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哺乳動物卵子学会誌

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哺乳動物卵子学会
Japanese Society of
Mammalian Ova Research

Vol. 11 No. 2

October 1994

第３６回　哺乳動物卵子学会開催のご案内

下記により第36回哺乳動物卵子学会および総会を開催致します。多数の会員の参加をお願い致します。

第36回　哺乳動物卵子学会
大会長　菅原　七郎

記

期　　日：1995年5月18日（木）・19日（金）

会　　場：仙台サンプラザ　3階　クリスタルルーム
〒980　仙台市宮城野区榴岡（ツツジガオカ）5丁目11－1 電話：022（257）3333

連 絡 先：〒981　仙台市青葉区堤通雨宮町1－1
東北大学農学部動物生殖科学講座内 第36回哺乳動物卵子学会大会事務局
電話：022（272）4321　内線231　Fax ：022（272）1870

特別講演：「マウス毛色形成と初期発生にかかわる遺伝子の分子機構」
東北大学名誉教授　竹内拓司（クラスター・コア・日本遺伝子研究所）

教育講演：「生殖細胞の起源とその特異性の維持機構」
東北大学助教授　松居靖久（東北大学・加齢医学研究所）

ワークショップ：「哺乳動物卵子の体外取扱い法の問題点－体外培養系を中心に」

講演申込要領：

講演原稿

（a）演題申込　：締切／1995年1月28日（土）必着

講演の申込は、葉書に演題名（和文および英文）、発表者名および共同研究者（ローマ字）を記入のうえ、下記宛お送り下さい。折り返し、発表内容を記載する原稿用紙をお送り致します。講演原稿の締切は、1995年2月28日（火）必着とさせていただきます。

一般講演は前回と同様に講演内容をオフセット印刷し、哺乳動物卵子学会誌12巻１号に講演要旨として掲載いたします。なお、今回から講演原稿は１ページＡ４版におまとめ頂きます。

〒252　神奈川県藤沢市亀井野1866
日本農獣医学部獣医生理学教室
哺乳動物卵子学会事務局　遠藤　克宛
電話：0466(81)6241　内線 2125

（b）講　演　：　会員1人1題、講演時間1題7分、討論時間3分、プロジェクター1台

事前申し込み　：　参加費　5000円（当日6000円）と懇親会費5000円（当日6000円）を前納できます。

同封の郵便振替用紙をご利用下さい。なお、申し込み後には払い戻しは致しませんのでご了承下さい。　締切／1995年2月28日（火）

総　　会：　5月19日（金）13:15〜13:45　仙台サンプラザ　3階　クリスタルルーム

懇親 会　：　5月18日（木）講演終了後、仙台サンプラザ　3階　宮城野　で開催致します。多数ご参加下さい。

宿　　泊　：　依託した東北大学生活協同組合・学会宿泊係からの案内（次頁）をご参照下さい。

大会実行委員：　石川　勇志（宮城県庁）　梅津　元昭（東北大学農学部）

佐々田　比呂志（東北大学農学部）　　寺田　幸弘（東北大学医学部）

沼辺　孝（宮城県畜試）　　深谷　孝夫（東北大学医学部）

松本　浩道（東北大学農学部）

当大会では日本産科婦人科学会の認定医シールが発行される予定です。

第３６回　哺乳動物卵子学会
―宿泊予約のご案内―

東北大学生活共同組合　学会宿泊係

謹啓

時下、ますます御清祥のこととお慶び申し上げます。

この度、仙台市におきまして第36回哺乳動物卵子学会が開催されますことを心よりお祝い申し上げます。つきましては、表記学会に参加される方々の宿泊に関してご案内いたします。

宿泊予約は東北大学生活共同組合、学会宿泊係でお取扱い致します。この時期は他の学会も開催されており、仙台市内の宿泊施設が非常に混み合うことが予想されます。申込書到着順に受け付けを行いますので、安心して会議にご参加頂けますよう、お早目にお申し込み下さい。　　敬具

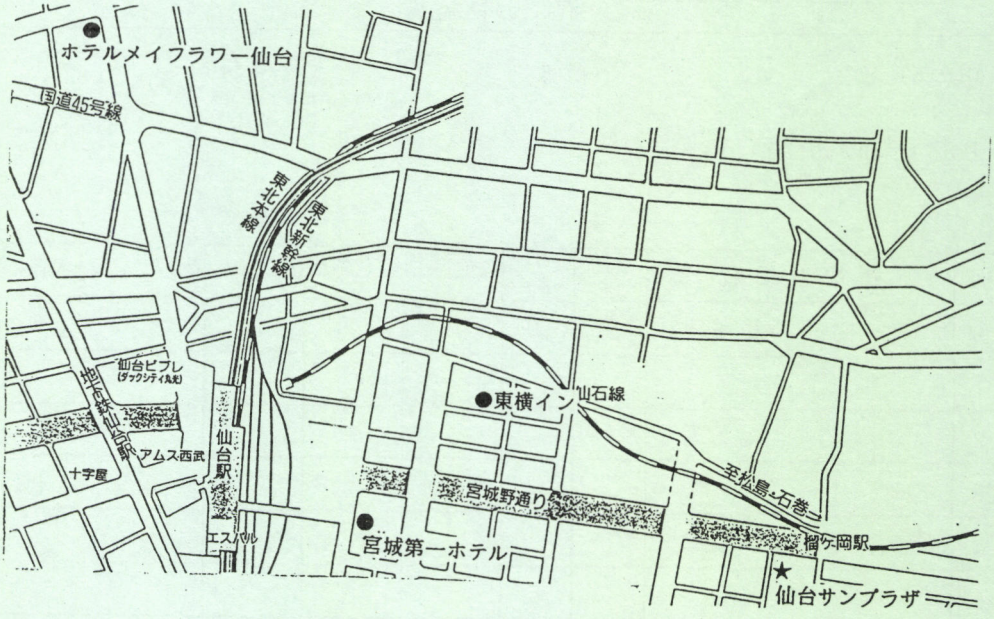
学会会場：仙台サンプラザ
仙台市宮城野区榴岡５-11-１（仙台駅東口より徒歩10分）

１．宿泊のご案内

（１）宿泊の取扱い期間　1995年５月17日（水）～19日（金）

（２）宿泊ホテル、料金（シングル）、場所は以下のようになっております。

記号	ホテル名	１泊朝食付	素泊		所在地
A	宮城第一ホテル	￥11,000	￥10,000	税別	宮城野区榴岡１-２-45
B	東横イン	￥8,500	￥7,500	税別	宮城野区榴岡３-４-31
C	ホテルメイフラワー	￥6,600	￥5,800	税込	青葉区本町１-３-28



2．お申し込み方法

申込用紙に必要事項（ホテル名は記号で記入）をご記入のうえ、コピーされ、下記の送り先まで郵送またはファックスでお申込みください。ツインルームご希望の方は備考の欄に記入してください。

（1）お申込締切日］1995年5月2日（火）必着

（2）宿泊券の送付・精算：ご出発の10日～15日前までに宿泊券をお送りします。

（3）同時に料金明細を同封いたしますので、恐れ入りますが、1週間以内にご送金下さい。尚、郵便通信料として、お一人様1件につき520円をいただいておりますので、ご了承下さい。

（4）お客様のご都合による予約変更・取消しに際しては、それぞれ210円づつ申し受けます。また、取消しの場合、取消日によって以下の違約金を申し受けます。

3日前取消しの場合　20%、　前日取消しの場合　50%

当日取消しの場合　80%、　無連絡不参加の場合　全額

（送り先）〒980　仙台市青葉区片平2－1－1

東北大学生活共同組合　学会宿泊係

（Tel. 022－261－5040　Fax. 022－261－1015）

宮城県知事登録国内旅行業第141号

営業時間　平日　9:45～13:15

14:15～17:30

土曜日　9:45～13:30

第36回哺乳動物卵子学会

宿泊申込書

申込者氏名（ふりがな）：　　所属：　　電話：

住所（宿泊券送付先）〒

電話：

No.	宿泊者氏名	性別	年齢	宿泊日と希望ホテル名			受付No.
例	生協　太郎	男	50	A	A	A	
1							
2							
3							
4							
5							

備考：

哺乳動物卵子学会誌

第１１巻　第２号　　平成６年１０月１日

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原著論文（和文）

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The meiotic resumption and cleaving ability of porcine oocytes matured in - vitro and followed by elctroactivation

Satoshi KUREBAYASHI[1)], Masashi MIYAKE, Kenji KOHNO,
Takashi MIYANO and Seishiro KATO

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Abstract: The optimum voltage for inducing activation by electrostimulation of porcine oocytes matured in vitro was determined. Oocytes matured for 42 h were exposed to a single squared pulse for 100 μsec at 100 to 3,000 V/cm DC and examined 12 h later. More than 90 % of eggs pulsed at 250 to 1,250 V/cm were activated, and the highest rate (98 %) was obtained at 750 V/cm. Oocytes matured for 36 to 54 h were subjected to the stimulation at 750 V/cm. The rates of activation in oocytes cultured for 36 and 38 h (47 and 44 %, respectively) were significantly lower than in those cultured longer than 42 h (94 to 99 %; $P<0.01$). Succeeding events were examined. Oocytes matured for 42 h were pulsed at 750 V/cm, and anaphase II (32 %) and telophase II (32 %) were observed 10 min and 30 min after stimulation, respectively. Nuclear formation was completed 4 h after stimulation in 95 % of the eggs. Meiotic resumption induced by electrostimulation is thus quite synchronous. Extrusion of the second polar body and formation of a nucleus was observed in 92 % of activated eggs. These eggs when further cultured developed beyond the 4 - cell stage. **Key Words:** Electrostimulation, Resumption of meiosis, Cleaving ability, Porcine follicular oocytes (Received 24 January 1994, Accepted 25 February 1994)

Introduction

The resumption of meiosis in mammalian secondary oocytes, so called the activation, is ordinarily triggered by the sperm penetration. The activation is also induced by various other stimuli[1)]. The details of the activation methods and succeeding development of activated eggs have been studied mainly in the mouse[1,2)]. Recently, these events are also investigated in the rabbit[3)], cattle[4-7)] and human[8)].

Only several reports described the development of porcine eggs matured and fertilized in vitro[9-11] because of high incidence of polyspermy in-vitro. The employment of the in-vitro activation instead of in-vitro fertilization may lead to elimination of such problems. Porcine follicular oocytes can be obtained from the almost unlimited supply of ovaries from commercially slaughtered pigs. There have been only several reports concerning the methods of activation[12-15] and the details of events that follow[16].

This study was designed to establish an effective condition for inducing the activation by electrostimulation of porcine oocytes matured in-vitro. Succeeding events in the activated eggs including the cleaving ability were examined.

Materials and Methods

Collection and maturation culture of follicular oocytes : The composition of media was based on Yoshida et al.[17]. Briefly, TCM-199 with Earl's salts (Nissui, Japan) supplemented with 0.35 mM D-glucose, 2.92 mM Ca-lactate, 0.91 mM Na-pyruvate, 100 U/ml penicillin-G potassium, 50 μg/ml streptomycin sulfate and 10 % (V/V) heat-treated fetal calf serum (Biocell Lab., U.S.A.) (mTCM) was used for culture of the eggs activated. mTCM including 10 IU/ml PMSG (Teikoku Zoki Japan), 10 IU/ml hCG (Sankyo, Japan) and 1 μg/ml estradiol-17β (Sigma, U.S.A.) was used for maturation culture of the oocytes collected as below.

Ovaries were collected from gilts at a local slaughterhouse and brought to the laboratory within 1 h. Temperature was maintained at 30-35℃. Ovaries were washed with Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate buffered saline (PBS), and the oocyte-cumulus complexes (OCC) were recovered from antral follicles, 2-5 mm in diameter, by puncture. OCC were washed three times with maturation medium, and oocytes possessing compact cumulus mass and evenly granulated ooplasm were selected for the experiments. Ten oocytes each were transferred to the droplets of maturation medium (100 μl) under paraffin oil (Nacarai Tesque, Japan) in a plastic dish (60 × 15 mm, Falcon, U.S.A.) and cultured for various durations in a CO_2 incubator at 39℃ in air containing 5 % CO_2 with high humidity.

After maturation culture, the oocytes with expanded cumulus mass were suspended in PBS containing 290 U/ml hyaluronidase (Sigma) for 2-3 min, and freed from the cells by pipetting in mTCM. The denuded oocytes were washed three times with mTCM and ascertained the extrusion of the first polar body under an inverted microscope. The oocytes with the first polar body were used for the experiments.

Induction of parthenogenetic activation : For electrostimulation, 0.3 M mannitol solution containing 0.1 mM $MgSO_4$ and 0.05 mM $CaCl_2$[18] was placed between parallel electrodes 2 mm apart in an electric chamber (FTC-03; Shimadzu, Japan). Ten to 20 denuded oocytes were placed in the solution and they were subjected to a single squared pulse for 100 μsec from an electric cell fuser (SSH-1; Shimadzu). They were transferred to mTCM within 1 min, washed three times with mTCM and then cultured in a CO_2 incubator.

Examination of eggs : After culture, eggs were whole-mounted, fixed in Carnoy's solution for 48 h and stained with aceto-orcein in all experiments except Experiment 4. The eggs thus prepared were then examined under a differential interference microscope (Nikon, Japan). In Experiments 1 and 2, the activation was judged on the basis of the formation of nucleus(ei). In Experiment 3, the presence of the nucleus(ei) and the stage of the second meiosis were evaluated. The activated eggs before formation of nucleus(ei) were classified into the following stages; metaphase II (Met II, Plate 1A), anaphase II (Ana II, Plate 1B), telophase II (Tel II, Plate 1C) and condensed chromatin (CC, Plate 1D). Some eggs were stained with 1 μg/ml of Hoechst 33342[19] (Polysciences, U.S.A.) and observed under an epifluorescent microscope (Nikon) in Experiment 3.

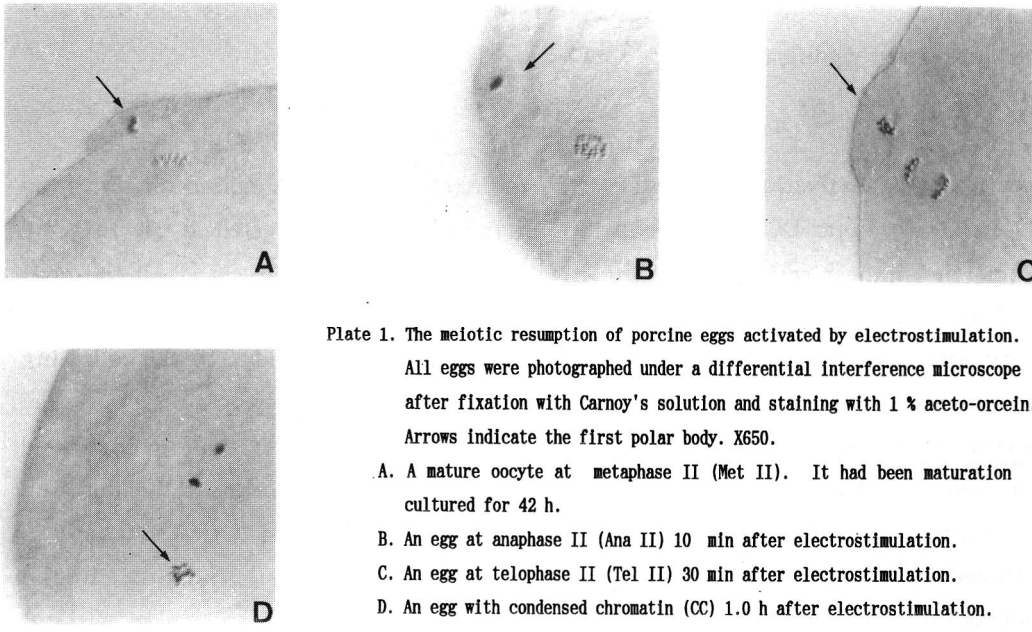

Plate 1. The meiotic resumption of porcine eggs activated by electrostimulation. All eggs were photographed under a differential interference microscope after fixation with Carnoy's solution and staining with 1 % aceto-orcein. Arrows indicate the first polar body. X650.

A. A mature oocyte at metaphase II (Met II). It had been maturation cultured for 42 h.

B. An egg at anaphase II (Ana II) 10 min after electrostimulation.

C. An egg at telophase II (Tel II) 30 min after electrostimulation.

D. An egg with condensed chromatin (CC) 1.0 h after electrostimulation.

Experiments :

Experiment 1: To examine effects of charged voltage on the rate of activation, oocytes cultured for 42 h were stimulated at 0 to 3,000 V/cm DC (see Fig. 1). Activation was determined 12 h after electrostimulation.

Experiment 2: Effects of the duration of maturation culture on the activation of oocytes were examined. Oocytes cultured for 36, 38, 40, 42, 48 and 54 h were electrically stimulated at 750 V/cm DC. As a control the occurrence of activation without electrostimulation was measured on the oocytes cultured for 54 h and placed for 1 min in mannitol solution.

Experiment 3: Oocytes cultured for 42 h and stimulated at 750 V/cm DC were further cultured for 10, 20, 30 min, 1.0, 2.0, 3.0, 4.0, 5.0, and 6.0 h. The timing of the resumption of the second meiotic division and nuclear formation were examined. Activated eggs were classified into four types according to the number of polar bodies and nuclei[1] (see explanation of Table 3).

Experiment 4: Cleaving ability of activated eggs with 2 polar bodies was examined. Oocytes cultured for 42 h and activated at 750 V/cm DC were cultured for 6 h, and examined for the presence of the second polar body on an inverted microscope. The eggs with 2 polar bodies were further cultured up to 60 h. Numbers of blastomeres in these eggs were counted 24, 48 and 60 h after electrostimulation.

Statistical analysis : The results of Experiment 2 and the types of activated eggs in Experiment 3 were analyzed using one-way ANOVA. Probability less than 0.01 was considered significant.

Results

Experiment 1: Activation was observed in the eggs treated with pulses ranging from 100 to 2,500 V/cm DC. More than 90 % of the eggs were activated at 250 to 1,250 V/cm, the highest rate (98 %) being obtained at 750 V/cm (Fig. 1). All the eggs not activated by the treatment between 0 to 750 V/cm were still being arrested at metaphase II. All the eggs treated at 3,000 V/cm degenerated. In the following experiments oocytes were thus stimulated at 750 V/cm.

Experiment 2: More than 90 % of the oocytes cultured for longer than 36 h showed maturation (Table 1). The rate of activation, however, increased as the duration of culture increased. The rates of activation were 47 and 44 % in the oocytes cultured for 36 and 38 h, respectively. The rate increased significantly in the oocytes cultured for 42 h or more ($P<0.01$). No oocytes were activated without electro stimulation.

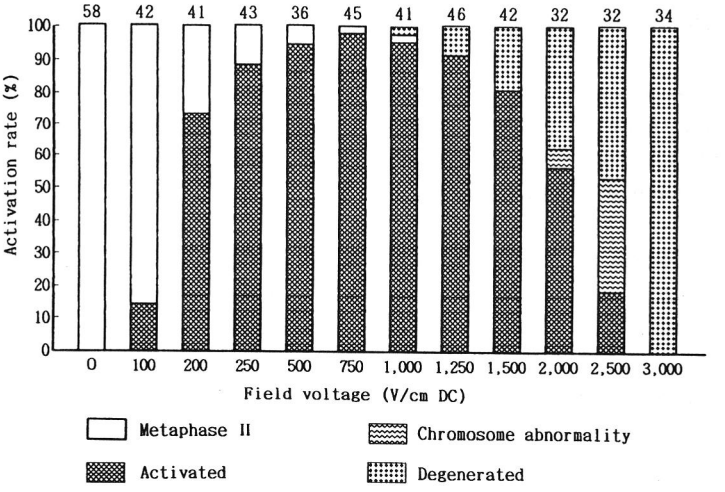

Fig. 1. Effect of field voltage on the rate of activation in porcine oocytes.

The figure on each column means the number of oocytes examined.

Table 1. The effects of duration of maturation culture on the activation of porcine oocytes

Duration of maturation culture (h)	Stimulation	Number of eggs		
		Examined	Matured(%)*	Activated(%)**
36	+	109	104(95)	49(47)[a]
38	+	69	64(93)	28(44)[a]
40	+	68	66(97)	48(73)[a,b]
42	+	70	70(100)	66(94)[b]
48	+	86	85(99)	84(99)[b]
54	+	78	78(100)	74(95)[b]
54	-	44	42(95)	0(0)

+: with electrostimulation.

-: without electrostimulation.

*: Rate to the number of eggs examined.

**: Rate to the number of matured eggs.

a,b: Figures with different superscripts are significantly different ($P < 0.01$). Oocytes without electrostimulation are not included in the statistical analysis.

Experiment 3: Table 2 shows the stage of meiosis and formation of nucleus(ei) in the activated eggs. Ana II (32 %) was observed within 10 min, and Tel II (32 %) emerged half an hour after activation. Resumed meiosis proceeded quickly, and almost all eggs (95 %) had CC 2.0 h after activation. Nuclear formation began at this time and completed 2.0 h later in 95 % of the stimulated eggs.

Table 2. Resumption of the second meiosis and nuclear formation after electrostimulation in porcine oocytes matured in-vitro

Duration of culture after stimulation	Number of oocytes		Number (%)** of eggs at stage of				
	Examined	Matured and activated (%)*	M II	A II	T II	CC	N
10 min	69	65(94)	44(68)	21(32)			
20	63	58(92)	22(38)	36(62)			
30	64	60(94)	5(8)	34(57)	19(32)	2(3)	
1.0 h	64	62(97)	2(3)	1(2)	43(69)	16(26)	
2.0	72	72(100)	0(0)	0(0)	0(0)	68(95)	4(5)
3.0	56	55(98)	0(0)	0(0)	0(0)	28(51)	27(49)
4.0	75	75(100)	1(1)	0(0)	0(0)	3(4)	71(95)
5.0	54	53(98)	0(0)	0(0)	1(2)	0(0)	52(98)
6.0	69	68(99)	0(0)	0(0)	1(1)	1(1)	66(97)

*: Rate to the number of oocytes examined.

**: Rate to the number of oocytes matured.

M II; metaphase II, A II; anaphase II, T II; telophase II, CC; condensed chromatin, N; nucleus(ei)

Types of activated eggs were presented in Table 3. Immediate cleavage (IC) was not observed in this experiment. Most of the activated eggs (92 %) had the first and second polar bodies and a nucleus (2PB1N; most probably haploid, Plate 2C), and the rate of eggs probably diploid (1PB1N and 1PB2N, Plate 2A and 2B, respectively) was only 8 %.

Table 3. Classification of activation according to the extrusion of the second polar body and number of nuclei in porcine eggs activated by electrostimulation

Number of eggs examined	Number (%) of eggs with			
	2PB1N	1PB1N	1PB2N	IC
66	61(92)*	3(5)	2(3)	0(0)

*: Significantly different from other types of activated eggs ($P < 0.01$).

2PB1N; 2 polar bodies and a nucleus, 1PB1N; a polar body and a nucleus, 1PB2N; a polar body and 2 nuclei, IC; immediate cleavage

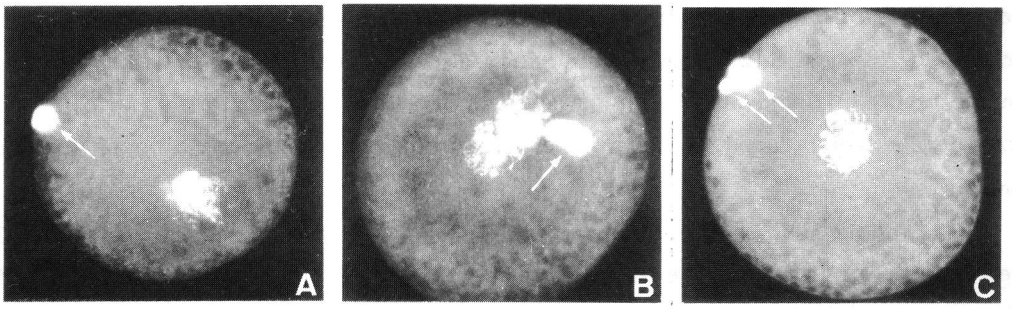

Plate 2. Nuclear formation of porcine eggs 12 h after electrostimultion. All eggs were photographed under an epifluorescent microscope after fixation with Carnoy's solution and staining with 1 ug/ml Hoechst 33342. X260.

A. An egg with the first polar body (arrow) and a single nucleus (1PB1N).

B. An egg with the first polar body (arrow) and 2 nuclei (1PB2N).

C. An egg with two polar bodies (arrows) and a single nucleus (2PB1N).

Experiment 4: The eggs with 2 polar bodies (2PB1N) were further cultured, and the cleaving ability of these eggs was examined (Table 4). Twenty percent of the 65 eggs cultured were degenerated within 24 h after activation, and the rate of degenerated eggs increased to 60 % at 60 h. The eggs composed of more than 4 blastomeres were observed 48 and 60 h after electrostimulation, but no compacted eggs were obtained.

Table 4. Cleaving ability of the activated porcine eggs with 2 polar bodies and a nucleus

Number of eggs cultured	Duration of culture after activation (h)	Number of eggs (%)						
		Remaining at 1-cell	Cleaved					Degenerated
			Total	2-cell	3-cell	4-cell	>4-cell	
65	24	34(52)	18(28)	17(26)	1(2)	0(0)	0(0)	13(20)
	48	8(12)	24(37)	5(8)	4(6)	12(18)	3(5)	33(51)
	60	3(5)	23(35)	2(3)	2(3)	11(17)	8(12)	39(60)

Discussion

It was reported previously that electrostimulation by multiple pulses was more effective than that by a single one in the mouse[20] and rabbit[3]. A single pulse, however, was shown to result in higher activation than double pulses in the pig[12]. In Experiment 1, porcine oocytes were activated in high proportions (more than 90 %) by a single pulse at 250 to 1,250 V/cm. The higher pulses resulted in

destruction and/or degeneration of cells, and the lower pulses failed to induce activation. The stronger electrostimulation also lead to abnormal or small nuclei in the rabbit[3]. Removal of cumulus cells by hyaluronidase treatment and exposure to the electrolyte used in the present study did not cause activation of porcine oocytes.

