخبرية (بواسطة التزاوج العادي ، الحمل الكاذب ، التنشيط الهرموني) ومدى قدرة نمو الأجنة في بيئـات مختلفة (بيئة معدلة في المعمل MCM، وبيئة تناضحية KSOM، وبيئتان تجاريتان M٢,M١٦ (من شـــركة ســـيجما) U.S.A Sigma . بشكل عام أظهرت النتائج أن عملية الإخصاب الصناعي الخارجي للبويضات ونموها فيما بعد يعتمـد على الطريقة التي تم الحصول بها على البويضات وكذلك على نوع البيئة المستخدمة . إذ أظهرت التنـــائج أن البويضـــات المأخوذة من إناث التزاوج العادي كانت أفضل في عملية الإخصاب الخارجي ونمو الأجنة فيما بعـــد في البيئـــات الختلفـــة (٣٥,٩%) من البويضات المأخوذة بالطريقتين الأخريتين (٨% للتلقيح الكاذب ، ١٢,٨% للحقن بالهرمونات) . كمـــا أظهرت النتائج أن الإناث المحقونة بالهرمونات تنتج أكثر عدد من البويضات (سواء السليمة أو غير السليمة) لكنها أقـــل قدرة على النمو فيما بعد . كذلك كانت البيئتان المعدلة لدينا في المعمل (MCM)(٦٣.٨%) والبيئـة (M٢) (٥٣,١%) (التجارية) أفضل في عملية النمو للأجنة من البيئتين الأخريتين (KSOM,M١٦) .

(*) هذا البحث تم دعمه من مدينة الملك عبد العزيز للعلوم والتقنية برقم (م- ص - ١-٩-١٤١٧)
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