マウス体外受精胚におけるimprinted genes発現の検討

誌名	Journal of mammalian ova research = 日本哺乳動物卵子学会誌
ISSN	13417738
著者名	福田,雄介 片桐,由起子 森田,峰人
発行元	日本哺乳動物卵子学会
巻/号	27巻1号
掲載ページ	p. 51-57
発行年月	2010年4月

Investigation of Imprinted Gene Expression for In Vitro Fertilized Mouse Embryos

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Abstract: *It has been shown that epigenetic abnormalities are involved in both abnormal fetal development, as represented by large offspring syndrome, and the onset of diseases affecting vital prognosis, such as metabolic syndrome and malignancy. And if assisted reproductive technology (ART) causes epigenetic abnormalities, then this must be avoided. We compared the expression of imprinted genes (Igf2, Peg3, Snrpn and Kcnq1ot1), which are expressed in early embryos and are involved in fetal and placental development, using in vivo and in vitro mouse blastocysts. In order to compare the changes in gene expression under different culture conditions, in vitro embryos were incubated using two culture media (KSOM/AA and M16) and two gas phases (O_2 5% and 20%). Between in vivo embryos and KSOM/AA embryos, no marked differences were seen in gene expression. However, between in vivo embryos and M16 embryos, changes in gene expression patterns were confirmed. By changing the incubator's O_2 concentration, even when using the same culture medium, changes in imprinted gene expression were confirmed, clarifying that the expression of imprinted genes in early mouse embryos is related to culture medium components and oxygen concentration. It is important for ART to improve culture conditions.*

Key words: *Epigenetics, Imprinted gene expression, ART, Culture condition, O_2 concentration, Medium components, Mouse blastocyst*

Introduction

In Japan, one in 55 infants is currently born as a result of assisted reproductive technology (ART) treatments [1]. A background factors of this statistic is that the safety of ART is widely accepted, socially. Studies have reported that changes in epigenetics, as represented by imprinting, are seen more often in ART children than in children conceived naturally [2, 3]. Epigenetics refers to changes in protein synthesis and gene expression unaccompanied by changes in DNA sequences, and epigenetic changes are caused by changes in DNA methylation, histone modification, and so on. Imprinting is one of the mechanisms of epigenetics, and it involves both maternal and paternal allelic genes; while one is expressed, the other is inactivated for correct expression. Many imprinted genes play important roles in the process of embryonic development, placental function and neonatal development [4], and many reports have documented that ART is involved in the onset of diseases caused by imprinting disorders. Although diseases related to imprinting disorders are relatively rare, the incidence of such diseases is increasing [5–7]. Furthermore, abnormal fetal growth caused by imprinted gene expression, as represented by large offspring syndrome (LOS), has also been reported in humans [2, 3, 8]. It has been clarified that epigenetics are involved in both developmental abnormalities and diseases that markedly impact vital prognosis, such as metabolic syndrome and malignancy [9, 10]. Imprinted gene expression is specific to tissues and organs, and ontogenetic stage (Table 1). Because epigenetics are reprogrammed during gameteogenesis and early embryo development, ART can impact on various processes involving gametes and early embryos, such as ovarian stimulation, oocyte collection, sperm collection, fertilization, *in vitro* culture, embryo transfer and freezing/thawing.

It has been reported that ovarian stimulation lowers

Received: November 26, 2009
Accepted: December 11, 2009
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Table 1. Expression of imprinted genes at each stage

Imprinted genes	Location			
	Gamete	Early embryo	Fetus	Placenta
H19	○	○	○	○
Igf2	○	○	○	○
Ascl2			○	○
Peg3	○	○	○	○
Peg1	○		○	○
Cdkn1			○	○
Phlda2			○	○
Grb10	○		○	○
Snrpn	○	○	○	○
Kcnq1ot1	○	○	○	○

Imprinted gene expression is specific to tissues and organs, and ontogenetic stage.

egg quality and impairs the imprint maintaining mechanism in the embryonic development process [11]. It has also been reported that the process of sperm collection (differences in maturity based on the sperm origin) causes RNA changes [12], and that even among embryos at the same stage, gene expression varies, depending on the embryo stage at the time of embryo transfer and for culture time [13]. In addition, during the process of embryonic development, elevated protein synthesis from the fourth to the fifth day after hCG administration is thought to facilitate development to the blastocyst [14]. Therefore, it is possible that culture medium components affect embryonic development and can concurrently affect imprinted genes. Different culture media are known to cause abnormal imprinted gene expressions in fetal placentas; however, it is not clear when the expression of imprinted genes changes during the actual process of embryonic development. Thus, the present study analyzed and compared the expression of imprinted genes (*Igf2*, *Peg3*, *Snrpn* and *Kcnq1ot1*) that are expressed in early mouse embryos and are known to be involved in fetal and placental development using individual *in vivo* and *in vitro* mouse blastocysts.