Kaufman[1] suggested that postovulately aged oocytes undergo time-related detrimental changes in the spindle at metaphase II, leading to dispersion of chromosomes which would result in aneuploidy in activated eggs. Culture for 36 h of porcine follicular oocytes is sufficient for nuclear maturation[17]. In the present study, it was shown that the rate of maturation was sufficiently high (95 %) after maturation culture for 36 h. The rate of activation in oocytes cultured for 36 h (47 %), however, was significantly lower than that in oocytes cultured for 42 h and longer periods (94 to 99 %). It has been reported that the spontaneous activation in bovine oocytes is triggered by aging of the oocytes[21]. In the present study, no oocytes were activated without electrostimulation even after a prolonged maturation culture for 54 h. It is most probably that the increase in the rate of activation as observed here is caused by maturational changes in ooplasm and/or plasma membrane, and not by aging of the oocytes. Procházka et al.[16] reported a drastic increase in the rate of the porcine oocyte activation during 34 (22 %) to 36 h (90 %) of maturation culture followed by gradual increase up to 42 h. In our study, a similar change was observed during 38 (44 %) to 42 h (94 %). These results indicate that physiological changes responsible for resumption of the second meiotic division occur in porcine oocytes at around the completion of nuclear maturation.

The eggs at Ana II were observed 10 min after electrostimulation at 750 V/cm, indicating the immediate resumption of meiosis. The meiotic resumption was confirmed in 92 % of treated oocytes within half an hour. These results show that the activation can be almost simultaneously triggered by the electrostimulation and succeeding behavior of chromosomes is quite synchronized. The duration of Ana II was estimated to be about 20 min, and the second meiotic division continued for 1 h or more after treatment. The nuclear formation began to be observed 2.0 h, after stimulation, and the eggs which formed nucleus(ei) accounted for 95 % at 4.0 h. The process of nuclear formation observed in this study coincide quite well with the result of in-vitro fertilization, in which the female pronucleus was observed in 75 % of porcine eggs fertilized 4.0 h after sperm penetration[22].

More than 92 % of activated eggs had second polar body and a single nucleus. They were most probably haploid. As the extrusion of the second polar body is easily confirmed on an inverted microscope, only activated eggs can be subjected to experiments concerned with development.

About one half of the eggs cultured were degenerated 48 h after treatment. Although activated eggs had cleaving ability beyond the 4-cell stage 48 and 60 h after electrostimulation, no eggs were compacted. This result suggests that development of eggs electrically activated is blocked at the 4-cell stage just as in-vivo fertilized and in-vitro cultured eggs[23]. It is shown, however, that the pattern of the intracellular Ca^{2+} oscillation induced by electrostimulation was different from that induced by sperm penetration[24].

The present results demonstrate that a large number of synchronously activated eggs can be provided by electrostimulation in the pig and they are a useful model for studies on fertilization.

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電気刺激により活性化されたブタ体外成熟卵母細胞の減数分裂再開ならびに分割能

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効率的に活性化を誘起する直流電圧を決定するため，42時間体外成熟培養を行ったブタ卵母細胞に，100～3,000 V/cm DC，パルス幅100 μsecの単矩形波を負荷した．200～1,250V/cmにおいて，90%以上の高い活性化が認められ，750V/cmでは最も高い活性化率（98%）が得られた．成熟培養時間が活性化に及ぼす影響を調べるために，36～54時間成熟培養した卵母細胞に750 V/cmの負荷電圧を与えた結果，成熟培養時間の増加とともに活性化率が上昇する傾向が認められた．培養時間が42時間以上の卵母細胞の活性化率（94～99%）は，培養36（47%）あるいは38時間（44%）より有意に高かった（$P<0.01$）．電気刺激後，卵子の減数分裂再開時期を明らかにするために，750 V/cmの負荷電圧を42時間成熟培養した卵母細胞に与え，経時的に固定・観察した．刺激の10分後にはAna IIの染色体が認められ（32%），30分後にはTel IIの染色体が観察された（32%）．その後，染色体の変化は卵子間で同調して進行し，4時間後には95%の卵子で核の形成が認められた．核形成後の活性化卵子の極体数と核数を調べたところ，92 %の卵子が第2極体と1つの核を保有する，半数体（2PB1N）と推測されるものであった．2PB1N卵子は，その後の培養により 4細胞期以降にまで卵割した．

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Effects of Oxytocin and Granulosa Cell Co - Culture on the Development of Mouse One - Cell Stage Embryos

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Abstract: Effects of oxytocin supplemented into medium on the development of mouse one - cell stage embryos were investigated. When the embryos with and without cumulus cells were cultured in the media containing 10^{-14} to 10^{-4}M oxytocin, the developmental rates of 10^{-10}M oxytocin - supplemented group in the cumulus - free embryos were significantly increased compared to those of control, but the rates of 10^{-4}M oxytocin group were decreased. There, however, was almost no effect on the developmental rates in the cumulus - enclosed embryos. In addition, effect of 10^{-10}M oxytocin on the embryo development was compared to that of co - culture with granulosa cells. There were no differences between oxytocin - supplemented and co - culture groups in the developmental rates to each stage from 2 - cell to expanded blastocyst. But co - culture system obviously increased the cell number of blastocysts. The additional effect of supplementation with oxytocin into co - culture system on the developmental rate and the cell number of blastocysts was not found. **Key words:** embryo development, oxytocin, co - culture, mouse
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Introduction

Oxytocin is synthesized by the hypothalamo - neurohypophyseal system, and secreted from the neurohypophysis. In addition, oxytocin has been elucidated to be released from the ovarian tissue of some species, sheep[1], human[2], cattle[3], non - human primate[4], swine[5]. It has been demonstrated that the sources of ovarian oxytocin are the granulosa cells[6] and granulosa lutein cells[7]. With regard to the physiological role of ovarian oxytocin, two main hypotheses have been proposed; 1) it may regulate the ovarian steroidogenesis by the paracrine actions[1,8], and 2) cause the luteolysis in ruminant through a positive feed back loop in which oxytocin and prostaglandin $F_{2\alpha}$ are mutually reinforced[9-11]. Recently Makimura et al.[12] have reported that oxytocin promotes the development of mouse embryos in vitro. However, research data on the effect of oxytocin on embryos is insufficient. The purpose of this study was to examine the effect of oxytocin on embryo development

in vitro, and to compare its effect with that of granulosa cell co-culture.

Materials and Methods

Collection of embryos: Female mice (7-12 weeks old) of ddY strain were superovulated by an intraperitoneal injection of 5 iu pregnant mare serum gonadotrophin (PMSG) followed by an injection of 5 iu human chorionic gonadotrophin (hCG) at 48 h after injection of PMSG. Then females were placed in the male's cages and the existence of vaginal plug was confirmed on the following morning. Female mice were sacrificed by the cervical dislocation at 18-20 h after injection of hCG. One-cell stage embryos were obtained by tearing the wall of ampulla in Hoppe and Pitts's medium[13]. Some of embryos were transferred to the medium containing 0.1% hyaluronidase to remove the cumulus cells and washed six times in the fresh medium.

Culture of granulosa cells: Granulosa cells were obtained from the antral follicles after the collection of embryos by the method of Sanders and Midgley[14]. Mass of the cells was transferred in TCM 199 with 60 mg penicillin and 50 mg streptomycin/100 ml. The erythrocytes in the medium were ruined by the method of Sirard et al.[15]. Then the cells were incubated for dispersion in 0.25% trypsin solution in phosphate buffer saline for 5 min. Viability of the cells was assessed by staining with the trypan blue. The cells were added to 0.2 ml of TCM 199 supplemented with 10% fetal bovine serum in tissue culture plate to give a final concentration of 0.6-2.5×10^6 cells/ml, and cultured under paraffin oil at 37 ℃ in 5% CO_2 in humidified air. Medium was changed at 24 h after the start of culture. The cells became almost confluent after about 96 h of culture. The monolayer exclusively consisted of granulosa cells was used for the co-culture experiment.

Culture of embryos in the medium with oxytocin: The cumulus-enclosed and cumulus-free embryos were cultured in the Hoppe and Pitts's media with 50 μM EDTA and supplemented with 10^{-10}, 10^{-8}, 10^{-6} and 10^{-4}M of oxytocin (Bachem Inc. Torrance CA). Furthermore, in order to determine the effects of lower concentration of oxytocin, the media with 10^{-14}, 10^{-12} and 10^{-10}M oxytocin were tested using the cumulus-free embryos.

Co-culture of embryos with the granulosa cell monolayer: At 96 h after culture of granulosa cells, the medium was changed for Hoppe and Pitts's medium with and without 10^{-10}M oxytocin. Then five to ten one-cell stage embryos without cumulus cells were co-cultured with the granulosa cell monolayer, and were simultaneously cultured in Hoppe and Pitts's medium alone with and without oxytocin.

These embryos were incubated at 37℃ in 5% CO_2 in air under paraffin oil. The developmental stage of embryos was examined every 12 h under phase-contrast microscope. Blastocysts at 120 h after the start of culture were fixed and the number of cells was counted by the method of Ushijima et al.[16]. Data were analyzed by a Chi-square or Duncan's multiple range tests.

Results

When one-cell stage embryos were cultured in the media with 10^{-14} to 10^{-4}M oxytocin, there was no difference in the developmental rates of cumulus-enclosed embryos between oxytocin-supplemented and control groups except 8-cell stage in 10^{-6}M oxytocin group (Table 1). On the other hand, in the cumulus-free embryos the developmental rates of 10^{-10}M oxytocin group were significantly higher than those of control, whereas the rates in 10^{-4}M oxytocin group significantly decreased in each stage from 8-cell to early blastocyst stage. There were no differences among groups in each experiment in the cell number of blastocysts at 120 h after the start of culture (data not shown).

Table 1. Development of mouse one-cell stage embryos with or without cumulus cells in the media supplemented with various concentrations of oxytocin

Concentration of oxytocin (M)	No. of embryos cultured	% of embryos developed to						
		2C[1)]	4C	8C	M	E1B	EpB	HtB
Cumulus-enclosed embryos								
0	70	97	89	66	66	60	53	3
10^{-10}	59	98	92	80	78	71	58	8
10^{-8}	56	95	93	77	68	59	48	5
10^{-6}	59	98	93	86**	80	68	54	5
10^{-4}	58	97	91	81	64	62	55	2
Cumulus-free embryos								
0	76	96	89	88	79	70	58	4
10^{-10}	83	98	96	94	90*	88**	75*	14*
10^{-8}	66	98	95	94	85	82	70	3
10^{-6}	70	97	91	87	80	70	59	7
10^{-4}	70	97	80	73*	61*	53*	49	3
Cumulus-free embryos								
0	80	96	81	80	68	60	53	5
10^{-14}	78	99	94*	90	79	68	58	12
10^{-12}	77	96	92*	86	77	69	60	19**
10^{-10}	80	99	94*	91*	85**	75*	69*	19**

1) 2C=2-cell, 4C=4-cell, 8C=8-cell, M=morula, E1B=early blastocyst (embryo with a small blastocoele), EpB=expanded blastocyst, HtB=hatched blastocyst

* Values are significantly different from value of each control in the same column. * $P<0.05$ ** $P<0.01$

Table 2 summarizes the developmental rates of one-cell stage embryos in the media with 10^{-10}M oxytocin and the co-culture system. Presence of oxytocin in the culture medium improved the developmental rates of one-cell embryo to morula, early blastocyst and expanded blastocyst as shown in former experiment. Co-culture with the granulosa cell monolayer promoted the rates to each stage from 8

-cell stage onward. By comparison of the effects between oxytocin and co-culture groups, there were no difference between the two groups in the developmental rates of all stages except hatched blastocyst stage. The effect of supplementation with oxytocin into the co-culture system on the developmental rates was not found.

Table 2. Effects of oxytocin and granulosa cell co-culture on the development of one-cell stage embryos

Oxytocin 10^{-10}M	Granulosa cells	No. of embryos cultured	% of embryos developed to						
			2C[1)]	4C	8C	M	ElB	EpB	HtB
−	−	76	100	89	76[a]	64[a]	57[a]	54[a]	18[a]
+	−	76	99	92	83[a,b]	79[b]	72[b]	70[b]	25[a]
−	+	200	100	92	87[b]	83[b]	75[b]	68[b]	41[b]
+	+	195	100	95	91[b]	86[b]	78[b]	70[b]	50[b]

[1)] 2C=2-cell, 4C=4-cell, 8C=8-cell, M=morula, ElB=early blastocyst(embryo with a small blastocoele), EpB=expanded blastocyst, HtB=hatched blastocyst
[a,b] Values within the same column with the different superscripts are significantly different ($P<0.05$).

The supplementation with oxytocin has no effect on increase of cell number of blastocysts. On the other hand, co-culture system obviously increased the cell number (Table 3). Additional effect on the cell number was not also found in the case of the supplementation with oxytocin to the co-culture system.

Table 3. Effects of oxytocin and granulosa cell co-culture on the cell number of blastocysts

Oxytocin 10^{-10}M	Granulosa cells	No. of embryos analyzed	No. of cells[1)]
−	−	25	75.4 ± 4.3 [a]
+	−	34	83.7 ± 4.3 [a]
−	+	103	109.7 ± 3.2 [b]
+	+	107	116.5 ± 3.3 [b]

[1)] Mean ± SE
[a,b] Values with the different superscripts are significantly different ($P<0.001$).

Discussion

The results of this study indicated that oxytocin had a beneficial effect on the development of mouse one-cell stage embryos in vitro and the concentration of 10^{-10}M was the most effective in supporting the development of cumulus-free embryos. Makimura et al.[12)] have reported that the developmental rates of in-vitro fertilized eggs in mouse to blastocyst stage are higher in 10^{-6}M oxytocin group than in 10^{-8}, 10^{-10}M oxytocin and control groups. Although the discrepancy in the effective concentration of oxytocin is unclear at present, it could be partly due to differences in the strain of mice and/or the conditions of culture. In this study oxytocin at any concentration had almost no effect on the development of cumulus-enclosed embryos and higher oxytocin concentration (10^{-4}M) reduced the developmental

rates of cumulus-free embryos, suggesting the possibility that the presence of cumulus oophorus or cumulus cells dispersed in the medium may inhibit the beneficial and deteriorative effects of oxytocin on the development of embryos.

The present study showed that both supplementation with oxytocin and co-culture system with granulosa cells had beneficial effects on the early embryo development. There were no differences between the two groups in the degree of the positive effect on the developmental rate of embryo. But co-culture system increased the cell number of blastocysts when compared to oxytocin-supplemented group. This suggests that, at least under condition of this study, co-culture system is superior to supplementation with oxytocin for embryo development, and that there is a possibility that the mode of action on the embryo is different between the two. In addition, it seems that more powerful effect by co-culture system masks the effect of oxytocin since the simultaneous combination of the two treatment does not increase the developmental rate and the cell number of blastocysts more than co-culture alone.

It is obscure why co-culture is beneficial for embryo development, although some hypotheses have been proposed; helper cells produce mitogenic substances, extracellular matrix products of the helper cells support the cell differentiation, or helper cells remove the embryotoxic substances from the culture medium[17]. Although there is a possibility that mouse granulosa cells secrete oxytocin in the same manner as bovine granulosa cells[18], it seems probable that granulosa cells produce other factor(s) than oxytocin under condition of this study.

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マウス１細胞期胚の発生に及ぼすオキシトシン及び顆粒層細胞との共培養の効果

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培地に添加したオキシトシンがマウス１細胞期胚の発生に及ぼす効果を検討した．卵丘細胞除去胚を10^{-14} ～10^{-4}M オキシトシン添加培地で培養した結果，10^{-10}M区の発生率は対照に比べて有意に増加し，10^{-4}M 区の発生率は低下した．しかし，卵丘細胞付着胚の発生には，オキシトシン添加の効果はほとんど認められなかった．さらに，10^{-10}Mオキシトシンの添加効果と顆粒層細胞との共培養の効果を比較した結果，２細胞期から拡張胚盤胞期への発生率には，両区の間に差は認められなかったが，共培養区ではオキシトシン添加区に比べて胚盤胞期の細胞数が有意に増加した．また，共培養区へのオキシトシン添加の効果は認められなかった．

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A Possibility for the Determination of the Sex of Preimplantation Porcine Embryos Using Polymerase Chain Reaction

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Abstract: We have attempted to sex preimplantation porcine embryos using a polymerase chain reaction (PCR) with the primers made from the SRY (sex-determining region of Y) conserved sequences of mammals. The sense primer was 5'-GTCAAGC GACCCATGAACGC-3' (20 mer) and the antisense primer was 5'-CTGTGCCTCCTG GAAGAATGGC-3' (22 mer). After amplification of male blood samples, the male specific DNA band was detected as a 165-bp fragment. These specific DNA bands were detected in 44-50% of the embryos from 16-cell to blastocyst stage, although the frequencies of positive samples were lower in 2-cell and 4-cell stage embryos. Furthermore, direct sequencing of prepared fragments revealed that there were considerable similarities among the porcine, human, mouse and rabbit sequences.
Key words: Porcine embryo, PCR, Sexing, DNA (Received January 19 1994, Accepted July 21 1994)

Introduction

Attempts to achieve embryo sexing in mammals have been made for a long time[1]. The successful establishment of such techniques would have a major impact on animal production. However, no reliable methods have been developed except for a few animals[2-5]. In porcine, it is yet difficult to control sex. The advent of the polymerase chain reaction (PCR) to amplify sequences of DNA has great potential for sex control. This technique apart from its accuracy[6] can be carried out rapidly to determine the sex of embryos.

Sinclair et al.[7] reported the discovery of the gene and its sequence from the sex-determing region of Y(SRY). Since then, the sequence of SRY gene has been used as one of the universal primers and template for embryo sexing in various species of mammals[2-3,8].

In this paper, we attempted to explore the method to detect the SRY conserved

region in porcine cells for sexing of preimplantation embryos using PCR. Moreover, the sequence of the amplified fragments was determined by direct sequencing.

Materials and Methods

In Vitro Fertilization and Embryo Culture : Immature oocytes derived from a local slaughterhouse were matured in TCM-199 medium supplemented with 100 mg/l dibekacine sulphate (Meiji, Japan), 10%(v/v) fetal bovine serum (Hazleton, USA), 10 IU/mℓ PMS, 10 IU/mℓ HCG and 1 μg/mℓ E_2 for 48 to 50h at 38.5C in 5% CO_2 in air. Semen was collected from Landrace boars by the glove method. Sperm samples were diluted with a basic medium consisting of TCM-199 supplemented with 10% fetal bovine serum, 100 mg/ℓ dibekacine sulfate to a final concentration of 2×10^8 sperms/mℓ. The diluted sperms were then incubated for 4 to 5h at 37C. About 50 μℓ sperm solution (1×10^7 sperm) were introduced into 200 μℓ fertilization medium. The oocytes and sperms were cultured at 38.5C in an atmosphere of 5% CO_2 in air for 5 to 6 days.

Preparation of Embryos for PCR-amplification : Two-cell to blastocyst-stage embryos were washed 3 times in drops of phosphate buffered saline and individual embryo was trasnsferred into a 10 μℓ lysate buffer (10mM Tris-HCl pH 8.3,50mM KCl, 1.5mM $MgCl_2$, 1mM DTT, 1% SDS and 100 μg/mℓ proteinase K) containing tube. The DNA was prepared for the amplification by digesting the samples for 40 to 50 min at 37 C.A negative control was prepared by replacing embryo sample with PBS into the lysate buffer tube.

DNA Extraction from Blood : DNA was extracted from whole blood using standard method[9].

Oligonucleotide Primers : One set of DNA-specific primers derived from the conserved motif of SRY was used in the sexing assay. The oligonucleotides were synthesized with a DNA synthesizer (Applied Biosystems Ltd., Warrington, Cheshire U.K) according to the region from 588 to 752 bp in the SRY conserved region. The primer sequences used for PCR were 5'-GTCAAGCGACCCATGAACGC-3' (5' primer : SRY 1) and 5' -CTGTGCCTCCTGGAAGAATGGC-3' (3' primer : SRY 2). These oligonucleotides were purified by oligonucleotide purification cartridges.

PCR Amplification of Embryo Samples : PCR was carried out according to previous report[10]. To each of the tubes containing an embryo, 27 μℓ of a reaction mixture consistiong of 100mM Tris-HCl (pH 8.4), 15mM $MgCl_2$, 500mM KCl, 0.1% gelatin, 8mM dNTP, 10 μM DNA primers, and 1 unit of Taq DNA polymerase were added

and the mixture was overlaid with 30 $\mu\ell$ of mineral oil. PCR amplification was carried out for 50 cycles each consisting of denaturation for 3 min at 93C, annealing for 2 min at 60C, and extension for 1 min at 72C.

PCR Amplification of Blood Samples : Reaction mixture was the same as for embryonic samples. Each blood sample (0.5 μg DNA sample) was assayed like an embryo sample.

Analysis of PCR Products : PCR products (20 $\mu\ell$) were electrophoresed on a 2.5% agarose gel, stained with ethidium bromide and visualized under ultraviolet light.

If Y chromosome-specific 165 base pair products were visible in samples, the sample was considered to be derived from a male.

Sequences of DNA Fragments : Sequence reaction was conducted with Taq Dye Deoxy Terminator Cycle Sequncing Kit (ABI) using 5 $\mu\ell$ DNA sample as a template.

Results

Figure 1 shows an actual gel electrophoresis after the PCR amplification. The DNA sample derived from the blood of a male geve a clear band and no band was detected with the blood of a female. This band was estimated as the male specific DNA band. The size was determined as 165 bp from molecular size marker. As shown in Figure 1, preimplantation embryos also gave a clear band.

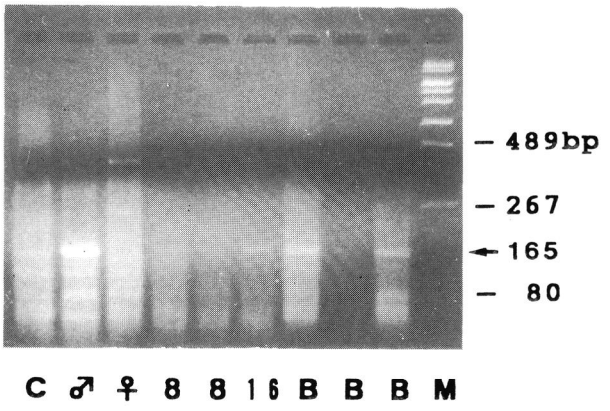

Figure 1. Agarose gel electrophoresis of PCR products from porcine embryos matured and fertilized . C,sample wituout DNA ; ♂, blood sample from male ; ♀, blood sample from female ; 8,8-cell porcine embryos;16, 16-cell ; B ,blastocysts ; M, molecular size marker (pHY marker).

The results of sexing are shown in Table 1. Thirty four of 103 embryos were identified as males by the presence of the male specific DNA band. The embryos from 2-cell to 8-cell resulted in low positive percentages (12~36%), but about half (43.8~50.0%) of the embryos from 16-cell to blastocyst stage showed the positive band. In these results, the statistical analysis was performed with the Chi-square test and more than 75% of confidence coefficient was obtained in 16-cell and morula as of the expected rateio of 1:1 (♂ : ♀).

Table 1. Sex determination by PCR analysis of in vitro fertilized embryos

Cell stage	Number of samples	No. and (%) of embryos detected with male specific band
2-cell	17	2 (11.8)
4-cell	24	5 (20.8)
8-cell	17	6 (35.5) a)※
16-cell	13	6 (46.2) b)
morula	16	8 (50.0) c)
blastocyst	16	7 (43.8) d)
Total	103	34 (33.0)

※ The statistical analysis was performed with the Chi-square test (expected value was ♂:♀ = 1 : 1).
a) $0.10 < P < 0.25$, b) $0.75 < P < 0.90$, c) $0.90 < P$, d) $0.50 < P < 0.75$

Subsequently, we determined the sequences of our fragment. Figure 2 presents the sequences obtained. The sequences were similar to the results of Kageyama et al.[11] in a portion of the conserved SRY. This amplified fragement was recognized to be the SRY conserved region. There was more than 75% homology among mouse and rabbit with 85% homology to humans (Figure 3).

```
                          20                                  40
5'-TTTCATTGTG  TGGTCTCGTG  ATCAAAGGAG  AAAAGTGGCT
                          60                                  80
   CTAGAGAACC  CTCAAATGCA  AAACTCAGAG  ATCAGCAAGT
                         100                                 120
   GGCTGGGATG  CAAGTGGAAA  ATGCTTACAG  AAGCCGAAAA  GCG-3'
```

Figure 2. DNA sequences of prepared fragments of porcine amplified by PCR. The sequences were obtained by directly sequencing from PCR products.

```
      1                  20                  40                  60        70
Pig   TTTCATTGTGTGGTCTCGTGATCAAAGGAGAAAAGTGGCTCTAGAGAACCCTCAAATGCAAAACTCAGAG
Mouse A··T··G·····C····G·G·G···CAC··GT····C·AGC····T··CAGC·····TA·····
Rabbit A·····G·····C·AAC····G··AC·CC·G·····GC····T··CA·····G·····C
Human A·····C·····C·····G···C·C··GA·····T··CAG·····G·····

      71                 90                 110         123
Pig   ATCAGCAAGTGGCTGGGATGCAAGTGGAAAATGCTTACAGAAGCCGAAAAGCG
Mouse ·····CA·····G·····GC·····AA·   93/123*  (75.6)
Rabbit ·····CA·····CA·C·····T·····T·····AT·   95/123   (77.2)
Human ·····CA·····A·C·····T·····AT·  103/123   (84.5)
```

Figure 3. Comparison of DNA sequences of SRY conserved region of mammals amplified by PCR.

* $\frac{\text{No. of similar bases}}{\text{No. of bases}}$ (%)

Discussion

The results of the present study indicate that porcine embryos are able to sex during preimplantation stage using PCR with the primers made from the conserved sequence of SRY. It seems that more than 10 cells is necessary for sexing using our PCR system. After amplification, the male specific DNA band which was similar in size to that from the blood sample was detected in embryo samples. However, Bradbury et al.[3] succeeded in detecting male specific repeated sequences in mouse single blastomere using PCR. Similar results have been reported in human[2] and cattle[5].

since 1990, SRY has been reported as a Y-specific gene over a wide range of mammals[6),12-16]. This study utilized primers made from the conserved sequence of SRY. The results obtained imply that this system would be useful to amplify specific DNA sequences in the Y-specific DNA region.

The results obtained in the DNA sequences of our fragment used were essentially similar to Kageyama et al.[11] described in pig.

Finally, there is a paucity of information regarding sexing porine embryos using PCR. This work therefore provides an efficient and suitable assay for sex control using PCR. Further studies into methods which may modify single assays to ensure complete accuracy are necessary.

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ＰＣＲ法による着床前ブタ胚の性判別の可能性

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PCR法を用いて，体外受精－体外培養した着床前豚胚の性判別を行なった．PCRに用いたプライマーは哺乳動物のSRY保存領域を基に作成した．使用したセンスプライマーは5'－GTCAAGCGACCCATGAACGC－3'（20mer）で，アンチセンスプライマーは5'－CTGTGCCTCCTGGAAGAATGGC－3'（22mer）であった．雄特異的DNAバンドは165bpで検出された．体外受精卵の33％において，雄特異的DNAバンドが検出された．16－細胞期から胚盤胞期の胚を検体とした時，供試胚の46.6％において雄サンプルで得られたものと同様なバンドが得られた．得られたDNA断片をダイレクトシークエンス法により塩基配列決定を行った結果，ウサギ及びマウスSRY保存領域とは約75％，ヒトSRY保存領域とは85％以上の相同性があることが確認された．

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The effect of bovine follicular fluid from 2-5mm follicles and its fractions on maturation and subsequent fertilizability and cleavage of the oocytes in vitro

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Abstract: In this study the bovine follicular fluid (bFF) from small follicles (2-5mm in diameter) or its fractions were added to maturation medium, and their effects on maturation, fertilization and cleavage of bovine follicular oocytes were examined. Oocytes from slaughtered Holstein cows were cultured in maturation medium supplemented with various concentrations of bFF or its fractions. After 22-24 hr of culture, the oocytes were coincubated with frozen-thawed spermatozoa, then cultured in development medium for 48 hr and at the end of culture evaluated for cleavage, nuclear maturation and fertilization. The bFF at 30% significantly enhanced maturation compared to bFF-free group (control); it also significantly enhanced cleavage beyond the 2-cell stage compared to bFF-free and 60% low molecular fraction (<10KD) groups. The 60% bFF group significantly enhanced fertilization compared to bFF-free group. In addition, the embryos derived from oocytes matured in the 60% bFF significantly proceeded to >2-cell stages. The fractions of bFF were without significant effect on the rates of maturation, fertlization or cleavage, except at 60% where the high molecular fraction (>10KD) significantly enhanced cleavage to 2-cells compared to bFF-free group, but the rates of embryos that cleaved beyond the 2-cell stage was significantly lower ($P<0.01$) than in the 60% bFF group. In conclusion, addition of whole bFF (2-5mm follicles) to maturation medium was beneficial for maturation, fertilization and cleavage beyond the 2-cell stage of bovine oocytes at 30% and 60% concentrations. On the other hand, the fractions of bFF did not show significant effect on the rates of maturation and fertlization. **Key words:** Bovine oocytes, Follicular fluid, Maturation, Fertilization
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Introduction

It was first demonstrated by Pincus and Enzmann[1]that rabbit oocytes removed from antral follicles and cultured in a serum-containing medium can undergo spontaneous nuclear maturation in the absence of gonadotropins. However, later it was shown that many of these spontaneously matured oocytes failed to be normally fertilized or to produce viable embryos[2,3]. On the other hand, Leibfried-Rutledge et al.[4] demonstrated that oocytes fertilized in vitro after maturation in vivo exhibit high rates of development to morulae and blastocysts. Moreover, in many cases, by supplementing the culture medium with gonadotropins and/or steroids[5,6,7],spontaneously matured oocytes could be fertilized and could unbergo cleavage.

These observations suggested that fertilizability and the ability of the oocyte to undergo cleavage and develoment is acquired during the course of oocyte maturation. In addition, the observations suggested that oocyte maturation consists of events occurring in both the nucleus and the cytoplasm.

The present study was designed to examine the influence of the addition of the fluid from small bovine follicles (2-5mm) to maturation medium on maturation, fertilization, the first cleavage division and the consecutive divisions of bovine follicular oocytes in a complete in vitro system.

Materials and Methods

Sterilization of culture media and solutions was done by filtration through 0.45 μm filters (Japan Millipore LTD., Tokyo, Lot No.GE 77), and all incubations were performed at 39℃ in an atmosphere of 5% CO_2 in air and 100% relative humidity. Oocytes and embryos were cultured in 100 μl of maturation, fertilization and development medium in a 35mm polystyrene dishes (Iwaki Glass, Japan), and were covered with mineral oil (E.R.Squibb and Sons, Inc., Princeton, NJ, US., 08540).

Bovine follicular fluid : From the ovaries of slaughtered cows follicles of 2-5mm in diameter were aspirated. The collected bovine follicular fluid (bFF) was centrifuged for 15 min at 1,600g (at 5℃) to remove granulosa cells, blood cells and oocytes. The supernatant was heated to 56℃ in a water bath for 30 min. Part of it was kept without any further treatment, and the other part was ultrafiltrated through filters whose filtrate was of <10 KD molecular size (Amicon Corporation, Ireland). The >10 KD fraction of bFF (high molecular fraction ; HMF) was freeze-dried, and the

dry matter in one ml bFF was 7.984mg. This amount was dissolved in one ml of TCM 199, and from this mixture the volume that makes the desired concentration in the basic maturation medium was taken (TCM 199 and the basic maturation medium are described below). The whole follicular fluid, the extract (<10 KD low molecular fraction ; LMF) and the HMF were stored at −20℃ until use.

Oocyte collection and maturation : Ovaries were collected from slaughtered Holstein cows and heifers, and were transgerred to the laboratory in an insulated flask containing 0.85% sterile saline (autoclave sterilization) at approximately 30℃ within 4hr. Oocytes were aspirated from 2-5 mm follicles using a 21-gauge needle and a 10-ml disposable syringe. Only oocytes with unexpanded cumulus oophorus cells and homogeneous ooplasm were chosen for culture[8]. The oocytes were placed in a watch glass containing Dulbecco's phosphate buffered saline (PBS) supplemented with 5% (v/v) heat-inactivated bovine serum (Nacalai Tesque, Inc., Kyoto, Lot No. 02, Japan) and 100 μg/ml kanamycin, and were then introduced into maturation medium within 1 hr. The basic maturation medium was composed of TCM 199 (Earle's salts with 25mM Hepes buffer and L-glutamine, Gibco, Cat. 380-2340, NY, U.S.) and 10% estrous cow serum. To this basic medium 1.3 μg/ml LH (Sigma, U.S., Lot 128F-0465), 0.6 μg/ml FSH (Sigma, U.S.A., Lot 128F-0521) and 50 μg/ml gentamycin were added. The medium was further supplemented with 0% (control), 10%, 30% and 60% bFF or its fractions.

In vitro fertilization (IVF) : Frozen semen of a proved Japanese Black bull was thawed in water bath at 35℃, and was washed two times by centrifugation (at 320g for 5min)in the modified Tyrode's medium (TALP)[9] supplemented with 10mM caffein and 100 μg/ml kanamycin. The sperm pellet was resuspended in the TALP (10×10^6 sperm/ml). At the end of in vitro maturation culture period (22-24hr), 10-15 oocytes were introduced into the 50 μl fertilization drops (TALP+20mg/ml BSA+20 μg/ml heparin). Aliquots of 50 μl from the sperm suspension were added to each drop (final concentration : 5×10^6 sperm/ml), and the spermatozoa and oocytes were coincubated for 7hr.

In vitro development : At the end of in vitro fertilization culture period, the oocytes were transferred into development medium (TCM 199 + 10% estrous cow serum + 50 μg/ml gentamicin) and cultured for 48hr. The oocytes were evaluated with Nomarski optics (100x) for cleavage to ≧2-cells, and the uncleaved were used to assess fertilization and nuclear maturation. The cumulus cells surrounding the oocytes were removed by repeated pipetting with finely drawn fire-polished pipettes, and were then fixed for 48 hr in a mixture of acetic acid and ethanol(1:3; v/v),

stained with 0.5% lacmoid in 45% acetic acid and examined by phase-contrast microscope (400x). Oocytes with one set of male and female pronuclei as well as cleaved embryos were regarded as normally fertilized. The stained oocytes with the chromosomal configurations of metaphase II, the cleaved embryos and normally fertilized oocytes constitute the total number of matured oocytes. In our evaluation for cleavage, only embryos with blastomeres of regular size were considered as cleaved in order to minimize the possibility of parthenogenetic activation.

Statistical analysis : All data acquired were analyzed by χ^2 test[10], and a level of $P<0.05$ was considered statistically significant.

Results

The results are shown in the table. Maturation rate was significantly higher in the 30% bFF group compared to the control ($P<0.05$), and fertilization rate was significantly higher in 60% bFF group than in the control ($P<0.05$). In addition, the cleavage index was significantly higher in the 30% bFF group than in the 60% LMF group and the control ($P<0.05$).

The oocytes matured in the medium supplemented with 60% bFF were fertilized at a significantly higher rate than the control ($P<0.05$). In addition, the cleavage

Table 1 The effect of bovine follicular fluid (bFF) from 2-5mm follicles and its fractions on maturation, fertilization and cleavage of bovine oocytes and embryos in vitro.

Concentration of bFF and its fractions	Matured[1] %	Fertilized[1] %	Polyspermy[2] %	Cleaved[1] (≧2-cells) %	Cleavage index[3] %
0%	74.4^{a} (67/90)	70.0^{a} (63/90)	5.7 (4/70)	54.5^{a} (55/101)	23.6^{a*} (13/55)
10%bFF	79.0^{ab} (79/100)	75.0^{ab} (75/100)	4.7 (4/85)	60.2^{ab} (68/113)	36.8^{ab} (25/68)
30%bFF	85.5^{b} (144/169)	79.3^{ab} (134/169)	2.1 (3/141)	66.3^{ab} (116/175)	44.0^{bc} (51/116)
60%bFF	84.5^{ab} (82/97)	82.5^{b} (80/97)	2.4 (2/83)	64.2^{ab} (70/109)	58.6^{c} (41/70)
30%LMF[4]	80.8^{ab} (97/120)	75.8^{ab} (91/120)	3.1 (3/97)	67.2^{ab} (82/122)	34.1^{ab*} (28/82)
30%HMF[5]	78.2^{ab} (122/156)	71.8^{ab} (112/156)	6.7 (8/120)	61.1^{ab} (96/157)	35.4^{ab*} (34/96)
60%LMF	77.2^{ab} (71/92)	75.0^{ab} (69/92)	2.7 (2/73)	65.6^{ab} (63/96)	27.0^{a*} (17/63)
60%HMF	80.6^{ab} (125/155)	76.8^{ab} (119/155)	4.6 (6/130)	68.4^{b} (108/158)	36.1^{ab*} (39/108)

1) As a proportion to inseminated oocytes.
2) As a propotion to penetrated oocytes.
3) No. of embryos cleaved beyond the 2-cell stage/total no. of cleaved embryos.
4) Low bFF molecular fraction (<10 KD).
5) High bFF molecular fraction (>10 KD).
a,b,c Different superscripts within the same column are significantly different ($P<0.05$).
* Significantly different from c ($P<0.01$).

index was significantly higher in the 60% bFF group than in the control, in the low and high molecular fractions of bFF at 30% and 60%($P<0.01$) and in the 10% bFF group ($P<0.05$).

The fractions of bFF did not show significant effect on the rates of maturation and fertilization. However, the positive effect of the fractions was noticeable only at 60% HMF. In this group, the rate of embryos cleavaged to ≧2-cells was significantly higher ($P<0.05$) than the bFF-free group (the control).

Discussion

The results of this experiment (Table) demonstrated that at 30%, bFF significantly improved maturation and cleavage beyond the 2-cell stage. Similarly, at 60%, bFF significantly enhanced fertilization ($P<0.05$), and greatly promoted cleavage of the embryos beyond the 2-cell stage ($P<0.01$). These results suggest that the heat-inactivated bFF from follicles of 2-5mm in diameter does not contain inhibiting substanse(s) at a concentration high enough to suppress the nuclear maturation of follicular oocytes. On the contrary, the oocyte's maturation, fertilizability and development beyond the 2-cell stage were enhanced, particularly at high concentrations (30%,60%) of bFF supplements. This enhancement is probably a result of improved cytoplasmic maturation, achieved by a substance(s) in follicular fluid.

These results are in agreement with the findings of Yoshida et al.[11] who reported that addition of pig follicular fluid to maturation medium significantly increased the rates of nuclear maturation, fertilization and cleavage of pig oocytes after in vitro fertilization. However, the present results are in disagreement with findings of Leibfried and First[12] who demonstrated that neither bovine follicular fluid nor granulosa cells affect the completion of the first meiotic divisions of bovine oocytes in vitro. Additionally, the results are in disagreement with findings of Ayoub and Hunter[13] who demonstrated that bovine follicular fluid from small, medium or large follicles inhibited the resumption of meiosis in bovine oocytes. It is to be noted that in their experiments follicular fluid (100%) was used as a maturation medium, and the effects of follicular fluid were compared with effects of maturation in TCM 199 supplemented with FSH and estradiol. This may give hints to explain the stimulatory effect in our experiments, that addition of follicular fluid to TCM 199 may have diluted the inhibitory substance(s) and hence giving a chance for stimulatory substance(s) to work.

The unfractionated bFF at 30% significantly enhanced maturation and the proportion of embryos that developed beyond the 2-cell stage compared to the

bFF-free medium (Table). Similarly, at 60%, the unfractionated bFF significantly enhanced fertilization and the proportion of embryos that developed beyond the 2-cell stage. On the other hand, the fractions of bFF did not show significant effect on the rates of maturation, fertilization or cleavage, except at 60% where the high molecular weight fraction (>10KD) significantly enhanced cleavage to 2-cells compared to the contral, however the proportion of embryos that developed beyond the 2-cell stage was significantly lower than in the 60% whole bFF (Table). Therefore, the results demonstrate that the fractions of follicular fluid from small follicles are not beneficial for development beyond the 2-cell stage. Hence, it could not be concluded whether the stimulatory substance(s) is contained in the fraction of follicular fluid smaller than or larger than 10,000 daltons. This is in agreement with the findings of Rigby et al.[14] who repoted that biological activity of follicular fluid is generally reduced with fractionation.

In conclusion, the results demonstrated that nuclear maturation, fertilizability and cleavage beyond the 2-cell stage were enhanced following maturation of bovine oocytes in a medium supplemented with high concentrations (30-60%) of bovine follicular fluid from 2-5mm-follicles. Additionally, the fractions of bFF did not show significant effect on the rates of maturation and fertilization.

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2-5㎜の細胞から採取した卵胞液及びその画分がウシ卵胞卵子の成熟，受精及び発生に及ぼす影響

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2－5㎜の牛卵胞から採取した卵胞液あるいはその画分を添加した培地で成熟させた牛卵胞卵子の成熟，受精及びその後の胚の発生率に及ぼす影響について検討した．基本培地（TCM199＋発情牛血清＋LH＋FSH）に卵胞液を30％添加した区の成熟率及び分割率（使用卵胞卵子の内，２細胞期以降へ発達した胚の割合）は，無添加区（対照区）より有意に高く，卵胞液60％添加区の受精率及び分割率も対照区に比較して有意に高い値を示した．また，卵胞液の高分子画分30％添加区，低分子画分（10KD未満）30％添加区及び低分子画分60％添加区では受精率，成熟率及び分割率において有意な増加は認められなかった．また，高分子画分60％添加区の分割指数（２細胞期胚の内，それ以降へ発達した胚の割合）は卵胞液60％添加区より有意に低かった．以上の結果から，２－５㎜の牛卵胞から採取した卵胞液の成熟培地への添加は，卵胞卵子の成熟，受精及びその後の胚の発生に有効であるが，その効果は分画により低下することが示唆された．

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In Vitro Fertilization of Bovine Oocytes With Serono Gamete Preparation Medium (GPM)

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Abstract: The purpose of the present study was to investigate the efficacy of Serono Gamete Preparation Medium (GPM), which is used as the medium for human in vitro fertilization, as the fertilization medium for bovine oocytes matured in vitro. Oocytes were inseminated in GPM or GPM supplemented with 5 mM caffeine and 10 μg/ml heparin (GPM-CH). Brackett and Oliphant's (BO) medium supplemented with 5 mM caffeine and 10 μg/ml heparin (BO-CH) was used as a control medium. There were no differences in the rates of cleaved embryos and development to blastocysts between GPM-CH and BO-CH (72.0% vs 65.0%, 22.0% vs 25.0%, respectively). However, the rates were significantly low when oocytes were inseminated in GPM alone. It was found that there were no differences in the rates of development to blastocysts by using frozen spermatozoa derived from two different bulls when GPM-CH was used. These results indicated that GPM was successfully used as the fertilization medium for bovine oocytes. **Key words:** GPM, in vitro maturation, bovine oocytes, in vitro fertilization, blastocyst (Received December 3 1993, Accepted February 4 1994)

Introduction

Progress in the development of techniques for in vitro fertilization of bovine oocytes has led to successful production of blastocysts and offspring derived from oocytes matured in vitro [1-4]. Brackett and Oliphant's (BO) medium [5] or TALP [6] supplemented with heparin, caffeine or ionophore [7-10] has been widely used for washing bovine spermatozoa and for in vitro fertilization of bovine oocytes. Serono GPM (Serono, Spain) is a commercially available culture medium for separating human active spermatozoa [11] and for human embryo transfer [12], which is produced in large batches and has a long shelf life of one year at room temperature. GPM is based on Earle's balanced salt solution and contains human serum albumin (HSA). In the present study, we examined the availability of GPM as the fertilization medium for bovine oocytes matured in vitro.

Materials and Methods

Collection of Oocytes: Ovaries of Holstein and Japanese Black cattle (JBC) were obtained at a slaughterhouse and were transported to the laboratory in saline at 37℃ within 4 hours after slaughter. The ovaries were washed several times in saline at 37℃. Each ovary obviated corpus luteum was placed into a 90 mm petri dish with 15 to 20 ml Ringer's solution containing 1% calf serum (CS, Gibco, N.Y), and visible small antral follicles (about 2 to 5 mm in diameter) on the ovarian surface were cut with a disposable surgical blade. Ringer's solution containing the oocytes from 5 to 7 ovaries were transferred into a 100 ml beaker. After a few minutes, the supernatant was discarded, and 20 ml of the sediment was diluted with 60 ml Ringer's solution. The supernatant was discarded again, and the sediment was transferred into petri dishes with a grid. Oocytes containing multilayered compact cumulus cells and with an evenly granulated cytoplasm were selected. They were washed 3 times in Ringer's solution containing 1% CS, and in the maturation medium (25 mM Hepes buffered Medium199 with Earle's salts; Gibco, N.Y.) supplemented with 5% CS, 0.1 to 0.25 mg/ml follicle stimulating hormone (FSH, Denka, Kawasaki, JAPAN) [13].

In Vitro Maturation: In Experiment 1, 30 to 50 cumulus oocyte complexes from Holstein cows and heifers were placed into a 1 ml aliquot of the maturation medium and cultured for 20 hours at 38.5℃ in 3.5% CO_2 in air. Oocyte cumulus complexes from individual JBCs were cultured separately in Experiment 2.

In Vitro Fertilization: < Experiment 1 > Commercially distributed frozen semen from one Japanese Black bull was used in Experiment 1. One straw of frozen semen was thawed at 37℃ for 30 seconds. The thawed spermatozoa were washed twice with one of the following media by centrifugation at 500 xg for 5 minutes. 1) GPM (4 ml×2 times) alone; 2) GPM-CH (4 ml×2 times) : GPM supplemented with 5 mM caffeine (Sigma, St. Louis, USA) and 10 μg/ml heparin (Sigma, St.Louis, USA). 3) BO-CH (8 ml×2 times) :BO without bovine serum albumin (BSA) and glucose, but supplemented with 10 mM caffeine and 20 μg/ml heparin. The final pellet of spermatozoa was resuspended in one of the fertilization media at a concentration of 5×10^6 spermatozoa /ml. Compositions of the fertilization media are shown in Table 1. In vitro matured oocytes were transferred to 35 mm petri dishes containing 100 μl drops of each sperm suspension under paraffin oil (20 oocytes /drop). At 18 to 20 hours after insemination, some oocytes were collected, stained with 1% aceto-orcein and examined for evidence of sperm penetration. The remainder were used to evaluate embryonic development of oocytes fertilized in vitro. < Experiment 2 >

Table 1. Composition of GPM, GPM-CH and BO-CH used for in vitro fertilization of bovine oocytes

	GPM	GPM-CH	BO-CH
NaCl	116.40mM	116.40mM	112.00mM
KCl	5.37mM	5.37mM	4.02mM
$CaCl_2 \cdot 2H_2O$	1.80mM	1.80mM	2.25mM
$NaH_2PO_4 \cdot 2H_2O$	1.02mM	1.02mM	-
$NaH_2PO_4 \cdot H_2O$	-	-	0.83mM
$MgSO_4 \cdot 7H_2O$	0.81mM	0.81mM	-
$MgCl_2 \cdot 6H_2O$	-	-	0.52mM
$NaHCO_3$	25.00mM	25.00mM	37.00mM
Na-Pyruvate	0.41mM	0.41mM	1.25mM
Glucose	5.55mM	5.55mM	-
HSA	5.00mg/ml	5.00mg/ml	-
BSA*	-	-	10.00mg/ml
Caffeine	-	5.00mM	5.00mM
Heparin	-	10.00μg/ml	10.00μg/ml
Penicillin	-	-	100I.U./ml
Streptomycin	-	-	100μg/ml

*Sigma, St.Louis, USA.

Oocytes from 24 individual JBCs were cultured separately. After maturation culture, oocytes were inseminated with the frozen semen from one of two Japanese Black bulls. GPM-CH was used for sperm washing and in vitro fertilization.

In Vitro Development: After 5 hours of sperm/oocyte incubation, the oocytes were washed twice in TCM199 supplemented with 5% CS, and were further cultured at 38.5℃ for 10 days in TCM199 supplemented with 5% CS, 100 I.U./ml penicillin, 100 μg/ml streptomycin and 0.1 to 0.25 mg/ml FSH. The culture medium was changed after 60 hours of insemination and the cleavage of embryos and development to the 2-4 cell, 5-7 cell and more than 8 cell stage was recorded. Development to the blastocyst stage was recorded after 7-10days' culture. The data were analyzed by χ^2-test.

Results

Experiment 1: The proportions of penetrated oocytes in GPM-CH and BO-CH were higher than in GPM ($P<0.01$, Table 2). Embryonic development is summarized in Table 3. There were no differences between GPM-CH and BO-CH in the cleavage rates at 60 hours after insemination and in the rates of development to the blastocyst stage 7-10 days after insemination. On the other hand, 93 of 236 (39.4%) embryos were cleaved, and only 19 (8.1%) embryos developed to the blastocyst stage in GPM.

Table 2. Effects of different fertilization media on the in vitro fertilization of bovine oocytes

Fertilization media*	Number of oocytes examined	fertilized(%)	monospermy	polyspermy
GPM	30	10(33.3)[b]	10	0
GPM-CH	30	26(86.7)[a]	16	10
BO-CH	30	25(83.3)[a]	17	8

*See Table 1

[a,b]Values with different superscripts are significantly different (I^2,P<0.01).

Table 3. Effects of different fertilization media on the development of in vitro matured and fertilized bovine oocytes

Fertilization media*	Number of oocytes examined	cleaved(%)	Number of embryos developed to 2-4 cells	5-7 cells	8≤ cells(%)	blasto-cysts(%)
GPM	236	93(39.4)[b]	43	20	30(12.7)[b]	19(8.1)[b]
GPM-CH	586	422(72.0)[a]	117	110	195(33.3)[a]	129(22.0)[a]
BO-CH	488	317(65.0)[a]	80	88	149(30.5)[a]	122(25.0)[a]

*See Table 1

[a,b]Values with different superscripts within the same column are significantly different (I^2,P<0.01).

Experiment 2: From 24 individual JBCs a total of 862 oocytes were obtained. The number of oocytes from individual JBCs varied from 11 to 91 (mean=35.9±19.3). The cleavage rates for the oocytes inseminated with the spermatozoa from bull A and bull B were 66.7% and 68.3%, respectively. The rates for the embryos developing to blastocysts were 18.5% and 21.1% for bulls A and B, respectively (Table 4).

Table 4. Development of in vitro matured and fertilized bovine oocytes from individual Japanese Black cattle inseminated with frozen spermatozoa from two different bulls

Bull	individuals	Number of oocytes examined	oocytes cleaved(%)[a]	blasto-cyst(%)[b]
A	13	502	335(66.7)	93(18.5)
		[14-91][c]	[24.0-83.7][d]	[0-34.9]
B	11	360	246(68.3)	76(21.1)
		[11-82]	[36.4-97.6]	[0-31.7]

a:60 hours after insemination.
b:7-10 days after insemination.
c:Minimum oocytes-Maximum oocytes.
d:Minimum%-Maximum%.

Discussion

GPM supplemented with 0.5% (w/v) HSA is used as a medium for separation of active spermatozoa for in vitro fertilization, gamete intrafallopian transfer and intrafallopian insemination in human[11]. It has been demonstrated that HSA is a safe and suitable replacement for serum, both in embryo culture and in the transfer medium [12]. In Experiment 1, GPM-CH has shown similar effects on the cleavage and the development to blastocysts of bovine oocytes to those in BO-CH. GPM-CH includes HSA and glucose, while BO-CH includes BSA, and is free from

glucose. Significant inhibition of fertilization in the presence of glucose has been reported in cattle [15]. However, in our experiments there were no differences between GPM-CH, including glucose and BO-CH without glucose, in the rates of cleavage and the blastocyst formation. It has been known that BSA induces capacitation of human spermatozoa [16]. Bovine spermatozoa treated with GPM, containing HSA free from caffeine and heparin, produced cleaved embryos and blastocysts. This indicates that HSA induces capacitation of bovine spermatozoa.

In Experiment 2, a variation in the numbers of blastocysts obtained from individual JBCs was observed. No blastocyst formation was observed in 3 of 24 JBCs. Goto et al. [17] reported that the number of blastocysts obtained from individual JBCs after in vitro culture varied from 0 to 11, when BO medium supplemented with caffeine was used. Our findings were similar to those reported by them.

Routinely, the washing medium for bovine spermatozoa and the in vitro fertilization medium for bovine sperm and oocytes, based on BO medium and TALP, are homemade. It is therefore difficult to maintain a consistently high quality of these media. It is possible that the quality of the medium affects the fertilization rate. The use of commercially available GPM could eliminate potential batch variability problems. To date we obtained two viable JBC offspring and two pregnant recipients by transferring frozen-thawed blastocysts that had been produced by the methods reported here. It is concluded that GPM can be used as a culture medium for the preparation of bovine spermatozoa and in vitro fertilization of bovine oocytes.

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Serono GPM (Gamete Preparation Medium) を用いたウシ卵子の体外受精

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ウシ体外成熟卵子の受精に，ヒト精子調整溶媒として市販されているGPMを用い，卵割，胚発生に対する影響を検討した．5mMカフェインと10μg/mlヘパリンを添加したGPMまたは修正BO液を用いた場合，媒精60時間後の卵割率および媒精10日後までに発生した胚盤胞の割合には，両者に差はなかった．また，同様にGPMを用いて黒毛和種の個体毎についても検討したところ，１頭当たり平均7.0個の胚盤胞が得られた．胚盤胞を凍結融解後移植を行い，正常な子牛が得られた．これらのことから，GPMはウシ体外受精の受精用培地に使用できると考えられた．

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Morphometrical assessment of the expanded porcine oocyte - cumulus complexes matured in vitro : comparison of measuring methods

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Abstract: Our previous studies revealed that porcine oocyte-cumulus complexes that matured in porcine follicular fluid (pFF) and its active fraction resulted in a significant increase in the degree of cumulus expansion as compared with those matured in a modified Krebs-Ringer bicarbonate solution (mKRB) supplemented with bovine serum albumin. In this study, the differeces between an image analyzer and calculation by a formula (LW π/4, where L and W is the length and width of cumulus, respectively) in measuring the degree of cumulus expansion were investigated. Photographs were taken of the oocyte-cumulus complexes matured for 24 h in the different medium and the areas were measured using these two methods. The areas occupied by expanded oocyte-cumulus complexes that matured in pFF-Top fraction were measured accurately only by the formula method. The image analyzer gave significantly lower values due to faint boundary of expanded cumulus. There was no significant difference between the two methods for those unexpanded or very little expanded complexes. Advantages and disadvantages of the two different methods were noted. The advantage in using the image analyzer is that it can measure the natural shape of the oocyte-cumulus complexes. The disadvantages of the image analyzer are: a) the well expanded cumulus cell layers can not be measured accuretely, b) it has difficulty in measuring the oocyte-cumulus complexes in the presence of detached cumulus cells and c) only clearly taken photographs can be used for measurement. The advantages noted for using the formula were: a) oocyte-cumulus complexes printed in the photographs can be measured separately from the detached cumulus cells that are present and b)photographs with either over-or under-exposed prints can be used as a subject for measurement. **Key words:** cumulus expansion, porcine oocytes, in vitro maturation (Received April 23 1994, Accepted June 13 1994)

Introduction

The mammalian egg is surrounded by layers of cumulus cells and their hyaluronate-rich matrix. These cumulus cells are the remnants of the granulosa cells of the ovarian follicle, which are shed along with the egg during ovulation[1]. It was earlier reported that cumulus cell expansion improved the fertilizability and developmental capacity thus, one of the parameters in choosing oocytes for in vitro fertilization[2,3,4]. Chen et al.[5] reported in mouse that successful fertilization was correlated with the quantity and quality of the expanded cumulus mass, and the spontaneous loss or mechanical removal of the cumulus was correlated with a loss of fertilizability[6]. Also, porcine oocytes exhibited a higher frequency of male pronucleus formation when inseminated in the presence of expanded cumulus cells as demonstrated by Kikuchi et al.[7]

Very few literature deals with the measurement of cumulus expansion. In the studies made by Vanderhyden et al.[8], Vanderhyden[9] and Eppig et al.[10], the degree of cumulus expansion was assessed according to a subjective scoring system using a Wild M5A stereomicroscope. In our previous study, the degree of expansion was measured by using the formula[11]. In this regard, this study was therefore conducted to compare the difference between using an image analyzer (Olympus SP 500 stereomicroscope) and a formula (A[area]=L[length] xW [width] x3.1416/4) in the measurement of the area occupied by an oocyte-cumulus complex and whether there are limitations in using both methods with regards to the degree of expansion.

Materials and Methods

Chemicals: Crystalline penicillin-G potassium and streptomycin sulfate were purchased from Meiji Seika Co., Tokyo, Japan. Bovine serum albumin (BSA: fraction V)and other chemical reagents were taken from Wako Pure Chemical Ind., Tokyo, Japan.

Collection of follicular oocytes: Ovaries were obtained from prepubertal gilts at a local slaughterhouse and transported to the laboratory within 30 min in saline maintained at 37℃ supplemented with 100 I.U./ml penicillin G potassium and 0.1 mg/ml streptomycim sulfate. The gilt ovaries were immediately freed from their hilus and connective tissues and washed five times in a sterile saline solution warmed at 37℃. The non-atretic follicles of 2-5 mm in diameter were punctured with a needle directly into a sterile Petri dish and cumulus-enclosed oocytes were collected and washed in modified Krebs-Ringer bicarbonate solution (mKRB)[11]. Modified KRB was supplemented with 4 mg/ml BSA and sterilized using 0.20 μm filters (Corning-Iwaki glass Co., Japan) just before use.

Collection of porcine follicular fluid: Freshly collected prepubertal gilts ovaries from

a slaughterhouse were transported in a chilled saline solution to the laboratory. Porcine follicular fluid (pFF) was aspirated from porcine follicles of 2-5 mm in diameter with disposable syringes fitted with a 21-gauge disposable needles (Terumo Co., Tokyo, Japan). Pooled follicular contents were then centrifuged to removed debris and blood cells at 1,000 g, 10℃ for 20 min. The supernatant was transferred to a sterile 50 ml centrifuge tube (Corning-Iwaki glass Co., Tokyo, Japan) and stored at -20℃ until use and for subsequent isolation procedures.

Ultracentrifugation: Ten ml of pFF was fractionated by means of ultracentrifugation (Beckman SW 41Ti rotor) at 220,000g at 10℃ for 48 h. Fractions resulted to four parts designated as Top, Second, Third and Bottom. Each fraction was separated by means of Pasteur pipette, placed into an individual sterile tube and reconstituted to 10 ml by adding mKRB. The fractions were then frozen and stored at -60℃ until required.

Assessment of cumulus expansion: The method for in vitro maturation of porcine oocytes was based on that of Naito et al.[3] Each group of 10 cumulus-enclosed oocytes was cultured in 100 μl of mKRB, porcine follicular fluid (pFF) or one of the pFF fractions for 24 h at 37℃ under 5 %CO_2 in air. Photographs were taken of oocyte-cumulus complexes (Nikon FE2 camera with Neopan F 100DX film) at 24 h of culture using a phase contrast microscope (x25). The area occupied by each oocyte-cumulus complex in the photographs was measured using a ruler and calculated from the formula: A (area)=L(length) xW(width) x3.1416/4[12]. Length was measured by taking the two most widely separated points and the width was measured from the two closest points and the mean±SE was determined. Also, the area of each oocyte-cumulus complex in the same photographs were measured using an image analyzer, Olympus SP 500. Both results were compared. At least, 5 replicates were performed in this experiment.

Statistical analysis: Differences in the degree of cumulus expansion between the experimental groups were analyzed by a modified Student's t-test as applied in Aspin Welch method of Statistical Analysis[13].

Results and Discussion

Measurements of the cumulus expansion were performed both by using an image analyzer and a formula through photographs of the oocyte-cumulus complexes after 24 hours in culture medium. In our previous report, fraction 1 (top fraction) of porcine follicular fluid showed the highest degree of cumulus expansion and followed by those matured in pFF[14]. Expansion was also observed in oocyte-cumulus complexes

matured in pFF but in a lesser degree compared with those from Top fraction. Oocyte-cumulus complexes matured in mKRB, Second, Third and Bottom fraction, however, exhibited very little or no expansion (Fig.1). As shown in Fig. 1, expansion in Top fraction measured by the formula gave a marked degree of cumulus expasion than those measured by the image analyzer. There was a great difference between the two methods when it comes to the measurement of the well expanded cumulus cells. In lesser expansion, as in mKRB, Second, Third and Bottom fractions, the degree of expansion measured by the two methods did not show any difference.

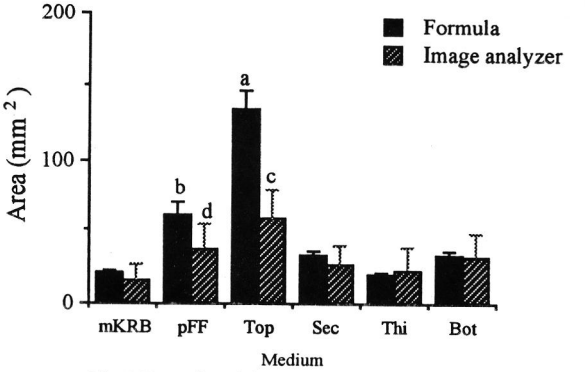

Fig. 1. Expansion of the cumulus cells calculated by the formula and assessed with an image analyzer. mKRB, modified Krebs-Ringer bicarbonate solution; pFF, porcine follicular fluid; Top, Top fraction; Sec, Second fraction; Thi, Third fraction; Bot, Bottom fraction.
a,b; b,d; a,c; c,d $P<0.01$, significantly different from each other.
n = 5

Some of the advantages and disadvantages were noted (Table 1). The advantages of using the formula are: a) oocyte-cumulus complexes printed in the photographs can be measured separately from the impurities, dirts and dettached cumulus cells that are present (Fig. 2a), and b) photographs with either over-or under-exposed prints can be used as a subject for measurement. For the image analyzer, it can measure the natural shape of the oocyte-cumulus complexes. The disadvantages of the image analyzer are: a) well expanded cumulus cells can not be measured accurately. When expansion becomes larger it appears to become very thin in the photographs. This kind of expansion can be hardly measured by the image analyzer, since measurements depends upon the color contrast of the machine. If the expansion appear thin in the photographs it resulted to a very light contrast giving a very low value of measurement, and if the contrast is thick as observed in unexpanded cumulus cells, the value of measurements is high, b) impurities, dirts and detached cumulus cells that appear in the photographs can be measured simultaneously with

Table 1. Advantages and disadvantages of using the formula and the image analyzer in the measurement of the area of expansion of the cumulus-oocyte complexes.

Methods	Advantages	Disadvantages
IMAGE ANALYZER (Olympus SP 500)	Can measure the natural shape of the oocyte-cumulus complexes Total number of oocyte-cumulus complexes that appears in the picture can be measured at one time	Well expanded cumulus cells can not be measured accurately Impurities in the glossy prints can be measured simultaneously with the oocyte-cumulus complexes Beautifully well taken pictures can be used as material for measurement only Using over- or under-exposed glossy prints are not advisable
FORMULA $A=\frac{LxWx3.1416}{4}$ *	Selectively measures the oocyte-cumulus complxes printed in the pictures (excluding dirts and other impurities) Much more easier to use Can be used to any type of glossy prints exposure (under or over expose prints), as long as the oocyte-cumulus complexes are visible	Irregular-shaped oocyte-cumulus complexes can not be measured accurately

* A = area, L = length, W = width

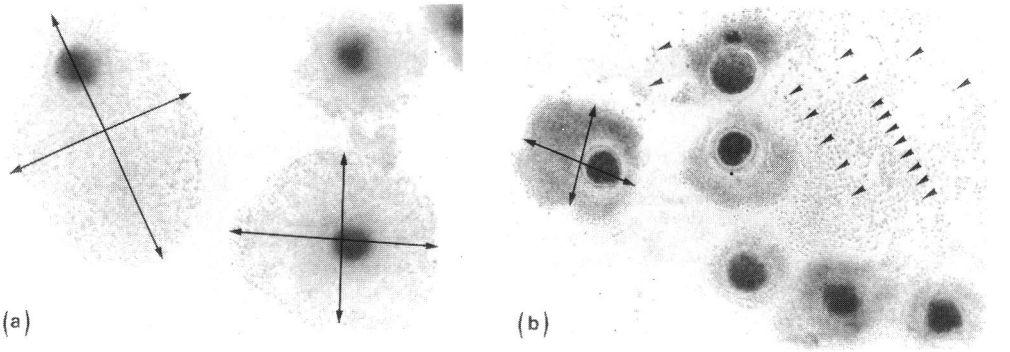

Fig. 2. Photomicrographs of porcine oocyte-cumulus complexes cultured for 24 h. (a) Calculations by means of the formula are shown by the directions of the arrows. Individual oocyte-cumulus complexes are measured carefully with this method. (b) Arrow heads pointing to some of the dettached cumulus cells. The area occupied by all of the detached cumulus cells (also dirts, impurities) are simultaneously measured together with the oocyte-cumulus complexes.When the detached cumulus cells are excluded during the measurement, cumulus cells surrounding or in the peripheral portion of cumulus-oocyte complexes are not measured. Magnification: X100

the oocte-cumulus complexes (Fig.2b). When trying to exclude the impurities, dirts and detached cumulus cells, the expanded layers of cumulus cells surrounding the cumulus-oocyte complexes that appear very thin in the photographs are not measured and c) only beautifully well taken photographs can be used as a subject for measurement. When a cumulus cells surrounding the oocytes appear light in the picture, it will shade light and give a very low value, while in a reverse situation, when the complex appears dark it will give a high value causing a high degree of expansion. There is no uniformity of the shade of the complexes, and the degree of expansion

depends upon the degree of shading. In compact cumulus cells, measurements is not much a problem and observed that percent error is very low as shown in mKRB, Second, Third and Bottom fractions. Also, the image analyzer gives different values to the photographs that are over expose and/or under expose developed prints.

It is therefore concluded in this experiment that by using the formula, gives accurate values in the measurement of well expanded cumulus complexes, and without limitations to the use of photographs of either the over-or the under-exposed prints.

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ブタ体外成熟卵の膨化卵丘の形態計測学的評価－計測方法の比較

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卵丘膨化の程度を表現する方法として，画像解析装置による方法と，卵子卵丘複合体の長径と短径を計測し，公式（長径×短径×π÷4）により算出する方法がある．私達は，ブタ卵胞液に卵丘膨化促進作用のあることを認めているが，卵丘膨化促進因子を分離するにあたって卵丘膨化の程度を正確に，さらに簡便に計測するためにどちらの方法が適しているかを明らかにしておく必要がある．私達は培養24時間後に卵子卵丘複合体集団を写真にとり，写真版を用いて画像解析装置と公式により卵子卵丘複合体の占める面積を計測し，それぞれの方法の利点と欠点を評価した．どちらの方法を使っても，部分的に精製したブタ卵胞液の活性分画は他の分画よりも強い活性を示したことから，計測方法によって最大活性を示す分画が異なることはないと考えられた．画像解析装置では，複雑な形を示す卵丘の占める面積を敏速に計測できたが，膨化の程度の著しいものについては計測がむずかしかった．また，遊離した卵丘細胞を除外して卵子卵丘複合体だけを計測することもむずかしく，写真版を用いて画像解析装置にかける場合には鮮明に撮影・現像されたものしか使えないことがわかった．公式により算出する方法は，膨化の程度の著しいものも簡便に計測でき，また，写真の質に影響されずに測定できるなど画像解析装置よりも優れた点があった．

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The Effect of Follicular Shells and Fluid on In Vitro Fertilization of Porcine Oocytes

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Abstract: The present experiment was designed to investigate the effect of follicular shell and fluid added to maturation medium on the porcine sperm penetration rate and subsequent male pronucleus formation in oocytes. Pig follicular oocytes were cultured for 47h, with follicular shells, 10% follicular fluid or their combinations added to a medium drop of TCM-199. All treatments had no effect on the proportion of oocytes developing to second metaphase of meiosis. Following insemination, the penetration of oocytes by frozen-thawed sperms was not significantly different among the treated groups. However, the proportion of polyspermic oocytes was significantly lower ($p<0.05$) when the oocytes were cultured with 10% follicular fluid (25.8%; 16/62) than when they were cultured in other conditions. The percentage of oocytes capable of sustaining male pronucleus formation was higher ($p<0.05$) when they were cultured with follicular shells combined with 10% follicular fluid (53.8%; 43/80) than in all the other maturation conditions. These results indicate that follicular fluid has an effective action on preventing polyspermy in oocytes and that the follicular shells combined with follicular fluid increases male pronucleus formation, inducing the cytoplasmic development of porcine oocytes during their in vitro maturation. **Key words:** pig oocytes, maturation, IVF, follicles, polyspermy
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Introduction

It has been reported that somatic follicular cells surrounding the oocyte are important for its complete maturation[10]. The soluble factor secreted by follicular cells plays a crucial role in the process of oocyte maturation, sperm penetration and male pronucleus formation in pigs[15]. The addition of follicular fluid to maturation medium is also effective in stimulating pronuclear formation in porcine oocytes matured in vitro[16] and the importance of maturational factors has further been confirmed by experiments which resulted in the birth of lambs, calves and piglets

after co-culturing oocytes with follicular cells during their in vitro maturation[2,7,9]. As the follicle matures, follicle cells undergo functional differentiation. Therefore, it is considered that follicle cells at different developmental stages may have different effects on oocyte maturation. With this idea in view, the present experiment was conducted to confirm the effect of follicular shells and fluid on the in vitro maturation and subsequent fertilization of porcine oocytes.

Materials and Methods

Ovaries were obtained from gilts in their late prepuberty, weighing approximately 110kg, which were aquired at a local slaughterhouse. Transported to the laboratory within 1.5h in a polystyren box at 35-37℃, they were washed once with 70% alcohol and twice with modified PBS medium. Follicles (2 to 5mm in diameter) were microscopically dissected in Tissue Culture Medium-199 supplemented with 3mg BSA/ml at room temperature. Subjected to the experiment were only those ovaries which contained no corpus luteum or albicans. Dissected follicular shells[4], after washed twice with the maturation medium, were everted and introduced into 400 μl of the maturation medium at a ratio of one per medium drop. Follicular fluid was aspirated from the follicles (2 to 5mm in diameter) with a 10-ml disposable syringe, attached to an 18-guage needle. Centrifuged twice at 530xg for 15min, the fluid was stored at -40℃ prior to being used for the in vitro maturation.

In Vitro Maturation : Oocyte complexes were aspirated from healthy follicles (2 to 5mm in diameter) with a 10-ml disposable syringe attached to an 18-gauge needle. Cumulus-enclosed oocytes were selected for the experiment from pooled oocyte complexes under a microscope. The basic medium (BM) for oocyte maturation consisted of TCM-199 supplemented with 15 IU PMSG/ml, 15 IU hCG/ml, 10% fetal calf serum, 3 mg BSA/ml and 100 μg penicillin-G/ml (control). Three experiments constituted the present study: (1) FS (one follicular shell added to a drop of BM), (2) pFF (10% follicular fluid added to a drop of BM) and (3) FS + pFF (these two elements added to a drop of BM). Oocytes were incubated at 37℃ for 47h in a humidified atmosphere containing 5% CO_2.

Preparation of Spermatozoa : Sperms derived from frozen semen that had been collected from Landrace boars weighing 200kg. Thawed at 39℃ for 50 sec., the semen was diluted with modified B.O. medium[1] containing 3mg BSA/ml, and washed twice by centrifugation at 600xg for 5min. The material was then preincubated for 2-3h at a concentration of $4-8\times10^8$/ml in B.O. medium, supplemented with 5 mM Caffeine and 3mg BSA/ml, in the same atmosphere conditions as in the oocyte

maturation.

In Vitro Fertilization : After the in vitro maturation cumulus-enclosed oocytes were co-incubated for 6-8h with preincubated boar sperms at a concentration of 2×10^6/ml in B.O. medium. Extra sperms and cumulus cells adhering to the surface of the zona pellucida were then removed by repeated pipetting. The oocytes thus obtained were washed twice with TCM-199, supplemented with 10mg insulin/ml and 3 mg BSA/ml, and further cultured for 9-12h in a developmental medium.

Examination of Nuclear and Fertilization Status : The oocytes denuded of the cumulus cells were mounted on a slide with a whole-mount technique and fixed for 3-5 days in acetic acid/methanol (1:3). Their nuclear status was examined according to the criteria of Hunter and Polge[6] under a phase contrast microscope after the staining with 1% orcein in a 45% acetic acid solution. Fertilization status was determined as described by Ding et al.[3]. Oocytes with swollen sperm head(s) and/or male pronuclei (MPN) deprived of sperm tail(s) were classified as being penetrated, irrespective of the number of sperm heads present in the cytoplasm. Oocytes with one or more full-sized MPN were considered to have undergone normal pronuclear development.

Results

The result with the in vitro oocyte maturation was shown in Table 1. The resumption of meiosis and maturation rate were not significantly influenced by each treatment. However, the proportion of oocytes remaining at germinal vesicle stage

Table 1. Influence of Culture Conditions on the In Vitro Maturation of Porcine Follicular Oocytes

Maturation condition	Total no. of oocytes examined	No. of oocytes (%)		
		Remaining at GV	GVBD	Matured*
Control	101	35 (34.7)[a]	8 (7.9)	58 (57.4)
FS	93	18 (19.4)[b]	15 (16.1)	60 (64.5)
pFF 10%	78	17 (21.8)[a,b]	11 (14.1)	50 (64.1)
FS+pFF 10%	80	13 (16.3)[b]	13 (16.3)	54 (67.5)

* No. of oocytes maturing to second metaphase.
GV=germinal vesicle, GVBD=germinal vesicle breakdown,
FS=follicular shells, pFF=porcine follicular fluid
[a,b] Different letters indicate significant difference between treatments ($p<0.05$).

was significantly lower in experimental groups than in the control ($p<0.05$). Table 2 shows sperm penetration and polyspermy in the oocytes which were cocultured with sperms for 6-8h and subsequently formed the male pronuclei in their cytoplasm. Sperm penetration was not significantly different among the treatments. Polyspermy in oocytes was significantly lower among those matured in the medium with 10% follicular fluid (25.8%; 16/62) than in those prepared in other conditions ($p<0.05$). The highest proportion for male pronuclear formation was obtained from the oocytes matured in the medium supplemented with follicular shells as well as 10% follicular fluid (53.8%; 43/80). However, all treatments had no effect on the coincidental formation of male and female pronuclei in the cytoplasm.

Table 2. Effect of FS and pFF Addition to Maturation Medium on Male Pronuclear Formation after Insemination in Porcine Oocytes

Maturation condition	No. of penetrated oocytes (%)		No. of oocytes with (%)		
	Total	Polyspermic	MPN	2MPN	MFPN
Control	77/ 99	53/77	22/77	3/22	7/77
	(77.8)	(68.8)[a]	(28.6)[a]	(13.6)	(9.1)
FS	70/102	39/70	22/70	4/22	10/70
	(68.6)	(55.7)[a]	(31.4)[a]	(18.2)	(14.3)
pFF 10%	62/ 93	16/62	21/62	4/21	9/62
	(66.7)	(25.8)[b]	(33.9)[a]	(19.0)	(14.5)
FS+pFF 10%	80/116	43/80	43/80	9/43	5/80
	(69.0)	(53.8)[a]	(53.8)[b]	(20.9)	(6.3)

MPN=Male pronuclei, MFPN=Male and female pronuclei
a vs b; $p<0.05$.

Discussion

Oocytes removed from the antral follicles of a wide variety of mammals resume meiosis in vitro. In over 95% of instances, however, they remain developmentally incompetent and fail to form normal fetuses after transfer[11]. The competence of oocytes, therefore, cannot be determined until a relatively late stage in embryogenesis, because various abnormalities may be expressed at a various stages of development[10,12]. It has been reported that complete maturation cannot be achieved without the direct support of follicular cells[13] and that the coculture of oocytes with follicular shells affects their penetration by sperms and the formation of male pronuclei[8].

Our results demonstrated that the presence of follicular shells together with follicular fluid during the maturation of porcine oocytes influenced the formation of male pronuclei. The findings were in agreement with the report of a marked increase in male pronucleus formation in penetrated oocytes matured in the medium

containing follicular shells[5]. Naito et al.[14] reported that the addition of follicular fluid to the culture medium greatly increased the rate of male pronucleus formation after insemination. Mattioli et al.[9] obtained piglets from the oocytes matured in vitro in the presence of follicular shells. In the present study, the polyspermy in penetrated oocytes was significantly decreased by the addition of follicular fluid to the maturation medium. On the other hand, the addition of either follicular shells or follicular fluid had no effect on male pronucleus formation. However, the addition of these two elements to maturation medium supported the maturation of cytoplasm and stimulated the development of male pronuclei. These findings indicated that follicular shells synergistically acted with follicular fluid and promoted cytoplasmic maturation.

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ブタ卵母細胞の体外受精に及ぼす卵胞細胞と卵胞液の影響

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体外でのブタ卵母細胞の成熟と，体外受精後の精子侵入，多精子侵入及び雄性前核形成に及ぼす卵胞細胞と卵胞液の影響を検討する目的で，実験を行った．成熟培養の基本培地はTCM-199を用いた．実験には4区を設け，(1)対照区では基本培地のみで培養を行った．他の区では同液に，(2)卵胞細胞，(3)卵胞液，(4)卵胞細胞＋卵胞液をそれぞれ加え，これらの条件で卵母細胞を47時間培養した．成熟培養後，半数の卵母細胞は固定し，残りは媒精に用いて受精状況を調べた．卵母細胞の第II減数分裂中期へ発育した割合及び媒精後の精子侵入率において実験区間有意差はなかった．多精子侵入率は，他の区に比べて卵胞液を添加した区で低くなった．雄性前核形成率においては，卵胞細胞＋卵胞液の添加区で有意に高い値を示した．これらの結果から，成熟培養時に卵胞液を加えることによって多精子侵入が減り，卵胞細胞と卵胞液を共に加え，雄性前核形成率が高くなることから，これら物質には相乗作用があると考えられた．

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Expression of Cyclins Genes during Preimplantation Development in mice.

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Abstract: To investigate the regulation of cell cycle during preimplantation development, the expression of cyclins was examined in early mouse embryos. We first isolated a cDNA fragment of mouse cyclin A, corresponding to cyclin box domain, and determined the sequence. Mouse cyclin A is highly homologous to human cyclin A (98.9% in amino acid). The comparison of amino acid sequence of cyclin A with those of B- and D- type cyclins revealed the sequences specific for A- and B- type cyclins and for A- and D- type cyclins. It is possible that these sequences are involved in the association with cdc2 and cdk2 kinases, since A- and B- type cyclins are known to be associated with cdc2 kinase and A- and D- type cyclins are with cdk2 kinase. The expression of cyclin mRNAs was exmined by the RT-PCR assay in unfertilized oocytes and the embryos at the 2-cell, 4-cell, morula and blastocyst stages. Cyclin A mRNA was expressed in unfertilized oocytes and the level was gradually decreased to the blastocyst stage. The B-type cyclins which regulate G2/M phase transition were expressed in a low level at the 2-cell stage. The level of cyclin B1 was almost constant during preimplantation develoment, except for at the 2-cell stage. The level of cyclin B2 was decreased at the 2-cell stage and then increased from the morula to blastocyst stage. The long G2 phase at the 2-cell stage may be attributable to the decrease in the level of expression of B-type cyclins. The D- type cyclins which regulate G1/S transition were not constantly expressed. The expression of cyclin D1 was detected in only 2-cell embryos and blastocysts. The expression of cyclin D2 was not detected all through the preimplantation development. Cyclin D3 was detected in unfertilized oocytes. The level was decreased to the 4-cell stage and then increased from the morula to blastocyst stage. It is possible that the regulation of G1 phase in early embryos is different from that in most somatic cells in which D- type cyclins are required for G1/S transition. **Key Words:** Cyclins, Cell cycle, Mouse embryos, Reverse transcription - polymerase chein reaction (Received 2 september 1994, Accepted 13 september 1994)

Introduction

Early embryos are different from most somatic cells in some aspects of cell cycle regulation. First, the length of G1 phase is different. Most cells require many hours to transit a series of G1 subphase during which the cells commit to enter S phase[1], whereas in early embryos G1 phase is short. In mouse embryos, only 1 to 2h of G1 phases were detected until the blastocyst stage [2,3]. During these short G1 periods, no molecular events which are observed in somatic cells, such as the expression of transcription factors and kinase activation concerning with signal transduction, have been detected. Second, G2 phase is very long in early embryos. In somatic cells, G2 phase is short and takes a few hours, whereas the length of G2 phase is about 15 h in mouse 2-cell embryos[2,3]. Although the composition of cell cycle is thus unique in early embryos, the molecular mechanism regulating cell cycle has not been examined.

Cyclins are regulatory subunits of the protein kinases in the cyclin dependent kinase (cdk) family which are key regulators of cell ceycle. There are several types of cyclin which have conserved domain, cyclin box[4,5]. They are associated with different partners of cdk family and play different roles on cell cycle regulation[6,7]. B- type cyclins are associated with cdc2 kinase and regulate G2/M transtion. A-type cyclin is associated with cdc2 and cdk2 kinases, and regulates G2/M transition and the progression of S phase[6,8-11]. D- type cyclins are associated with cdk2, cdk4 and cdk5 and regulate G1/S transition[12-14].

To understand the regulation of cell cycle during preimplantation development, we examined the expression of cyclins in early mouse embryos.

Materials and Methods

In Vitro Fertilization and Culture of Embryos : Female B6C3F1 mice, 3 weeks old, and mature male ICR mice were purchased from SLC Japan (Shizuoka, Japan). Female mice were injected with 7.5 IU pregenant mares' serum gonandotropin (Sankyo, Tokyo) and 48h later with 7.5 IU human chorionic gonandotropin (hCG; Sankyo). The ovulated oocytes were collected from the ampulla of oviducts 15-16hr after hCG injection. Spermatozoa were obtained from the cauda epididymis. The ovulated oocytes were inseminated with capacitated spermatozoa which had been incubated for 2h at 38.5℃. The embryos were cultured in a humidified atmosphere of 5% CO_2-95% air at 38.5℃ in Whitten's medium 514[15] with slight modifications (109.5 mM NaCl, 4.8mM KCl, 1.2mM KH_2PO_4, 1.2mM $MgSO_4 \cdot 7H_2O$, 22.6mM $NaHCO_3$, 5.6mM glucose, 0.23mM Na-pyruvate, 1.5mM Ca-lactate,

75 μg/ml penicillin, 50 μg/ml streptomycin) and 0.3% bovine serum albmin (fraction V; Boehringer Mannheim, Mannheim).

Cloning of cyclin A fragments : The degenerate oligonucleotides, corresponding to conserved amino acid sequences in the cyclin box of cyclin A[16], were used for the PCR primers to amplify the fragments of cyclin A expressed in mouse oocytes. The sequences of primers were as follows:

5'-ATGAARAARCARCCDGAYAT-3' (sense primer)

5'-AARTCAAAAGTAARNACYTT-3' (antisenese primer)

(R:A/G,Y:C/T,D:G/A/T,N:A/G/C/T)

mRNA were isolated from about 40 oocytes by using mRNA Isolation Kit (Invitrogen, San Diego) and converted to cDNA using Microfast Track Kit (Invirtogen). The PCR reaction were performed through 40 cycles (94℃/20s, 37℃/30s, 72℃/70s). The PCR products were separated on 1.5% agarose gel electrophoresis. The anticipated size (320bp) of band was cut out, and cDNA fragments were eluted from the gel and subcloned into pCR™ Vector (Invitrogen). DNA sequnces were determined by the dideoxy method[17] using the DNA Sequencing system of Pharmacia LKB Biotechnology.

Detection of cyclins transcripts by RT-PCR : The relative changes in the expression level of mRNA were determined by RT-PCR assays. mRNAs were isolated from either 20 unfertilized oocytes or embryos by using the Acidganidium Phenol-Chloroform (AGPC) method[18] and reverse-transcribed in 40 μl reaction mixture containing 20 units of avian myeloblastosis virus reverse-transcriptase (Invirogen), 0.2 μg oligo dT primer (Invirogen) and 1mM dNTP at 42℃ for 1h. Amplificaton of cDNA by PCR was carried out in a 25 μl of reaction mixture consisting of 20mM Tris-HCl, pH8.4, 50mM KCl, 0.2mM of each dNTP, 2 μM of each primer, 1 unit of Taq DNA polymerase (Gibco BRL/Life Technologies, Tokyo), and 2 μl of cDNA, except for the detection of cyclin B1 in which cDNA was diluted by 40-folds. The primers for PCR and expected sizes of the products were shown in Table 1. The PCR were

Table 1. Primers used for PCR amplification

Target mRNA	5' primer	3' primer	product(bp)
cyclin A	GAGGTGGGAGAAGAATATAA	ACTAGGTGCTCCATTCTCAG	236
cyclin B1	ATTGACTGGCTAATACACCT	GATGCTCTACGGAGGAAGTG	314
cyclin B2	ACACTTCTTAGATGGAAGAG	CGGATTTGGGAACTGGTGTA	282
cyclin D1	CTGGAGCCCCTGAAGAAGAG	CTGCCCAGGTTCAGGCCTTG	386
cyclin D2	GTCCCGACTCCTAAGACCCA	TCATCCTGCTGAAGCCCACA	365
cyclin D3	CCCCACCCGAAAGGCGCAAT	CATCCGCAGACATAGAGCAG	395

performed through 40 cycles consisting of denaturation at 94℃ for 20s, annealing at 55℃ for 30s and extension at 72℃ for 60s, and 1 cycle of final extension at 72℃ for 5 min, unless otherwise specified. The products of PCR were separated on 1.5% agarose gel electrophoresis and visualized by staining with ethidium bromide.

Results

Since the cDNA sequence of mouse cyclin A had not been published, we first attempted to isolate it. cDNA fragment of mouse cyclin A, corresponding to the cyclin box domain, was amplified by PCR with degenerate primers, and sequenced (Fig 1A). The mouse cyclin A has 89.6% homology in nucleotides sequence and 98.9% homology in amino acids to human cyclin A (Fig 1B). The homology of amino acids sequence between different members of cyclin family in mouse are shown in Table 2. When the homologies between different types of cyclins were compared, the homologies of cyclin A to B-type and to D-type cyclins were relatively high. The homologies of the sequences between A-type and B-type and between A-type and D-type are higher than those between B-type and D-type. This feature is more obvious when the sequences identical between B-type cyclins and cyclin A and between D-type cyclins and cyclin A are listed up (Fig.2). The percentage of the amino acids identical between B-type cyclins and cyclin A is high (12.9% of total), whereas those identical between B-type cyclins and cyclin D1, cyclin D2 or cyclin D3 are 1.1, 1.1 or 3.2% respectively. Similarly, the percentage of amino acids identical between D-type cyclins and cyclin A is 7.5% of total, whereas those identical between D-type cyclins and cyclin B1 or cyclin B2 are 1.1 or 1.1%, respectively.

Table 2. Percentage homology of amino acid sequence in cyclin box between different members of cyclin family.

	B1	B2	A	D1	D2	D3
B1	100	60.2	47.3	29.0	26.9	30.1
B2	60.2	100	52.2	26.9	25.8	32.3
A	47.3	52.2	100	39.6	37.8	38.9
D1	29.0	26.9	39.6	100	76.3	62.4
D2	26.9	25.8	37.8	76.3	100	71.0
D3	30.1	32.3	38.9	62.4	70.0	100

The sequences of mouse cyclin B1, B2, D1, D2 and D3 were obtained from database of DNA Data Bank of Japan. Relatively higher score of homology is covered with more dense screen.

During preimplantation development, the patterns of the changes in expression levels were different among cyclins (Fig.3). The expression of cyclin A was detected in unfertilized oocytes. The level was gradually decreased to the blastocyst stage. The level of cyclin B1 expression was almost constant from unfertilized oocytes to blastocysts, except for 2-cell embryos in which the level was decreased. Cyclin B2 mRNA was decreased from unfertilized oocytes to 4-cell embryos and increased from morulae to blastocysts. Cyclin D1 mRNA was not detected in unfertilized oocytes. The expression level was transiently increased at the 2-cell stage. Then, it was decreased at the 4-cell stage and increased again at the blastocyst stage. Cyclin D2 mRNA was not detected all through the preimplantation developmental stage. Cyclin D3 mRNA was detected in unfertilized oocytes. The level of expression was gradually decreased until the 4-cell stage, and then increased at the morula stage. In all RT-PCR assays, the anticipated sizes of products amplified were derived from cDNA after RT reaction but not from genomic DNA, since omitting the reverse-transcriptase from RT reaction did not generate any those products (Fig.3, lane C in each panel).

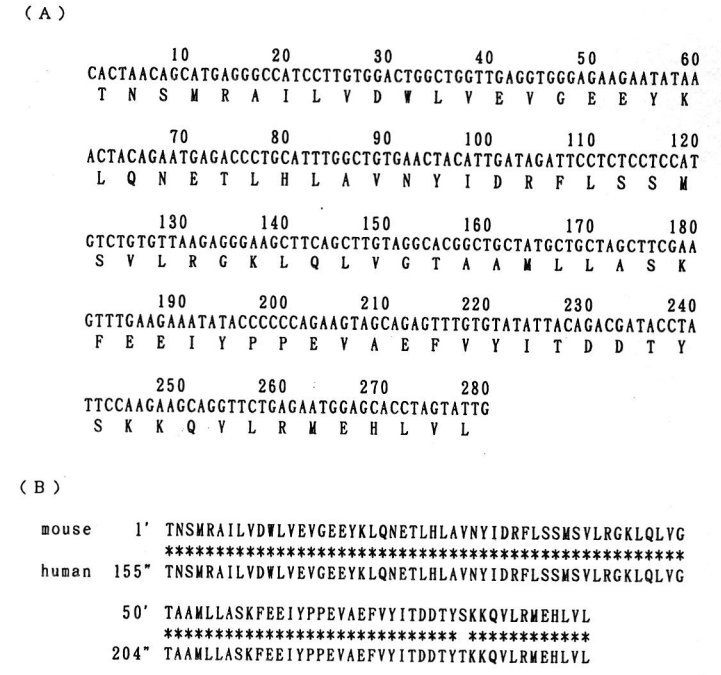

Fig 1

Partial sequence of cDNA and deduced amino acid of mouse cyclin A. (A) The nucleic acids (upper) and amino acid (lower) sequence of mouse are shown. (B) Comparison of amino acid sequences of cyclin A between mouse (upper) and human (lower). Amino acids conserved between two species are indicated by asterisks.

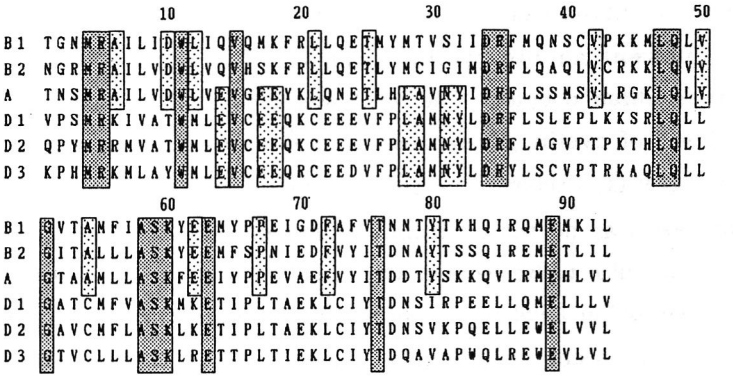

Fig 2

The sequences specific for cyclin A and B−type cyclins, and for cyclin A and D−type cyclins. The amino acids conserved in both cyclin A and all B−type cyclins but not in D−type cyclins, and conserved in both cyclin A and all D−type cyclins but not in B−type cyclins are boxed with sparse screen. The amino acids conserved in all cyclins listed up are box with dense screen.

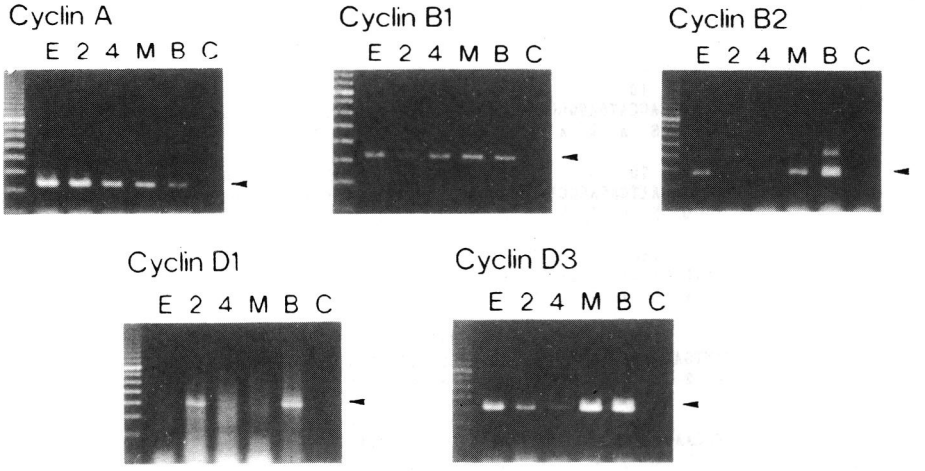

Fig 3

Expression of cyclins transcripts during preimplantation development of mouse embryos. mRNA was isolated from 20 unfertilized oocytes (E), 2−cell embryos (30 h after insemination; 2), 4−cell embryos (48 h after insemination; 4), morulae (72 h after insemination; M) and blastocysts (126 h after insemination; B), and subjected to RT−PCR. To control the amplification of genomic DNA, RT step was performed without reverse−transcriptase in the sample of blastocysts (126 h after insemination; C). The products were separated by electrophoresis. Three experiments were performed and the representative results are shown.

Discussion

The part of mouse cyclin A cDNA, which is the domain corresponding to cyclin box, was cloned and its sequence was determined. Since this part of cyclin A sequence was conserved well between mouse and human, cyclin box should be important in its function.

The necessity of cyclin box is suggested for the association of cyclin with cdks[19]. B-type cyclins are associated with cdc2 but with no other members of cdk family[20,21]. On the contrary, D-type cyclins are associated with the member other than cdc2[14]. A-type cyclin is associated with both cdc2 and cdk2[14]. These suggest that in cyclin box cyclin A contains the sequnces homologous to B-type and D-type cyclins. This is consistent with our results that the homology of cyclin box between B- and D-type was low and relatively high homologies were observed both between A- and B-type and between A- and D-type . The sequence identical between B-type cyclins and cyclin A and between D-type cyclins and cyclin A, shown in Fig.2, may be involved in the association with cdc2 and cdk, respectively.

At the 2-cell stage, the periods of G2 phase is very long and thereafter become shorter during development to the blastocyst stage[2,3]. In general, G2/M transition is driven by the complex of B-type cyclins with cdc2 protein[7,20]. During early development. the level of both cyclin B1 and B2 mRNAs were decreased at the 2-cell stage and then increased until the blastocyst stage. It is possible that the levels of expression of B-type cyclins is responsible for the length of G2 phase in early embryos.

In early embryos, the duration of G1 phase is very short when compared to most somatic cells[2,3]. G1 phase is indispensable periods during which the events necessary for the commitment to enter S phase occur. To explain short G1 period in early embryos, it has been suggested that the G1 events would begin during previous cycle[1]. If this is the case, the regulators of G1/S transition should be expressed in the embryos. However, our results showed that any D-type cyclins were not constantly expressed during preimplantation development. This suggests that G1 events, at least dependent on D-type cyclins, do not occur and that the regulation of G1 phase in early embryos is different from that in most somatic cells.

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マウス初期胚におけるサイクリン類の発現

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着床前の胚における細胞周期の制御について調べるため，初期胚におけるサイクリン類の発現を調べた．はじめに，マウスの卵子からサイクリンAのcDNAの一部を分離し，その塩基配列を決定した．マウスのサイクリンAは，ヒトのサイクリンAと極めて相同性が高かった（アミノ酸で98.9%）．サイクリンAとサイクリンB，Dのアミノ酸配列を比較すると，AとBに共通する配列およびAとDに共通する配列がそれぞれ認められた．サイクリンAとBはCDC2蛋白質に，サイクリンAとDはCDK2蛋白質に結合することが知られていることを考えると，前途の配列は，それぞれCDC2とCDK2に結合するために必要な配列であることが考えられる．次にRT-PCR法を用いて，未受精卵，2細胞期胚，4細胞期胚，桑実胚，胚盤胞それぞれにおけるサイクリン類のmRNAの発現を調べた．サイクリンAのmRNAは未受精卵で発現しており，その後，胚盤胞になるまで，徐々に減少した．サイクリンB1のmRNAは2細胞期で減少した以外は，ほぼ同じ量が発現していた．サイクリンB2のmRNAは，2細胞期に減少しており，その後，桑実胚期から胚盤胞期まで増加した．2細胞期でのG2期が長いことの原因は，M期への移行を制御するサイクリンB類の発現がこの時期に減っていることによるのかもしれない．サイクリンD類は，G1／S期移行の制御に関与していることが知られている．サイクリンD1のmRNAは，2細胞期と胚盤胞期でしか発現が確認できなかった．サイクリンD2のmRNAは，着床前のどの時期においても発現が確認できなかった．サイクリンD3のmRNAの発現は，未受精卵で認められ，その後，2細胞から4細胞期にかけて急激に減少し，桑実胚から胚盤胞期にかけて再び増加した．サイクリンD類のいずれについても恒常的な発現が認められなかったことから，初期発生のG1期は，通常の分裂細胞で見られるようなサイクリンDを必要とするようなものではなく，それとは異なった機構で制御されている可能性がある．

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性腺刺激ホルモン添加がブタ卵細胞の成熟・受精と ^{3}H－メチオニンの取り込みに及ぼす影響について

Effects of Gonadotrophins on the In vitro Maturation and Fertilization of Pig Oocytes

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Abstract: This study was conducted to determine the effect of PMSG and/or hCG(each 15 IU/ml) added to TCM－199 supplemented with 10% FCS and 3mg/ml BSA on the maturation, fertilization and the incorporation of ^{3}H－methionine of pig oocytes. The follicular oocytes were cultured for 48hr with or without supplementation of PMSG, hCG, given each alone or in combination to maturation medium. The addition of PMSG with or without hCG to maturation medium was effective in inducting meiotic resumption in pig oocytes. Fertilization rate was significantly improved by addition of hCG alone or with PMSG, compared to the control. From these results, it is suggested that the sperm penetration rate of oocyte was increased by hCG added to the maturation medium. The proportion of polyspermic oocytes, however, was not different among the treatments. Incorporation of methionine into oocytes measured after maturation culture was increased by addition of PMSG and/or hCG as compared to the control. These results indicate that PMSG and/or hCG added to medium during maturation is related to the development of cytoplasm of pig oocytes. **Key Words:** pig oocyte, maturation, IVF, gonadotrophin

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緒　言

体外での卵母細胞の成熟培養(IVM) では，第2減数分裂中期に達するとともに，受精能および発生能をもつ卵子が得られるか否かが課題となる．そのためには in vivoにおいて減数分裂再開に関わっている FSHとLH，あるいは，これらFSH と LH 様作用を有する PMSG，hCG の培養液への添加がその成熟に必要であると考えられる．牛の卵胞卵子の成熟は，TCM

-199 培養液に 10%前後の血清を加えた培養液で成熟卵子を得ている報告[1]もあるが，性腺刺激ホルモンを添加している例も見られる[2,3]．さらに，成熟用培養液に性腺刺激ホルモンを添加することにより，第2減数分裂中期に達することに加えて，受精能，発生能を含んだ総合的な卵子の能力が高められることが，マウス[4,5]，ラット[6,7,8]，山羊[9,10]，羊[11,12,13]および牛[14,15,16]において報告されている．

一方，豚卵母細胞の IVMにおいても一般的に成熟用培養液に性腺刺激ホルモンが添加されており[17,18,19]，FSH と LH の添加が卵子の成熟を高めることを Mattioliら[20]が報告している．しかし，FSH，LH 様作用をもつ PMSG，hCG に関しては，近年一般的に成熟用培養液に添加されてはいるものの，それら自体が体外成熟・受精・培養の過程にどのように影響しているのか詳細に調べた報告は少ない．そこで本実験では，成熟用培養液に添加した PMSGとhCG が，卵母細胞の第2減数分裂中期への成熟と受精，多精子侵入およびアミノ酸の取り込みに与える影響について調べることを目的とした．

材料および方法

卵母細胞の採取：食肉センターにて，性成熟豚から黄体のない卵巣のみを採取し，39℃に保温した 0.85%生理食塩水(100mg/lの硫酸カナマイシンを含む)に浸した状態で研究室に持ち帰り，38 ℃の生理食塩水で 3回洗浄した後，直径 2－5mmの卵胞の内容物を 18G針付ディスポーザブルシリンジで吸引採取した．緊密な数層の卵丘細胞に包まれ，黒く均一な細胞質をもつ卵母細胞のみを実験に供した．培養液は，TCM-199 に10% FCS と 3mg/ml BSA （ナカライテスク株式会社)を添加したものを基礎培養液(BM)とし，これに15IU/ml PMSG（ピーメックス，三共ゾーキ）と15IU/ml hCG（プベローゲン，三共ゾーキ）を添加する組み合わせによって以下の 5区の実験区を設けた．［1］ BMのみ （Control区），［2］ BM＋PMSG（＋PMSG区），［3］ BM＋hCG（＋hCG区），［4］ BM＋PMSG＋hCG(＋PMSG,＋hCG区），［5］ PMSG添加培養液で24時間培養後，hCG 添加培養液で24時間培養(PMSG →hCG 区)．流動パラフィン下に各実験区の培養液ドロップ(0.4ml）を作り，卵母細胞を20－30 個ずつ導入し，37℃，5% CO_2，95% 空気の条件下で48時間成熟培養を行った．

凍結精子の処理と前培養：体外受精に供した精子は長野県畜産試験場のランドレース雄の射出凍結精液を使用した．精子の洗浄，前培養および受精用の培養液にはBO液(5mMカフェインと3mg/ml BSAを含む）を用いた．液体窒素中から取り出した凍結精液をストロー(精液 0.5 ml/本）ごと40℃の湯に 50－60秒間漬けて融解した．さらに遠沈管に移し，あらかじめ38℃に温めておいたBO液1.0 mlを加え十分撹拌後，500gで 5分間遠心分離した．上清液を捨て，同様の操作で 2度洗浄し，2×10^8/mlの濃度に調整し，37℃，5%CO_2，95% 空気の条件下で3時間前培養を行った．

体外受精とその後の培養：流動パラフィン下に用意したBO液のドロップ(0.4ml）に成熟培養した卵母細胞20-30 個を移し，前培養を終了した精子を 2×10^6/mlの濃度になるように導入し

て媒精を行った。媒精後 7-8時間目に卵子を取り出し，ピペッティングにより周囲に付着している精子を取り除いた後に10%FCSを加えたTCM－199 のドロップへ移し，媒精後18-20 時間目まで培養した．

成熟率と受精率，多精子侵入率の算定：成熟培養終了後の卵子の一部はカルノア液(メタノール: 酢酸＝ 3:1)で脱脂・固定し，成熟率の判定のための観察に供した．また，媒精後18から20時間目の卵子は同様に固定し，受精率および多精子侵入率の算定のための観察に供した．固定はいずれも 3－5 日間行い，1%アセトオルセインで染色した後にノマルスキー微分干渉装置付きの倒立顕微鏡を用い観察を行った．

アミノ酸の取り込み：卵胞からの採取直後のGV期および，成熟培養直後，顕微鏡下で極体の放出の観察された MII期の卵母細胞を選び，アミノ酸取り込みの実験に供するためピペッティングにより卵丘細胞をとり除いた．5 μCi/ml の ^{3}H－メチオニンを100 μl 入れた0.5ml 容マイクロチューブに， 1サンプル当たり10個の卵子を入れ，さらにBO液を加え合計 200μl になるように定容した．マイクロチューブを37℃のインキュベーター内に入れ 1時間培養した後に，10%TCAを等量(200μl)加え反応を停止させた．各サンプルは －20℃のストッカーで保存後，ミリポアフィルター上で吸引濾過し，5%TCA と 70%エタノールで洗浄しタンパク分画とした．フィルターを乾燥させた後，液体シンチレーションカウンターでカウントした．単位はcpm/oocyteで表した．

統計分析：成熟率，受精率，多精子侵入率は χ^2 検定で，アミノ酸取り込み量のデータは t検定により比較し，不等分散であるものは Cochran－Cox検定を用いて比較した．

結　果

各実験区で48時間の成熟培養を行った卵母細胞の成熟率ならびに受精率を表１に示した．成熟率は，Control区に比べ他の実験区では有意に高かった($P<0.05$)．また，＋hCG区に比べ，＋PMSG 区，＋PMSG,＋hCG区， PMSG →hCG 区は有意に高い成熟率を示した($P<0.05$)．

Table 1 : Effect of PMSG and/or hCG added to maturation medium on in vitro maturation and fertilization of pig oocytes

Experimental groups	Total No. of oocytes	Maturation rate(%)	Fertilization rate(%)	Polyspermic rate(%)
Control	127	6/46(13.0)^{c}	25/81(30.1)^{c}	14/25(56.0)
+PMSG	99	32/61(52.5)^{a}	14/38(36.8)^{c}	6/14(42.9)
+hCG	103	19/61(31.1)^{b}	34/42(81.0)^{a}	22/34(64.7)
+PMSG, +hCG	168	48/98(49.0)^{a}	43/70(61.4)^{b}	22/43(51.2)
PMSG→hCG	128	33/64(51.6)^{a}	52/64(81.3)^{a}	31/52(59.6)

$^{a-c}$Values with different superscripts are significantly different($P<0.05$).
+PMSG, +hCG : Addition of PMSG and/or hCG in maturation medium.
PMSG→hCG : Addition of hCG followed by PMSG in maturation medium.

受精率は，Control 区に比べて ＋PMSG区では有意な差は見られなかったが，＋hCG区，＋PMSG,＋hCG 区，PMSG →hCG 区では有意に高い受精率が得られた(P<0.05).また，＋PMSG,＋hCG区に比べて＋hCG区と PMSG →hCG 区の受精率は有意に高かった(P<0.05).また，受精卵における多精子侵入率については，各実験区の間に有意な差は見られなかった．各実験区の成熟培養後のアミノ酸の取り込み量(平均値±標準誤差)を図１に示した．その結果，GV期の卵子に比べ，48時間成熟培養後の MII期卵子において，全ての実験区でアミノ酸の取り込み量は増加する傾向が見られ，＋PMSG 区では有意に高い値であった(P<0.05).また，Control 区に比べ＋PMSG 区では取り込み量は有意に高かった(P< 0.05).

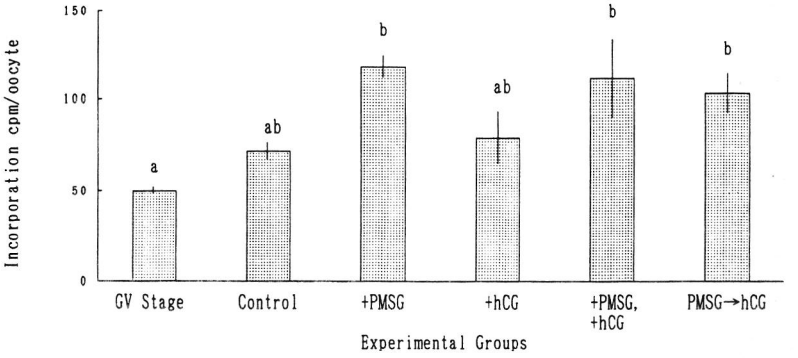

Figure 1 : Incorporation of ^{3}H-methionine into oocytes matured in addition of PMSG and/or hCG.
(M±S.D, a, b ; p < 0.01)

+PMSG,+hCG : Addition of PMSG and/or hCG in maturation medium.
PMSG→hCG : Addition of hCG followed by PMSG in maturation medium.

考　察

Mattioliら[20] は性腺刺激ホルモンを含まない培養液で豚の卵母細胞を成熟させた時の成熟率(35%) に比べ，FSH 及び LH を添加した培養液では成熟率が 86%, 76% と向上したと報告している．本実験における成熟率は Control区に比べ，PMSG, hCG を添加した他のどの実験区においても有意に高まり， PMSG 及び hCGが豚卵母細胞の減数分裂再開始誘起に有効であることが示された．また，＋PMSG, ＋hCG区，＋PMSG 区，PMSG→hCG 区に比べ，＋hCGで有意に成熟率が低かった．これは，in vivo において LH サージによって減数分裂の再開が起こることを考えると，やや矛盾しているように思われる．このことに関しては，Galliら[11] が， PMSG を投与した羊の卵巣卵母細胞を採取し，hMG(human menopausal gonadotropin)を添加した培養液で成熟培養することによって第２減数分裂中期卵子 83.8% を得ていることや，また，in vivo において卵母細胞は LH サージの作用を受ける前にFSH の作用を受けており，さらに，排卵の前後には FSHサージが発現し，血中 FSHレベルも上昇することから，卵母細胞の成熟には LH またはhCG による減数分裂再開の誘起作用を受ける前に FSHや PMSG の作用を受ける必要がある．これは卵丘細胞における減数分裂分裂再開の誘起因子[21]に FSH や PMSG が作用する必要があるのではないかと思われた．

一方，PMSG とhCG の両方の作用を受けた＋PMSG,＋hCG区および PMSG →hCG 区では成熟率は高く，PMSG，hCG の相互作用が核成熟に必要であると示唆された．また＋PMSG 区においても＋PMSG,＋hCG区，PMSG→hCG 区と同様に高い成熟率が得られた．PMSG はFSH と同様の生物学的作用に加えて弱いながらも LH 様作用も有することから，結局＋PMSG,＋hCG区と同様の効果が得られたのではないかと推察された．

Eppig ら[4] はFSH を添加した成熟用培養液で成熟させたマウスの卵母細胞は，体外受精後の２細胞あるいは胚盤胞までの発生率が高まると報告している．また Zhangら[6]は PMSG を投与したラットから得た卵母細胞および，FSH を添加した成熟用培養液で成熟させたラット卵母細胞で高い受精率を得たと報告している．これらは卵母細胞の受精能に関してFSH が有用であることを示している．一方，豚に関しては Mattioli[20] および Naitoら[22] の報告の中で，FSH 添加培養液で成熟させた卵母細胞の受精率の効果があるが，性腺刺激ホルモン無添加区を含む他のどの実験区とも有意な差はなく，高い受精率を得ている．本実験では，＋PMSG 区の受精率は低く，むしろ，hCG の添加が受精率の向上に必要であると推察された．

多精子侵入率は各実験区の間で有意な差はなかった．豚は多精子侵入が起こり易い種で，人工授精で卵管内に多数の精子を注入すると多精子侵入率が極端に高くなると言われている[23]．そのため一度に多数の精子が周りに存在する体外受精では多精子侵入が頻繁に起こり，その後の発生率が極端に低くなると言われている[24]．in vivoでは，多精子侵入を拒否するために，卵子は一精子の侵入刺激によって透明帯反応を起こすが，これは卵子細胞質の作用が強く関係している．本実験では全実験区の卵子とも同様な率で多精子侵入が起こっており，細胞質の成熟度合いは各実験区間で同様であったと考えられる．また，Yoshida ら[25] の体内成熟卵子を用いた体外受精率の結果によると，卵子を提供する雌個体によっても多精子侵入率に差があることから，本実験での多精子侵入率の高低について考察することは難しく，従って細胞質の成熟が充分であるかどうかは他の方法によって検討しなければならない．一方，多精子侵入に関する精子側の問題として，Nagai ら[26] は精子前培養濃度に違いよって受精率が変わり，受精率が向上するとともに多精子侵入率が増加すると報告し，Rathら[24] は媒精時における一卵子あたりの精子数が増加すると多精子侵入率も増加すると報告している．従って，多精子侵入を防ぐには精子側の処理についても検討すべきであると考えられた．

^{3}H-メチオニンの取り込みは，物質代謝，特にタンパク合成の指標となり得るものであり，卵母細胞や胚の生存力ひいては核や細胞質の成熟と非常に関係が深いと考えられる．加えて雄胚のグルコース代謝は高い[27] とされている．また，マウス胚では潜在的活性が高い胚は，代謝率を高め[27]，さらに異常発生や生存率低下を防ぐ[29] という報告もある．これらのことから，アミノ酸等の代謝活性を調べることで卵子の生存力の高低をある程度推察し得ると考えられる．

本実験の結果，核の成熟が進んだ卵子ほど，生存活性が高く，また細胞質の成熟も進んでいると考えられた．今後，受精前後の透明帯の変化などを的確に測定できる方法等を検討することによって，多精子侵入防御の機構が解明されると思われる．

要　約

本実験は TCM−199に 10% FCSと 3mg/ml BSA を添加した基礎培地に PMSG と hCG 添加が豚卵母細胞の成熟，受精および^{3}H−メチオニンの取り込みに及ぼす影響について調べた．実験区は，①基礎培地，②PMSG(15IU/ml) 添加，③hCG(15IU/ml)添加，④PMSG+hCG(+P，h) 添加および⑤PMSG 24 時間後,hCG 24 時間(P→h)の 5区を設け，48時間培養を行った．この結果，PMSGG および hCGを基礎培養液に添加することは，豚卵母細胞の減数分裂再開誘起に有効であった．受精率は対照に比べ，+hCG 区，+P，h区，P→h区で有意に高く，hCGの添加が受精率の向上を促進することが示された．しかし，多精子侵入率について，実験区間で差は見られなかった．上記 5区の成熟培養後の^{3}H−メチオニンの取り込みを測定した結果，対照区より PMSG，hCG添加した区で高くなる傾向が見られ，PMSG および hCG添加が，卵母細胞の細胞質の成熟に関与していることが明らかになった．

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1.5Mエチレングリコールで凍結されたウシ体外受精胚からの耐凍剤除去条件の検討

Effect of dilution procedures on the survival of IVF bovine embryos frozen by 1.5M ethylene glycol

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Abstract: The present study was designed to investigate the effect of temperatures and duration during removal of ethylene glycol as a cryoprotectant after thawing on the survival of frozen bovine IVF embryos. Bovine blastcysts (7 to 8 days after IVF) were frozen in 1.5M ethylene glycol, and then thawed and diluted by the different dilution procedures as follows. A group : Embryos were expelled from straw within 5 min after thawing and placed directly into Dulbecco's modified PBS supplemented with 20% calf serum (CS-PBS) at room temperature (22-23℃). B group : Embryos were expelled within 5min and placed directly into warm CS-PBS at 37℃. C group : Embryos were kept in the straw for 40min after thawing and placed into warm CS-PBS for dilution. After washing twice, embryos were co-cultured with granulosa cells in TCM-199 supplemented with 5% calf serum for 48hrs. Blastcystes developed to the hatching stage were estimated as viable ones. Survival rate in B group was slightly higher than A group, although the difference was not significant. Survival rate in C group was significantly lower than B group. These date suggest that embryos frozen with 1.5M ethylene glycol should be diluted in the warm CS-PBS to obtain better survival rate and the duration in straw after thawing might affect the survival and pregnancy rate in direct transfer. **Key Words:** Bovine IVF embryo, direct transfer, ethylene glycol, (1994年6月2日受付, 1994年7月17日受理)

要　約

体外受精由来胚をethylene glycolを耐凍剤として凍結保存し，融解後体外で耐凍剤を除去する際の環境温度及び融解後の経過時間が生存性に与える影響について検討した．その結果，融解5分後に胚をCS-PBSに直接浸漬して耐凍剤除去を行う場合，室温下で行ったA区（39.2％：20／51）よりもCS-PBSを加温して行ったB区（53.5％：23／43）の生存率が高かった（$P<0.10$）．しかし，融解40分後に加温CS-PBSに胚を浸漬して耐凍剤を除去したC区（24.4％：10／41）の生存率は，B区よりも有意に低かった（$P<0.05$）．以上のことから，ethylene glycolを耐凍剤として凍結された体外受精胚をCS-PBSに直接浸漬して耐凍剤を除去する場合，その環境温度に留意する必要があるとともに，融解後耐凍剤除去あるいは移植までの経過時間が長くなることで生存性が損なわれる可能性が示唆された．

緒　言

わが国の牛胚移植技術の普及のためには，凍結胚の融解，移植技術の簡易化が重要であり，当初融解後ストロー内でショ糖と混和することにより耐凍剤を除去する一段階ストロー法（One Step法）［1，2］の利用が野外においても検討されてきた［3－5］．しかし，この方法は融解後に煩雑な操作を必要とし，これが受胎率の不安定要因と考えられた［6］ことから，最近では融解後の操作を必要とせず直接移植可能なDirect Transfer法［7－9］あるいはノンステップ法［10,11］と呼ばれる凍結胚の移植方法（Direct法）が検討され始めている．Direct法はethylene glycol［7－9］，propylene glycol［10,11］，sucrose ＋ glycerol［12］など様々な耐凍剤が用いられ，融解後段階希釈として耐凍剤を除去後移植する従来法と同等の受胎率が得られると報告されている．また，Direct法で凍結した胚の生存性を検討するために，融解した胚を受胚牛に移植する代わりに，体外で耐凍剤を除去した後に培養して発生能が調べられているが，移植試験の成績を支持する生存が得られない場合がある［8，13，14］．そこで，本研究では，ethylene glycolを用いて凍結された牛体外受精由来胚の体外での耐凍剤除去条件が生存性に及ぼす影響を検討した．

材料及び方法

体外受精胚の作出：体外受精胚の作出は基本的には既報［15］に従って，成熟培養，媒精，発生培養を行い，媒精日を0日として7～8日目に胚盤胞期に達した胚で，形態的品質がBランク以上と判定された計191個を以下の実験に供試した．

凍結方法：体外受精由来胚135個を20％子牛地清加Dulbecco修正リン酸緩衝液（CS-PBS）を基本媒液とし，1.5Mのethylene glycolを耐凍剤として用いたVoelkelら［9］の方法を一部修正して凍結した．即ち，室温で1.5Mのethylene glycolに胚を直接浸漬し，20分後にストローに吸引して－7℃に保持したプログラミングフリーザー（富士平工業製，ET－1）に移し，約1分経過後に植氷し，10分間保持した．その後は毎分0.5℃で－35℃まで冷却し，15分間保持

した後，液体窒素に投入した．

凍結胚の融解，耐凍剤除去：凍結胚の融解は，液体窒素からストローを取り出し，空気中で約7～8秒間保持した後，30℃の温湯に投入して実施した．ついで，以下の3区に分けて耐凍剤を除去し，生存性を判定した．

（A区：5分-室温区）：融解5分後にストローから胚を取り出し，室温（22～23℃）下で直径35mmの小型シャーレ内の3mlCS－PBSに胚を直接浸漬して10分間静置することにより耐凍剤を除去した．

（B区：5分-加温区）：融解5分後に37℃に加温した3mlCS－PBSに直接浸漬し，10分間静置して耐凍剤の除去を行った．CS－PBSの加温は直接35mmの小型シャーレに3mlのCS－PBSを入れ，胚を浸漬する15分前から37℃に設定した加温板（北里メディカルサプライ）上に置いて行った．

（C区：40分-加温区）：融解後40分間ストローを室温に静置後，ストローから胚を取り出し，B区と同様に37℃のCS－PBSに浸漬して耐凍剤を除去した．なお，対照実験として，体外受精胚56個を用いて新鮮胚の生存性に及ぼすethylnene glycolの影響を調べた．即ち，胚を無作為に2群に分け，室温下で1.5Mのethylene glycolに浸漬して25分間及び60分間経過後に37℃に加温したCS－PBSに移し，10分間静置して耐凍剤の除去を行い，その後の胚の生存性を調べた．

生存性の判定法：凍結胚，新鮮胚とも耐凍剤を除去した後CS－PBSで1回，25mM HEPES緩衝TCM－199（m－199）培地で1回洗浄し，その後顆粒膜細胞を単層状に発育させた5％子牛血清加m－199培地の小滴内で48時間培養した．生存性の判定は，透明帯から脱出中の胚盤胞期以上に発育した胚を生存胚とした．

結　果

融解5分後に耐凍剤（ethylene glycol）除去を室温と37℃で行い，その後の生存性を比較した。その結果，37℃に加温したCS－PBSに直接浸漬して耐凍在を除去したB区の生存率は53.5％（23／43）で，有意差はみられないが室温下で耐凍剤を除去したA区（39.2％：20／51）よりも高かった（$P<0.10$）．

融解40分経過後ストローから胚を取り出し37℃に加温したCS－PBSに浸漬して耐凍剤除去を行ったC区の生存率は24.4％（10／41）で，B区よりも有意に低かった（$P<0.05$）(Table-1)．

Table 1　Survival of frozen-thawed bovine IVF embryos by using 1.5M ethylene glycol(EG) as a cryoprotectant after various EG dilution procedures

Group	Dilution temperature	Duration after thawing to dilution	No.of embryos	No.of survived	%
A	room temp.	5min	51	20	39.2[a]
B	37°C	5min	43	23	53.5[bc]
C	37°C	40min	41	10	24.4[d]

a vs b　P<0.10　　c vs d　P<0.05

融解後の耐凍剤内での経過時間が胚の生存性に影響を与えることが確認されたので，対照実験として，新鮮胚をethylene glycol中に一定時間浸漬し，その後加温したCS－PBSに直接浸漬して耐凍剤を除去した後の生存性について比較した．その結果，平衡時間20分間及び耐凍剤除去前の保持時間５分間の計25分間ethylene glycolに浸漬した新鮮胚の生存率は64.3％（18／28）であり，さらに35分間静置し，計60分間浸漬した胚の生存率は60.7％（17／28）で，両区の生存率に有意な差は認められなかった（Table－2）．

Table 2　Survival of fresh bovine IVF embryos after immersion to 1.5M ethylene glycol(EG)

Dilution temperature	Duration of immersion to EG	No.of embryos	No.of survived	%
37°C	25min	28	18	64.3
37°C	60min	28	17	60.7

考　察

ethylene glycolあるいはpropylene glycolなどを耐凍剤として用いたDirect法では，融解後耐凍剤を除去せずに胚を受胚牛に直接移植できることが知られている．Direct法で直接移植が可能な理由として，これらの耐凍剤は分子量が小さく，子宮内で容易に細胞外に拡散できるので，細胞内に水分が急速に流入することがなく，胚が変性しないためであると考えられている［９］．これに対して移植前に耐凍剤除去が必要なglycerolは，Direct法に用いられる上記の耐凍剤に比べて分子量が大きく，耐凍剤を除去しないまま移植した場合には細胞内外に大きな濃度勾配が生じ，胚細胞内への急速な水の流入が起こるので，細胞が膨満して変性するため受胎しないとされている［10］．

ethylene glycolはglycerolに比べると細胞内透過性が高いが［16］，今回の試験の様に体外で耐凍剤除去を行う場合に室温下で除去操作を行うと，子宮内と比べて環境温度が低いためethylene glycolの分子の動きが抑制され，細胞内に耐凍剤分子がとどまる時間が長くなり，細胞内への水分の急速な流入が起こり細胞が膨満し変性する割合が多くなると考えられた．CS－PBSを加温し子宮内に近い温度環境条件を設定して耐凍剤除去を行うことにより，細胞内のethylene glycolの分子の動きは活発となり細胞外に容易に拡散するので室温下に比べて細胞内外に大きな濃度勾配が生じ難く，胚細胞内への水分流入速度とその量が生理的状態に近くなり，生存性の向上がみられたと考えられた．

Direct法で凍結した胚について多くの研究者が子宮内直接移植を想定して融解胚を等張液（例えばCS－PBS）に直接浸漬して耐凍剤除去を試みている．Voelkelら［８，９］はethylene glycol，Suzukiら［10］はpropylene glycolを耐凍剤としてPBS直接浸漬でも耐凍剤除去が可能であったとしているのに対して，Voelkel［８，９］はpropylene glycol，堂地ら［13］はethylene glycol，体外受精由来胚を使った筆者ら［14］はethylene glycol及びpropylene glycolとも

に,直接浸漬では移植試験の受胎成績を支持するような高い生存性が得られなかったと報告している．このようにDirect法に用いる耐凍剤を使って凍結した胚の体外で耐凍剤除去後の生存性が報告者によって一致しない原因は，今回の試験結果から推察すると耐凍剤除去を行った環境温度の差が影響している可能性が考えられた．

つぎに，耐凍剤を除去するまでの経過時間については，対照実験で示したようにethylene glycolは新鮮胚に対しては毒性を示さないが，融解後は胚をethylene glycol溶液内に一定時間以上とどめると生存性が低下することが明かとなった．ethylene glycolで凍結された胚の融解直後の形態は凍結前の形態と変化はないが，時間の経過とともに胚が収縮し胞胚腔を確認できない胚が出現する．融解後の胚はethylene glycol中で経過時間が延長するに従って生存性の低下がみられた原因は，時間の経過とともに胚はethylene glycol溶液内で収縮し，胚の細胞内の塩類濃度が高い状態で長時間保たれたことにより塩害が生じたためと考えられた．このような融解後の胚の形態変化の原因は明かではないが，融解後の経過時間の延長による生存性の低下は他のDirect法あるいはOne Step法による凍結方法においても認められている［17，18］．今回の実験結果から，ethylene glycolを耐凍剤として凍結された胚をCS－PBSに直接浸漬して耐凍剤を除去する場合，融解後の経過時間が生存性に影響することが示され，このことから実際に受胚牛に移植する際にも融解後の経過時間に留意する必要があることが明らかになった．

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卵透明帯の生合成部位と発現時期に関する免疫組織学的解析

Immunohistochemical studies for the developmental expression of a zona pellucida antigen in ovarian follicles.

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Abstract: The developmental expression of a zona pellucida antigen was examined by use of immunofluorescent stainings and electromicroscopic observations. A monoclonal antibody (MAb-5H4) which recognized an amino acid sequece of Cys-Thr-Val-Leu-Asp-Pro-Glu-Asn-Leu of pZP1 was used for detection of the zona pellucida antigen. Electromicroscopic observations revealed that the zona pellucida antigen was deposited around oocytes in the primary follicles in a discontinuous pattern. The amount of the zona pellucida antigen increased according to the development of ovarian follicles in immunofluorescent stainings, suggesting that the zona pellucida antigen is produced continuously through the whole period of follicular development. The antigen was found not only outside but also inside of oocytes in primary follicles with 1-4 layers of granulosa cells. In addition, secretory vesicles containing the zona pellucida antigen were also observed in the cytoplasm of oocytes under the electromicroscope. It is thus concluded that during folliculogenesis the zona pellucida antigen was synthesized in the oocytes, transported to the plasma membrane by secretory vesicles, and was secreted. **Key Words:** zona pellucida, folliculogenesis, immunohistochemistry, monoclonal antibody, secretory vesicle. (1994年8月3日受付,1994年8月11日受理)

要　約

哺乳動物の卵細胞を取り囲む透明帯の生合成部位と時期について，免疫蛍光抗体法と免疫電顕法を用いて調べた．ブタ透明帯の一成分であるZP１のCys-Thr-Tyr-Val-Leu-Asp-Pro-Glu-Asn-Leuを認識することが判明しているモノクローナル抗体（MAb-5H4）を一次抗体として用い，ブタ卵巣組織の透明帯抗原を検出した．免疫電顕により，透明帯蛋白は一次卵胞の卵母細胞周囲にすでに不連続に形成されていることが観察された．一方，蛍光染色法では卵胞の成熟過程に伴って，卵母細胞の周囲に観察される透明帯の厚みと蛍光強度が増加した．この蛍光は卵母細胞の周辺のみならず，１～４層の顆粒膜細胞を伴う未熟な卵胞の卵母細胞の細胞質内にも認められた．このことから，透明帯蛋白は成熟過程にある卵母細胞の細胞質内で合成されることが示唆された．さらに，電顕観察では透明帯抗原を含む分泌小胞が卵母細胞の細胞質内に観察された．以上の結果より，透明帯蛋白は卵胞成熟の初期から後期にかけて卵母細胞で合成され，細胞質内の分泌小胞によって運搬されるものと考えられた．

緒　言

哺乳動物の卵細胞を取り囲む透明帯は，受精に際して，精子の動物種の認識，先体反応の誘起，進入過程における精子の保持，多精子受精からの防御などの機能を担うことが知られている[1]．透明帯は，一般に３種の糖蛋白からなる細胞間マトリックスの一種で，機能と構造とのかかわりが分子レベルで明らかになりつつある．しかし透明帯糖蛋白が生合成される部位については，卵母細胞自身あるいは顆粒膜細胞あるいはその両方など様々な報告があり一定の結論は得られていない．これまでの研究の多くは，ムコ多糖体の染色あるいは，複数抗原を認識するポリクローナル抗体を用いての免疫染色法を用いて行われたものである．今回我々は対応抗原エピトープの明らかなモノクローナル抗体（MAb-5H4）[2]を用い，透明帯の卵巣内局在性と発現時期について検討した．MAb-5H4の認識するアミノ酸配列はCys-Thr-Tyr-Val-Leu-Asp-Pro-Glu-Asn-Leuで，ブタ透明帯を抗原として作成されたが，ブタ以外にヒト，ウシ，ウサギ，イヌ，ネコの透明帯に交差反応する．しかし，透明帯以外の臓器との交差反応性は極めて低く，臓器特異抗原としての性格をもつ．

ブタ透明帯はpZP1，pZP3α，pZP3βの３種の糖蛋白から成るが，MAb-5H4が認識する配列はpZP1のアミノ酸50～59番の配列に対応する．pZP1はマウス透明帯のmZP2のアミノ酸配列と類似しているので，mZP2と同様の第２精子レセプターとしての機能[1]，すなわち透明帯進入過程における精子の保持の役割を担っていると考えられる．本研究では透明帯抗原の局在を，MAb-5H4を用いて光学顕微鏡及び電子顕微鏡にて観察した．

材料と方法

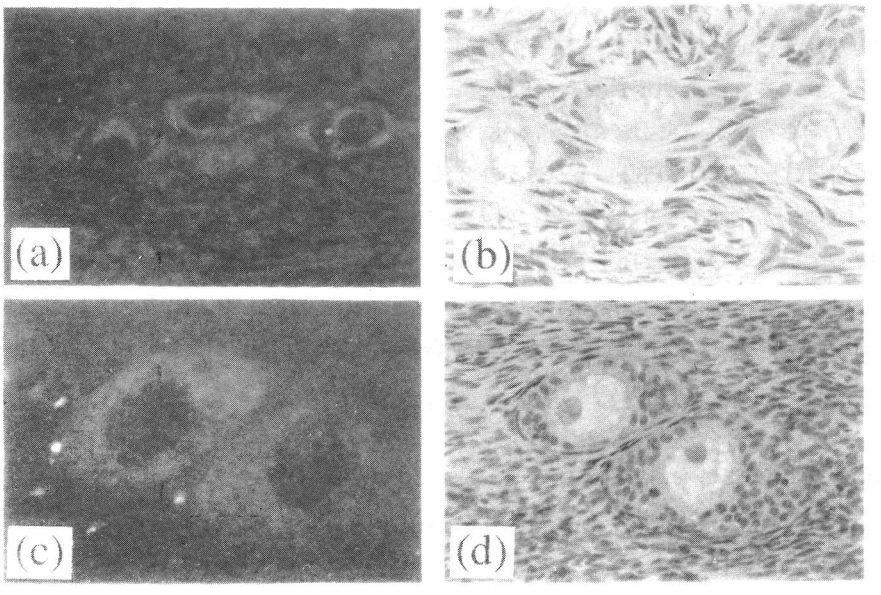
蛍光抗体法：ブタ卵巣を４％パラホルムアルデヒドで固定した後，アルコール上昇系列で脱水し，メタクリレート系樹脂JB-4（Polysciences）に包埋し，３μmの切片を作製した．この切片に，第１反応としてMAb-5H4のハイブリドーマ培養上清を室温で１時間処理し，PBSで

洗浄後，第２反応としてFITC標識抗マウスIgG（100倍希釈）を室温で１時間処理した．PBSで洗浄後，蛍光顕微鏡にて観察した．その後，同一切片をヘマトキシリン－エオジンにより対比染色した．

免疫電顕法：ブタおよびヒト卵巣を４％パラホルムアルデヒド－0.25％グルタールアルデヒド固定液中で10分間固定後，マイクロスライサー（DTK-1000DSK）により300μmの切片とした．この切片をさらに氷温中で１時間上記の固定液で固定し，アルコール上昇系列で脱水した後，切片を１mm角に細切し，Lowich wicry1系樹脂Lowicry1 K4M（Chemische Werke GMBH & Co.）中に，UV照射により包埋した．ミクロトーム（NOVA）で100nmの超薄切片を作成し，ホルムバール（Ladd）支持膜を張ったニッケル単孔メッシュ（Veco）上に，押し付け法により標本を調整した．第１反応として，MAb-5H4のハイブリドーマ培養上清を室温で１時間処理し，PBSで洗浄後，第２反応として粒子径15nmの金コロイド結合プロテインA（EY LABS）を室温で１時間処理した．PBSで洗浄後，2.5％グルタールアルデヒドで再固定し，ウラン－鉛の二重染色を施した後，電子顕微鏡JM1200EX（日本電子）で観察を行なった．

結　果

種々の発育段階の卵胞を含むブタ卵巣切片を，MAb-5H4により蛍光染色し，透明帯特異抗原の発現時期とその局在について観察した．扁平な卵胞上皮細胞を伴う原始卵胞，および１～４層の顆粒膜細胞を伴う未熟な１次卵胞においては，透明帯抗原の存在を示す蛍光は認められなかった（Fig. 1-a,c）．これらの時期は，ヘマトキシリン－エオジンによる対比染色においても，透明帯構造は認められなかった（Fig. 1-b,d）．５～６層の顆粒膜細胞層を伴うやや発育した卵胞においては，透明帯構造と一致する部位に明らかな陽性反応が見られた（Fig. 1 e,f）．さらに発育した胞状卵胞においては，透明帯の厚みとそれに一致して見られる蛍光の強度が増加した(Fig. 1-g,h)．



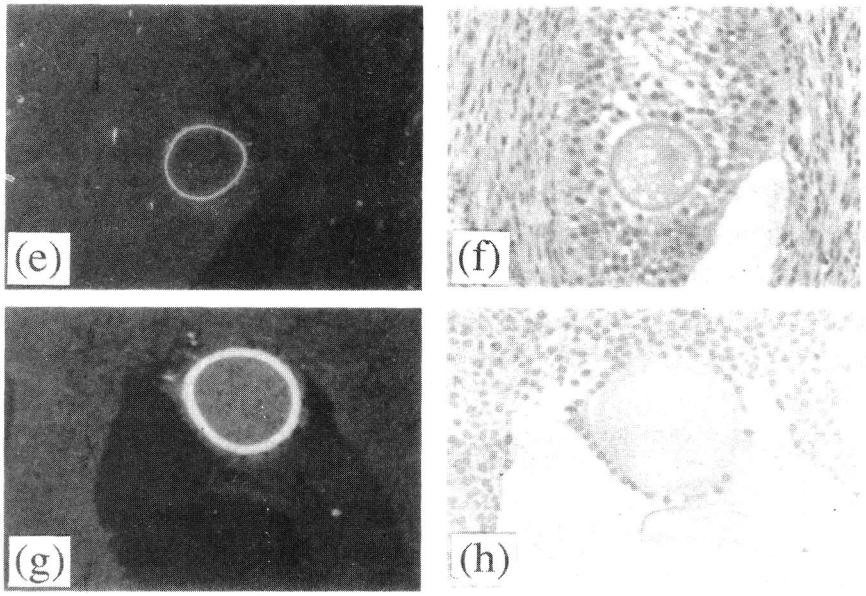


Fig.1 Immunofluorescent study of the localization of porcine zona pellucida in four different stages of growing follicles.
(a)(b) : primordial follicles; (c)(d): growing follicles with 1-4 layers of granulosa cells; (e)(f): preantral follicle; (g)(h) : antral follicle (a)(c)(e)(g): The sections were stained with MAb-5H4 and FITC-conjugated anti mouse IgG. (b)(d)(f)(h): The sections were stained with hematoxylin-eosin. Magnification ×200.

ホルムアルデヒドによる固定標本では形態の保持はよいが，抗原抗体反応を利用した免疫染色をやや阻害する傾向があるので，未固定の卵巣組織を直接凍結切片とし，MAb-5H4で染色した．Fig. 2-a,bは，発育段階の異る３種の卵胞を含む切片で，卵胞の発育に伴って透明帯抗原の存在を示す傾向の強度が増しているのがわかる．中央の卵胞は１～４層の顆粒膜細胞層を伴う比較的早期の発育段階の卵胞であるが，蛍光が卵母細胞表面のみならず，細胞質内にも認められる．

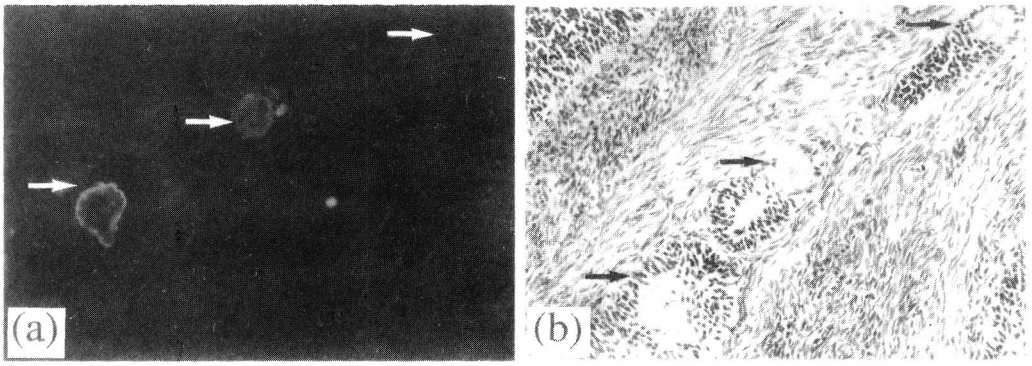


Fig.2 Immunofluorescent staining of a frozen section from a porcine ovary. This section contains three growing follicles with different stages as shown in arrows (b). The upper right follicle shows no immunofluorescence, the middle one shows weak immunofluorescence not only in zona pellucida but also in oocyte's cytoplasm, and the left one shows strong immunofluorescence in zona pellucida outside of oocyte (a). Magnification ×100.

また，免疫電顕による観察でも原始卵胞には，透明帯抗原の存在を示す金コロイドの沈着が全く認められなかった．１～４層の顆粒膜細胞層を伴う発育初期の卵胞では，卵母細胞と顆粒膜細胞層の間に断続的な間隙が存在し，この部分に金コロイドの沈着が認められた（Fig.3-a)．５～６層の顆粒膜細胞層を伴う発育段階の卵胞では，卵母細胞の周囲に厚さ約４μmの均一な透明帯が観察され，これに一致して金コロイド粒子の沈着が認められた（Fig. 3-b)．また，卵母細胞の細胞質内に直径１μmの金コロイドの付着した小胞が存在した（Fig. 3-c)．同様な小胞が，ヒト卵母細胞の細胞質内にも認められた（Fig. 3-d)．顆粒膜細胞の細胞質内およびその周囲には，いずれの発育段階の卵胞においても，全く金コロイドの沈着は認められなかった．

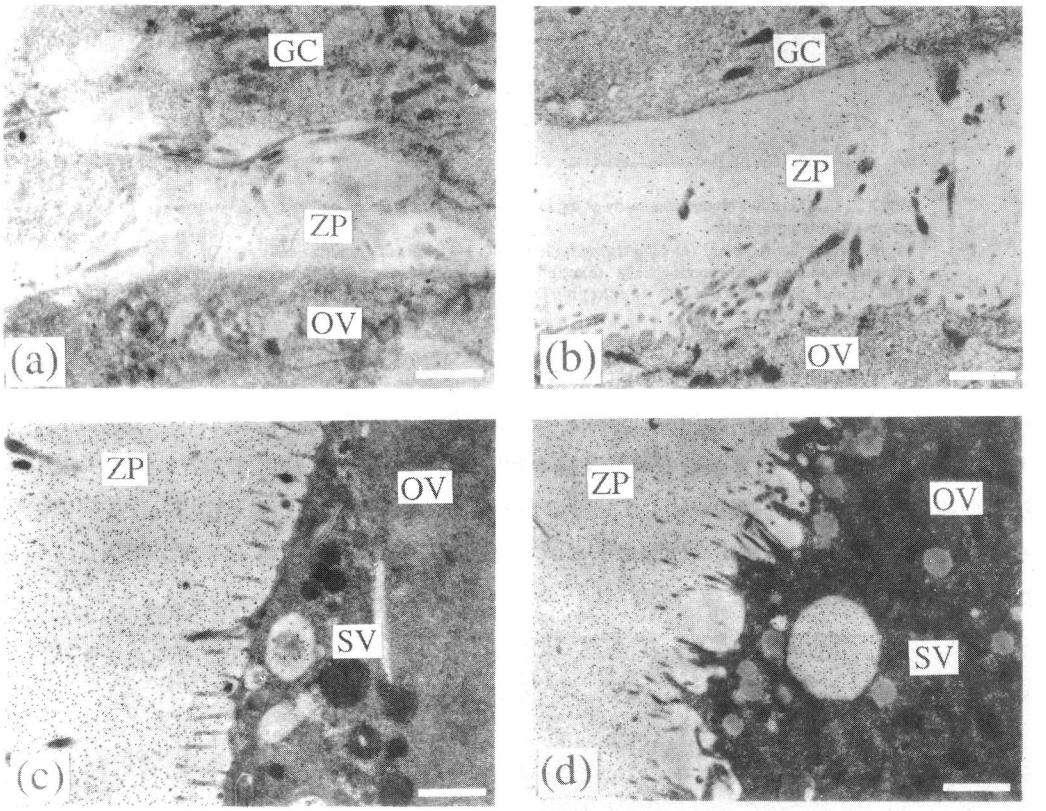


Fig.3 Electron micrograph of ultra thin sections of porcine (a)(b)(c) and human (d) ovarian tissues. (a) Porcine primary follicle. Note that gold colloid particles were located only between the oocyte and the granulosa cell in a discontinuous pattern. This area appears to be premature zona pellucida. (b) Porcine growing follicle with 5-6 layers of granulosa cells. Note that gold colloid particles were located in the zona pellucida with a width of 4μm.(c) Porcine preantral follicle. Note that the density of gold colloid particles located in the zona pellucida increased in a comparison to (a)(b). (d)Human preantral follicle. Both oocytes(c)(d) contained secretory vesicles(SV) with gold colloid particles. GC: granulosa cell; ZP: zona pellucida; OV: oocyte; SV: secretory vesicle
Bar is 1 μm

考　察

哺乳動物卵透明帯に特異的に反応するMAb-5H4を用いて透明帯抗原の生合成部位について検討した．蛍光抗体法によるブタ卵巣の観察では，透明帯抗原は5～6層の顆粒膜細胞層を伴う発育段階の卵胞ではじめて検出されたが，免疫電顕による微細な観察では，1～4層の顆粒膜細胞を伴うごく初期の発育段階でも，顆粒膜細胞と卵母細胞の間に断続的な間隙が存在し，この部分に金コロイドの沈着が見られた．このことから透明帯は，発育開始直後の卵胞において，すでに合成が開始され分泌されていることが判明した．固定処理を行なわない凍結切片では，透明帯のみならず，未熟な発育段階の卵母細胞の細胞質内にも蛍光が認められ，透明帯抗原が卵母細胞により合成されていることが示唆された．電顕観察からも，これを裏づける金コロイドの付着した分泌小胞が，卵細胞内に認められた．顆粒膜細胞層にはいずれの観察においても，全く透明帯抗原の存在は認められなかった．従って，MAb-5H4で認識される透明帯抗原は，原始卵胞には存在しないが，発育を開始したごく初期の卵胞の卵母細胞により合成され，分泌小胞により運搬され，この間に糖鎖の結合やその他の修飾を受け，分泌されるものと考えられる．Wassarmanらも，マウスの透明帯成分mZP3に対するポリクローナル抗体を用いて，同様な分泌顆粒の存在を認めている[1]．

透明帯の生合成部位と時期に関する組織学的な研究は古くから行われているが，ウサギ[3]，ラット[4]，ハムスター[5,6]，ヒト[5]において，いずれも卵母細胞によって合成されると報告されている．In vitroでの透明帯合成実験では，卵母細胞の培養により，培養液中に添加したアイソトープが透明帯内に取り込まれることが報告されている[7,8]．透明帯に非常に特異的に反応するモノクローナル抗体を用いた本研究もこれらの報告と一致するものであった．

また近年，メッセンジャーRNA（mRNA）を直接，組織の上で検出するin situ hybridization法を用い，マウスの透明帯成分のmZP3が卵母細胞に特異的に検出されることが報告された[9,10]．我々もMAb-5H4の対応抗原エピトープをコードするcRNAを用いて，ブタ卵巣組織のin situ hybridizationを行い，発育卵胞の卵母細胞にのみ陽性反応を認めている（未発表）．

一方，ウサギ透明帯の一成分R55Kは，発育のごく初期には，顆粒膜細胞にも対応するmRNAが検出され，この透明帯蛋白は卵細胞と顆粒膜細胞の両方で合成されると報告された[11]．透明帯は複数の糖蛋白により構成されているので，成分によっては顆粒膜細胞で作られるものが存在する可能性がある．事実，排卵期の卵母細胞透明帯には，卵管上皮から分泌される糖蛋白が特異的に結合し，あたかも透明帯固有成分のようにふるまうことが報告されている[12,13]．またLeeら[11]の指摘のように，発育時期により同じ蛋白が2種以上の細胞で合成される可能性もある．

現在のところ，透明帯を構成する糖蛋白の主たる合成部位は卵母細胞自身であり，その合成は卵胞発育直後から開始し，卵胞発育の間ずっと続いているものと考えられる．卵胞の発育，特に卵母細胞の減数分裂再開にあたっては，種々の蛋白の合成開始と停止が高度な制御の下に行われている．その中には，c-mosのようにmRNAが転写後翻訳されず，長時間保存されるものもあれば，α-tubulinやβ-actinのように，転写後直ちに翻訳される蛋白もあるが[14]，透

明帯蛋白は後者に属する蛋白のようである．我々は以前，透明帯で動物を免疫すると多数の卵母細胞を含まない小卵胞が卵巣内に発生し，明らかに卵胞の正常な発育が阻害されることを報告した[15]．すなわち，透明帯は受精において機能するのみならず，卵胞発育や卵母細胞自身の成熟にも何らかの役割を担っていると考えられるので，今後検討したいと考えている．

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マウス4-細胞期胚の体外発生におよぼすプロスタグランジンの影響

Effects of Prostaglandins on the Development of Mouse 4 - Cell Embryos

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Abstract: The effects of prostaglandins(PGs) on the development of mouse embryo in vitro was examined. Four cell embryos were obtained from mice after mating following PMS-hCG treatment. The embryos were cultured in the mBWW under 5% CO_2 in air at 37℃ with indomethacin (IND), PG (E_2, $F_2\alpha$, I_2) (0-200 μM) and adenosine 3', 5'-cyclicmonophosphathiate sp-isomer (Sp-cAMPS), and the subsequent development of embryos was observed. In the control, 77-87% of 4-cell embryos developed to blastocysts (BL). BL formation was suppressed to 57.1 and 25% by 5 and 10 μM of PGE_2, 52.3% by 100 μM of IND, 25% by 50 μM of $PGF_2\alpha$, while 200 μM of PGI_2 showed no effect. Sp-cAMPS also suppressed BL formation, but BL formation of these embryos was observed after release from Sp-cAMPS. These results indicate that PGs have a suppressive effect on the develoment of early mouse embryo, and PGE_2 was most effective among the agents used in this stydy. Sp-cAMPS also showed some effect, but its effect was seemed to be different from that of PGs. (1994年8月22日受付, 1994年9月12日受理)

要　約

PGの初期胚発生におよぼす影響についてマウス4細胞期胚を用い, in vitroで検討した.

マウス胚はPMS-hCG処置後の卵管より採取した．IND，PG（E_2,$F_2\alpha$，I_2)(0-200 μM)およびSp-cAMPS（0.3-2.5mM)添加のmBWWを用い5％CO_2 in air，37℃で培養し，その後の胚発生を観察した．無添加では胚盤胞の形成は77-87%であったが，PGE_2 5 μM添加では57.1%，10 μMで25%に低下した．IND100 μM添加では52.3%，$PGF_2\alpha$ 50 μMで25%低下したが，PGI_2は200 μM添加でも胚盤胞の形成率は無添加と同様であった．Sp-cAMPS(0.3mM）添加で胚盤胞の形成は抑制されたが，Sp-cAMPS暴露後の胚を無添加で培養すると66.7%に胚盤胞の形成の回復が認められた．これらより，PGがマウス初期胚発生を抑制することが明らかとなった．Sp-cAMPSも同様の効果を認めたが，PGによるものとは異なることが推察された．

緒　言

生体の各種の組織で産出されるプロスタグランジン（PG）は，局所ホルモンとして種々の働きを持っている．生殖器官においてPGは，卵胞液[1]や卵管液[2]などにも存在しすることが知られており，卵の成熟，受精，胚発生，着床などの一連の現象がPGの存在下で進行している．卵の成熟においては，PG合成酵素阻害剤であるインドメタシン（IND）を処置した幼若ラットにPGE_2や$PGF_2\alpha$を投与すると，第一極体形成やPFK活性抑制が回復すること[3]，またマウスではIND投与でhatchingや着床が阻害され，これらがPGの添加や投与で回復すること[4]などよりPGが生殖現象に深くかかわっていることが推察される．しかし，一方ヒトでは，子宮内膜症などで不妊が高頻度に認められ，マウス卵と卵管の共培養系に内膜症患者の腹腔内液を添加すると受精卵の卵割が抑制され，これがINDの同時添加で回復する[5]など生殖現象におけるPGの影響については不明な点が多い．そこで我々は胚発生におけるPGの影響を検討することを目的として，マウスを用いて初期胚（4-細胞期胚）の発生に対するPGの影響をin vitroで検討した．

材料および方法

実験動物には8-9週齢のICR系雌および同系雄マウス（東京実験動物）を用いた．PMS・hCG（帝国臓器製薬より恵与），PGE_2・$PGF_2\alpha$（小野薬品より恵与），PGI_2・IND（Sigma, USA)，Sp-cAMPS（BIOLOG,USA）および他の試薬は特級のものを用い，各種PGおよびINDは95%エタノールに溶解して用いた．マウス受精卵の培養にはBWW培養液を用い，組成はNaCl 94.59mM，KCl 4.78mM，Ca-lactate-5H_2O 1.71mM，$MgSO_4$-7H_2O 1.19mM，KH_2PO_4 1.19mM，$NaHCO_3$ 25.07mM，glucose 5.56mM，Na-pyruvate 0.25mM，Na-lactate 21.58mM，BSA 1mg/mlで超純水を用い，37℃，大気下5％CO_2で行なった．雌マウスにPMS10単位投与後，48時間後にhCG5単位をそれぞれ腹腔内に投与し，同系雄と交配した．膣栓が確認されたマウスはhCG投与48時間以降に屠殺し，卵管を摘出，BWW液で灌流して4-細胞期胚を採取し実験に用いた．PG，IND添加実験：各種PGおよびINDはBWW液で0.5-200 μM濃度に調整し，controlとしては0.8%エタノール含有のBWW液を用いた．採取した胚をそれぞれの培養液に移し，48時間培養後の胚盤胞の形成を観察した．さらにIND添加培養液に各種PGを添加して同様の実験を行った．

Sp-cAMPS添加実験：Sp-cAMPSは超純水に溶解後BWW液で0.3-5mMに調整した．48時間培養した胚は状態を観察した後，無添加BWW液に移してさらに培養を継続した．尚，胚盤胞形成の成績の統計学的処理には，X^2検定を用いた．

結　果

INDを25-200 μM添加した時の胚発生への影響はTable 1に示すごとくである．controlでは，80.6%の胚盤胞の形成が認められた．添加例ではINDの添加濃度の増加に伴い胚盤胞の形成率が低下し，IND100 μM添加で形成率は52.3%で有意に低下した．IND添加による胚盤胞形成抑制を回復する目的で，IND100 μMの培養液にPGE_2，$PGF_2\alpha$，PGI_2が各々100 μMとなるように調整して3系統（IND＋PGE_2．IND＋$PGE_2\alpha$，IND＋PGI_2）を作製してマウス胚の発生を検討したが，胚盤胞形成の回復を認められずその率は更に減少した．

Table 1. Effects of Indomethacin on the Development of Mouse Embyro

concentration (μM)	No of 4-cell	4-cell	8-cell· morula	blastocyst (%)
control	36	0	7	29(80.6)
25	43	0	10	33(76.7)
50	39	2	13	24(61.5)
100	44	5	16	23(52.3)**
200	43	13	16	14(32.6)**

Observation was performed after 48hr incubation with or without IND.

(blastocyst formation vs control, **:$p<0.01$)

Table 2には，PGE_2(0.5-200 μM)，$PGF_2\alpha$（25-100 μM）およびPGI_2（50-200 μM）添加48時間培養後の胚盤胞の形成状況を示した．controlでは78.7%に胚盤胞の形成を認めたが，PGE_2 5 μMでは57.1%，10 μMで25%と有意に減少し，50 μM以上では胚盤胞の形成は認めなかった．Figure 1はPGE_2 100 μM）添加培養後の胚の状況で，細胞質が凝集し粗大顆粒状に観察された．$PGF_2\alpha$添加では，50 μMで胚盤胞の形成が25%と有意に減少し，100 μMでは認めな

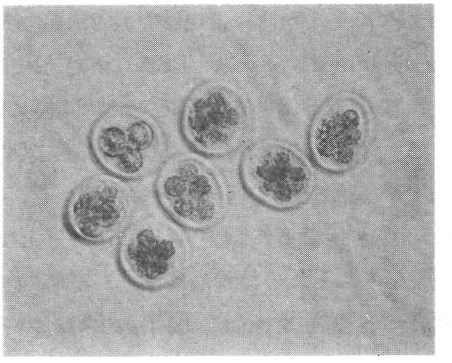

Figure 1. Photograph of degenarated mouse embryos. This photograph shows the changes of the embryos after 48hr culture of 4-cell embryos in the BWW medium containing 100 μM of PGE_2.

Table 2. Effects of PGs on the Development of Mouse Embyro

	concentration (μM)	No of 4-cell	4-cell	8-cell· morula	blastocyst (%)
PGE_2	control	75	2	14	59(78.7)
	0.5	36	1	8	27(75.0)
	1	45	0	18	27(60.0)
	5	42	2	16	24(57.1)*
	10	32	20	4	8(25.0)**
	50	34	16	18	0
	100	43	28	15	0
	200	38	32	6	0
$PGF_2\alpha$	control	47	1	10	36(76.6)
	25	28	0	8	20(71.4)
	50	28	0	21	7(25.0)**
	100	24	14	10	0
PGI_2	control	36	0	7	29(80.6)
	50	19	1	4	14(73.7)
	100	22	0	5	17(77.3)
	200	23	0	4	19(82.6)

Observation was performed after 48hr incubation with or without PGs.
(blastocyst formation vs control, *: $p<0.05$, **: $p<0.01$)

かった．PGI_2添加では胚盤胞の形成には影響を認めなかった．Sp-cAMPSの影響を検討した結果をTable 3に示した．細胞内に取り込まれた後，代謝されないこの物質の添加（0.3mM）によって胚盤胞の形成は認められなかったが，ほとんどの胚が８細胞期以降にまで発生した．顕微鏡下の観察では細胞質の変性の所見は認めず，48時間培養後の胚をSp-cAMPS無添加のBWW液に移し更に培養した結果，8/12(66.7%）に胚盤胞の形成が確認された．

Table 3. Effects of Sp-cAMPS on the Development of Mouse Embyro

concentration (mM)	No of 4-cell	4-cell	8-cell· morula	blastocyst (%)
control	30	2	2	26(86.7)
0.3	27	3	24	0
2.5	29	11	18	0

考　察

近交系マウスの受精卵は，in vitroで2細胞期より先に発生が進みにくい2細胞期ブロックがある[6]．このブロックの解除にEDTA，SODやEGFなどが有効であることが報告されているが，本実験は添加物質の効果をみるものであるので2細胞期ブロックの解除物質を用いずに4細胞期胚を実験対象とした．4細胞期はすでに卵管内にあり着床にむけて重要な準備段階にあり，また容易に胚盤胞まで観察することが可能で実験対象として非常に有用である．

生殖過程においてPGは多くの重要な働きをしている[4]．そこで，初期胚発生におよぼすPGの影響についてマウスを用いて検討した．INDは強力なPG合成酵素阻害剤で，多くの研究でPG産生抑制に用いられている[4,7]．本研究ではIND添加で胚盤胞の形成抑制が認められPGの関与が推察された．マウスの受精過程でも同程度の濃度のINDによる阻害効果が報告されているが，これはPG添加で回復している[8]．INDにより阻害効果がPG合成酵素の働きによるものであるか否かをみるためには，PG添加による阻害効果の回復をみることにより判定できるが，4-細胞期胚のINDによる影響は各種PGの同時添加培養では胚盤胞形成阻害の回復が観察できなかった．これはINDを培養期間中存在させているためか，あるいはINDがシクロオキシゲナーゼ阻害作用だけでなく，ホスホジエステラーゼやcAMP依存性プロテインキナーゼ等の酵素にも阻害作用を有することによるかもしれないが，マウス胚におけるこれら酵素の存在や役割は明かではなく検討する必要がある．

PGの添加により胚発生が阻害されることを認めたが，PGI_2では変化がなかった．これは物質の構造などの違いによるものか，その不安定性によるものかは結果から確認出来なかったが，PGI_2は溶液中では数10秒で変化する6-keto-$PGF_1\alpha$などを用いることでその違いが明かになるかもしれない．Prasad[9]はPGEによるマウス神経芽細胞の分化誘導現象を観察し，PGが細胞増殖に関与するとの報告している．ヒトの腫瘍細胞においても増殖抑制効果がPGJ_2，PGD_2，PGE_2などで認められ[10]，その作用はcAMPを介さず，無差別かつ直接的であろうと報告され，核酸合成阻害作用も認められていることや，マウス着床前の胚では^{14}C-glucose代謝がPGE_2や$PGF_2\alpha$で影響されること[11]より，これらのPGの作用が実験で認められた胚発生の抑制現象を引き起しているとも推察される．更に，PGにはセカンドメッセンジャーとしての作用があり，cAMPやCa代謝を調節する．我々もcAMPのアゴニストで細胞内で代謝されないSp-cAMPSを用いて検討した結果，PGとSp-cAMPSによる発生阻害は胚盤胞の形成率からみると同様であるが，その形態の違いや発生の回復がSp-cAMPS添加例で認められたことから，PGとSp-cAMPSの胚発生阻害効果は異なる作用によると思われる．一方，cAMPがマウスの4細胞期胚の発生を抑制したことより，この時期の胚でも卵の成熟過程におけるのと同様にcAMPが重要な働きをしていることを示している．PGは作用発現のステップとして受容体を介する系も考えられ，近年それらのマウスcDNAがクローニングされてきている．現在，我々もPGE_2や$PGF_2\alpha$[13]の受容体についてprimerを設計しRT-PCRを用いてmRNAの発現を検討している[14]．

今回，マウス初期胚に対するPGの影響を検討した結果，胚発生抑制効果が明かとなった．この様な効果の発現については直接的に作用すると推察されるが，この様な現象が生体内の局

所で発生しているか否かについては，PGの作用機構をさらに検討することによって明らかになるものと思われる．

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日本学術会議だより　No.33

第15期最後の総会開催される

平成6年6月　日本学術会議広報委員会

今回の日本学術会議だよりでは，5月25日から27日まで開催された第118回総会の概要と同総会で採択された「新しい方式の国際研究所の設立について(勧告)」，「公的機関の保有する情報の学術的利用について(要望)」，「女性科学研究者の環境改善の緊急性についての提言(声明)」についてお知らせします。

日本学術会議第118回総会報告

日本学術会議第118回総会（第15期・第6回）が，5月25日～27日の3日間にわたって開催されました。

総会の初日（25日）の午前は，会長からの前回総会以降の経過報告に続いて，各部，各委員会等の報告が行われました。次いで，今回総会に提案されている13案件について，それぞれ提案説明と質疑応答が行われました。午後からは，各部会が開催され，総会提案案件の審議及び各部会個別案件について審議が行われました。

総会2日目（26日）の午前は，前日提案された13案件のうち，9案件の審議・採択が順次行われました。

まず，「日本学術会議会則の一部を改正する規則」，「日本学術会議の運営の細則に関する内規の一部改正」，「日本学術会議の行う国際学術交流事業の実施に関する内規の一部改正」，「副会長世話担当研究連絡委員会の運営について（申合せ）の一部改正」及び「第16期における研究連絡委員会委員の在任期間等に関する規定の適用について（申合せ）」について一括して討論が行われ，採決の結果，いずれも可決されました。これらの会則，内規等の改正は，

1．運営審議会の構成員等の見直し

常置委員会と運営審議会の連絡を緊密にし，運営審議会の議論をより充実させるため，常置委員会委員長が常時運営審議会に出席することとし，併せて，運営審議会の構成員の見直しを行うこと。

2．第7常置委員会の設置及び第16期に向けての研連の見直し

国際対応委員会の改組について（申合せ）（平成5年4月22日第116回総会決定）に沿って第7常置委員会を設置し，併せて，各部等での検討結果を踏まえ，第16期へ向けての研連の見直しを行うこと。

3．研連委員の在任期間等関係

研連委員の在任期間に関する運営内規の解釈をより一層明確化するとともに，将来に向けての研連活動の継続的発展・活性化を図るため，研連委員の在任期間等についての関係規定を整備すること。

を趣旨とするものです。

次に，「運営審議会附置会員推薦手続検討委員会の設置」についての討論・採決が行われ，可決されました。これは，会員推薦制度導入以来，今回で4度目となり，会員推薦手続の過程において，幾つかの問題点がみられたことから，これらの諸問題について審議するため，新たな委員会を運営審議会に附置するものです。

続いて，「新しい方式の国際研究所の設立について（勧告）」，「公的機関の保有する情報の学術的利用について（要望）」，「女性科学研究者の環境改善の緊急性についての提言（声明）」についての討論・採決が行われ，可決されました。午後は，「第6常置委員会報告～国際学術交流・協力の飛躍的発展のために～」，「人口・食糧・土地利用特別委員会報告～21世紀の人口・食糧問題に対する全人類的取組に向けて～」，「学術国際貢献特別委員会報告～学術国際貢献のための新たなシステムについて～」及び「死と医療特別委員会報告～尊厳死について～」の4件の対外報告について討論が行われ，それぞれ承認されました。

総会3日目（27日）は，午前は各常置委員会及び国際対応委員会が，午後は各特別委員会がそれぞれ開催されました。

新しい方式の国際研究所の設立について（勧告）（抄）

近年，学術の国際交流がますます盛んになるとともに，新しい方式の研究所が世界の国々に設立されている。それらの新しさは，固有の研究員をほとんどもたず，国内外から招請した客員研究員による共同研究を企画し実行する点にある。この方式にふさわしい分野としては，自然科学のみならず，人文科学，社会科学を含め様々な領域が考えられるが，理論構築を主眼とする研究領域においては，研究テーマを学際的、機動的に選択する上で特に有効である。これは，また国を異にする若手研究者が相集い，生活と研究ないし研修を共にする場としても大きな効果を生むであろう。実際，世界的には，この意味で成果をあげている新研究所も少なくない。

さらに，いま国際貢献が基礎科学においても強く求められているが，それは，学術研究の推進と相互に強め合うべきものであって，このためにも新しい方式は最適である。

こうした観点から，新しい方式の国際研究所の設立が必要であり有用であるとの結論に達したので，ここにその設立を勧告する。

公的機関の保有する情報の学術的利用について（要望）（抄）

研究者が学術研究のために必要とする情報には、極めて広範囲なものが含まれており，その内容は，学問分野によっても多種多様である。学問分野によっては，公的機関の保有する情報が学術研究にとって極めて重要ないしは不可欠な意味をもつことになる場合も少なくないが，多くの場合に，かかる公的機関の保有する情報を学術情報として利用することには困難が伴っている。それは，公的機関の保有する情報の少なくない部分が公開されておらず，学術情報としての利用についてもその開示を求めることができないからである。

このような公的機関の保有する情報の学術的な利用のためにも，まず基本となるのは，国民の基本的な権利に基づく公的機関の保有する情報の公開制度である。この制度の確立によって，公的機関の保有する情報の学術情報としての利用も同時に保障されることになるからである。公的機関としては，国家機関及び地方公共団体機関を挙げることができるが，国家機関の保有する情報についての公開制度が設けられていないことは，学術研究にとっても特に重大な障害となっている。国民の「知る権利」を中心とする基本的権利を保障するための国家機関の保有する情報の公開制度は、学術研究にとっても極めて重要な意味をもっているということができる。国民の基本的な権利を保障するために，また学術研究の推進のためにも，原則公開を基本とし的確な内容を持つ国の情報公開制度の確立が不可欠であると考えられるので，ここに情報公開法の制定を要望する。

なお，公的機関の保有する情報の学術的利用については，情報の保存及び研究者による非公開情報の利用についての検討が必要である。

女性科学研究者の環境改善の緊急性についての提言（声明）（抄）

女性の社会的地位の向上を目指す取組が，国際的にも国内的にも種々行われているが，日本学術会議においても第10期及び第12期に女性科学研究者の地位の向上に関する「要望」を決議した。今期，すなわち第15期の発足に当たり，日本学術会議は「女性研究者の地位の向上」に留意することを再確認し，今期の活動計画の一つにこの課題を取り上げ審議してきた。その結果，女性科学研究者の地位の向上の必要性は理念的には一般化したものの，科学者全体の対応の遅れもあって，その地位は実質的に余り改善されていないことが明らかになった。

このため，特に基礎科学分野における科学研究者不足の事態が目前に迫っている現在，我が国における科学の調和のある発展のために，第10期，第12期での男女平等の視点を前提としつつ，日本学術会議は，改めて女性科学研究者の環境改善の緊急性を指摘するとともに，関係方面に環境改善の促進を強く訴えるものである。

「日本学術会議だより」について御意見，お問い合わせ等がありましたら，下記までお寄せください。
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日本学術会議広報委員会　電話03(3403)6291

日本学術会議だより　No.34

第16期最初の総会開催される

平成６年８月　日本学術会議広報委員会

日本学術会議の第16期が平成６年７月22日(金)からスタートし，７月25日から７月27日までの３日間，第119回総会が開催されました。今回の日本学術会議だよりでは，総会の概要等についてお知らせします。

日本学術会議第119回総会報告

平成６年７月22日から，第16期が開始されましたが，この第16期会員による最初の総会である，日本学術会議第119回総会が，７月25日から27日までの３日間にわたって開催されました。

初日（25日）の午前は，辞令交付式が，総理大臣官邸ホールで行われ，210名の会員のうち海外出張中等の22名を除く188名の会員が出席しました。式は，村山内閣総理大臣，五十嵐内閣官房長官，石原官房副長官，文田総理府次長等の出席を得て行われ，第１部から第７部までの全会員の名前が読み上げられた後，会員を代表して最年長である中田易直第１部会員が，村山内閣総理大臣から辞令を受け取りました。この後，村山内閣総理大臣が「会員の皆様には独創性豊かな学術研究の発展等のため，総合的観点に立って学術研究に係わる諸問題の解決に御尽力いただきたい」とあいさつし，これに応えて，中田易直第１部会員が「微力ながら全力を尽くし，重要な職責を全うし，国民の期待に応えたい」とあいさつしました。午後は，日本学術会議講堂において，総会が開催され，会長，副会長（２名）の互選が行われました。その結果，会長には，伊藤正男第７部会員が，人文科学部門の副会長には，利谷信義第２部会員が，自然科学部門の副会長には，西島安則第４部会員が，それぞれ選出され，伊藤会長及び利谷副会長（西島副会長は海外出張中）からそれぞれ就任のあいさつを行いました。続いて，各部会が開かれ，各部の部長，副部長及び幹事の選出等が行われました。（第16期の役員については，別掲を参照）

２日目（26日）は，午前10時から総会が開催され，近藤前会長が海外出張中のため代理として川田前副会長が第15期の総括的な活動報告を行い，続いて，会員推薦管理会報告として，久保亮五委員長の代理として高岡事務総長が，第16期会員の推薦を決定するまでの経過報告を行いました。引き続き，事務総長から第16期会員対して実施した「第16期の日本学術会議が取り組むべき課題について」のアンケートの結果について説明がありました。総会終了後は，各運営審議会附置委員会，各部会，各常置委員会等が開催されました。また，夕方には，総理大臣官邸ホールにおいて，村山内閣総理大臣主催の日本学術会議第16期会員との懇談会が初めて開催されました。懇談会は，村山内閣総理大臣のあいさつで開会し，五十嵐内閣官房長官の発声による乾杯，伊藤会長の答礼のあいさつの後，懇談に入りました。来賓として，与謝野文部大臣，田中科学技術庁長官，吉田農林水産政務次官，藤田日本学士院院長ほか大勢の方が出席され，あふれんばかりの人々で歓談が続き盛会となりました。

３日目（27日）は，午前10時から総会が開会され，会長から「第16期活動計画の作成について」の申合せ案について提案があり，原案どおり可決されました。続いて，第16期の活動計画についての自由討議が行われ，各部長から各部会での意見が披露されるなど活発な発言がありました。総会終了後は，地区会議合同会議，各運営審議会附置委員会，各常置委員会等が行われました。その後，運営審議会が開催され，第16期の活動計画の素案作成のために，運営審議会構成員の中から起草委員を選出し，審議に入りました。

第16期日本学術会議役員

会　長　　伊藤　正男（第７部・生理科学）
理化学研究所国際
フロンティア研究システム長
副会長　　利谷　信義（第２部・基礎法学）
お茶の水女子大学（生活科学）教授
副会長　　西島　安則（第４部・化学）
日本ユネスコ国内委員会会長

〔各部役員〕

第１部	部　長	中田　易直	（歴史学）
	副部長	戸川　芳郎	（哲学）
	幹　事	堀尾　輝久	（教育学）
	幹　事	森岡　清美	（社会学）
第２部	部　長	中山　和久	（社会法学）
	副部長	山口　　定	（政治学）
	幹　事	兼子　　仁	（公法学）
	幹　事	山中永之佑	（基礎法学）
第３部	部　長	柏崎利之輔	（経済政策）
	副部長	岡本　康雄	（経営学）
	幹　事	河野　博忠	（経済政策）
	幹　事	二神　恭一	（経営学）
第４部	部　長	伊達　宗行	（物理科学）
	副部長	竹内　郁夫	（生物科学）
	幹　事	井口　洋夫	（化学）
	幹　事	新藤　静夫	（地質科学）
第５部	部　長	内田　盛也	（応用化学）
	副部長	大橋　秀雄	（機械工学）
	幹　事	増子　　曻	（金属工学）
	幹　事	松尾　　稔	（土木工学）
第６部	部　長	志村　博康	（農業工学）
	副部長	北村貞太郎	（農業工学）
	幹　事	島田　淳子	（家政学）
	幹　事	平田　　熙	（農芸化学）
第７部	部　長	渥美　和彦	（内科系科学）
	副部長	金岡　祐一	（薬科学）
	幹　事	入江　　實	（内科系科学）
	幹　事	細田　泰弘	（病理科学）

〔常置委員会〕

第１常置　委員長	利谷　信義	（第２部）
第２常置　委員長	中塚　　明	（第１部）
第３常置　委員長	村上　英治	（第１部）
第４常置　委員長	増本　　健	（第５部）
第５常置　委員長	山中永之佑	（第２部）
第６常置　委員長	鹿取　廣人	（第１部）
第７常置　委員長	井口　洋夫	（第４部）

（注）カッコ内は，所属部・専門

第16期日本学術会議会員の概要について

この度任命された210人の第16期日本学術会議会員の概要を以下に紹介します。（カッコ内は第15期）

1　性別　　男性209人　　女性１人

2　年齢別

45～49歳	１人	50～54歳	３人
55～59歳	26人	60～64歳	93人
65～69歳	72人	70～74歳	12人
75～79歳	１人		

最年長　　75　歳（74　歳）
最年少　　47　歳（54　歳）
平均年齢　63.6歳（63.3歳）

3　勤務機関及び職名別

(1) 大学関係	国立大学		59人
	公立大学		２人
	私立大学		111人
	公私立短期大学		２人
	計		174人
(2) 国立私立試験研究機関・病院等			９人
(3) その他	法人・団体関係		５人
	民間会社		６人
	無職		14人
	その他		２人
	計		27人

4　その他の分類

(1) 前・元・新別	前会員		82人
	元会員		３人
	新会員		125人
(2) 地域別（居住地）			
	北海道		３人（ ５人）
	東　北		９人（ ８人）
	関　東		136人(133人)
	中　部		14人（ 19人）
	近　畿		41人（ 34人）
	中国・四国		３人（ ５人）
	九州・沖縄		４人（ ６人）

（注）詳細については，日本学術会議月報７月号を参照

「日本学術会議だより」について御意見，お問い合わせ等がありましたら，下記までお寄せください。

〒106 東京都港区六本木7-22-34
日本学術会議広報委員会　電話03(3403)6291

編集後記

このところ大学では，学部改組，教養部解体，大学院改組等が行われ，ここ数年間で大きく変革しようとしております．勿論，組織形態が変わっても内容はそれ程変化しようもないのですが，その度に会議に追われ，会議に出席するために大学に通っている感に陥ることも多々あります．早朝大学へ来て，実験室のインキュベーターから卵子の入っているディッシュを取り出し，顕微鏡下で発生している卵子の像をみると，何となく安らぎを覚える今日この頃です．真珠のように輝きながら，分裂を繰り返して発生を続けるこのちっぽけな細胞が，我々に何かを語りかけているように思えてなりません．卵子からのこのシグナルを聞き取りたいと思い，形態学的手法を駆使して研究しておりますが，未だ聞き取れないのが現状であります．微力ながらこの会誌を立派なものにしたいと思い，編集の手伝いをしている次第でありますので，宜しくお願い申し上げます．（新村末雄）

近年生殖医学領域ではassisted reproductive technolgy（ART）が花盛りである。本学会誌にもARTに関する多くの論文が投稿されている。不妊症治療や畜産の分野でARTが人類の幸福に多大な貢献を果たしてきたことは論を待たない。しかし自分自身を1個の生物としてとらえるとき、「生殖の無い性行為」を追求するcontraceptionや「性行為の無い生殖」を追求するARTが限りなく進歩していくことにはなんともいえない複雑な思いも感じる。reptrodutionの本質をもう一度原点に戻って考え直すことも必要なのかもしれない。（星　和彦）

哺乳動物卵子学会誌
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【効能・効果】

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【用法・用量】

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【使用上の注意】

1．**一般的注意**
1）本療法の対象は不妊症患者のうちの，間脳又は下垂体前葉の機能・器質的障害に由来する性腺刺激ホルモン低分泌無月経患者であるので次の点に注意すること．
ア．エストロゲン・プロゲステロンテストで初めて反応する第2度無月経又は抗エストロゲン療法（クエン酸クロミフェン，シクロフェニル等）が奏効しない第1度無月経の患者に投与すること．
イ．患者の状態（例えばエストロゲン・性腺刺激ホルモン・プレグナンジオール分泌，頸管粘液，基礎体温等）を詳細に検査し，子宮性無月経の患者には投与しないこと．
ウ．原発性卵巣不全による尿中性腺刺激ホルモン分泌の高い患者，副腎・甲状腺機能の異常による無月経患者，頭蓋内に病変（下垂体腫瘍等）を有する患者，及び無排卵症以外の不妊症患者には投与しないこと．
2）本療法の卵巣過剰刺激による副作用を避けるため，投与前及び治療期間中は毎日内診を行い，特に次の点に留意し，異常が認められた場合には，直ちに投与を中止すること．
ア．患者の自覚症状（特に下腹部痛）の有無
イ．卵巣腫大の有無
ウ．基礎体温の異常上昇の有無（毎日測定させること．）
エ．頸管粘液量とその性状
3）本療法による卵巣過剰刺激の結果として多胎妊娠が起こることがあるので，使用に際しては，その旨をあらかじめ患者に説明すること．
〔全国36病院における本療法による多胎妊娠についての調査で双胎以上の多胎妊娠は，妊娠総数454例中93例（20.5%）で，その内，双胎59例（13.0%），3胎20例（4.4%），4胎8例(1.8%)，5胎5例(1.1%)，6胎1例（0.2%）であったとの報告がある．〕
4）妊娠初期の不注意な投与を避けるため，投与前少なくとも1ヵ月間は基礎体温を記録させること．
5）産婦人科・内分泌専門医師の管理のもとに投与すること．

2．**次の場合には投与しないこと**
1）卵巣腫瘍及び多のう胞性卵巣症候群を原因としない卵巣の腫大を有する患者
2）妊婦

3．**次の患者には投与しないことを原則とするが，やむを得ず投与する場合には，慎重に投与すること**
1）児を望まない第2度無月経患者
2）多のう胞性卵巣を有する患者

4．**相互作用**
胎盤性性腺刺激ホルモンとの併用により，卵巣の過剰刺激による卵巣腫大，腹水・胸水を伴ういわゆるMeigs様症候群，ひいては血液濃縮，血液凝固能の亢進等があらわれることがある．

5．**副作用**
1）**卵巣過剰刺激**　卵巣腫大，下腹部痛，下腹部緊迫感，腹水・胸水を伴ういわゆるMeigs様症候群等があらわれた場合には，血液濃縮，血液凝固能の亢進等を併発することがあるので直ちに投与を中止し，循環血液量の改善につとめるなど適切な処置を行うこと．
2）**そ の 他**　ときに悪心，頻尿，しびれ感，頭痛，浮腫があらわれることがある．また尿量が増加することがある．

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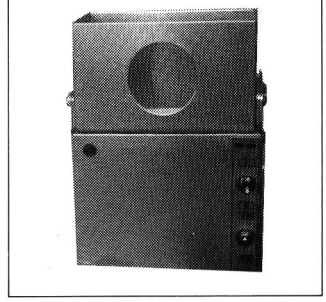

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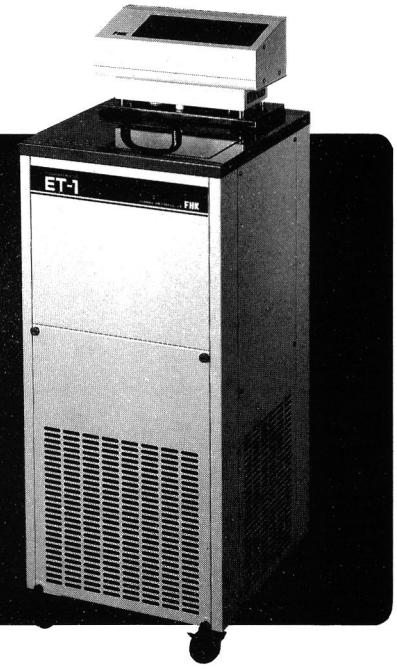

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