Materials and Methods

Animals

Male and female ICR mice (Japan Charles River) were used. The female and male mice were 8 and 10 weeks of age, respectively.

Oocyte and embryo collection

For ovarian hyperstimulation, 7 IU of pregnant mare serum gonadotropin (PMSG) (Sigma G4877) was administered intraperitoneally to 8-week-old female mice, and after 48 hours, 7 IU of human chorionic gonadotropin (hCG) (Sigma C8534) was administered intraperitoneally. For *in vivo* blastocysts, after intraperitoneal administration 7 IU of hCG, one female mouse and one male mouse were placed in the same cage overnight to mate, and 72 hours later, blastocysts were collected from the uterus of the female mouse. For *in vitro* blastocysts, 7 IU of hCG was administered intraperitoneally, and 19 hours later, oocytes were collected from the fallopian tubes of female mice, and swim-up sperm were collected from the caudal area of the epididymis of male mice for *in vitro* fertilization. Blastocysts were obtained by incubating for 72 hours using two types of culture medium [KSOM/AA (MR-121-D) and M16 (Sigma M7292)] (Table 2) and two gas

Table 2. Components of each culture medium

Components (mg/L)	KSOM/AA	M16
$CaCl_2$-$2H_2O$	250	251.37
KCL	186.38	356.35
KH_2PO_4	47.99	162
$MgSO_4$ (anhyd)		164.9
$MgSO_4$-$7H_2O$	49.3	
NaCl	5551.8	553.193
$NaHCO_3$	2100.25	2101
EDTA	3.72	4.00
D-Glucose	36.03	1000
Sodium Lactate 60% (ml/L)	1121	2610
Sodium Pyruvate	22	36
BSA	1000	4000
Phenol Red		10
Amino Acids	466.51	

Components of each culture medium (KSOM/AA and M16).

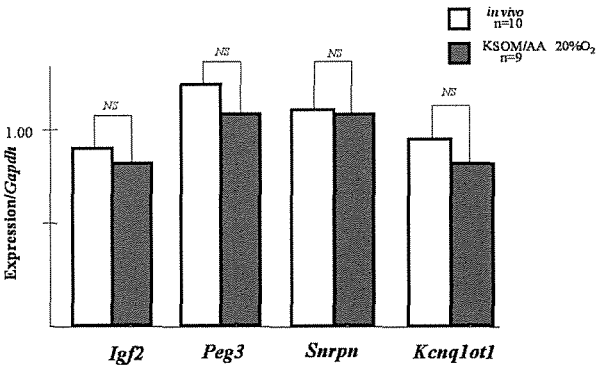

Fig. 1. *In vivo* and *in vitro* embryos (KSOM/AA 20% O_2). No significant differences were seen in the expression of each study gene between *in vivo* and *in vitro* embryos (KSOM/AA 20% O_2).

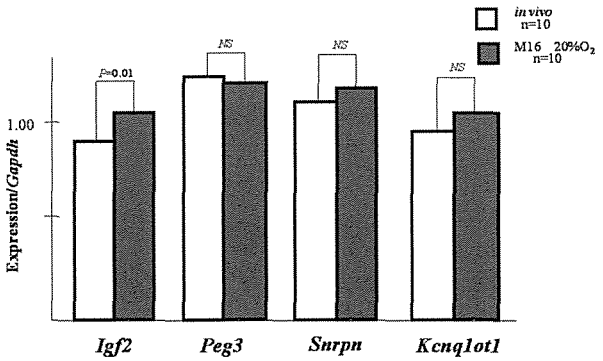

Fig. 2. *In vivo* and *in vitro* embryos (M16 20% O_2). Expression of *Igf2* was significantly higher by *in vitro* embryos.

phases (37°C under 5% CO_2, 20% O_2 and 75% N_2; or 37°C with 5% CO_2, 5% O_2 and 90% N_2) in an O_2/CO_2 incubator (SANYO O_2/CO_2 incubator MCO-175M). With regard to culture media, 20-μl drops were prepared on a culture dish (FALCON #353652), covered with mineral oil (Fisher Scientific O121-1) and used after stabilizing the gas phase inside the incubator for 12 hours. Ten fertilized embryos were incubated in each drop. To prevent culture medium degradation, embryos were transferred to another drop after 48 hours.

RNA extraction, cDNA synthesis, and gene expression analysis

Total RNA was extracted from individual blastocysts, and real-time PCR was performed. RNA was extracted using the Nanoprep Kit (Strategene #400753), and cDNA was synthesized using SuperScript III Platinum Two-Step (Invitrogen 11735-032) to investigate the expression of the following four imprinted genes, which are known to be involved in fetal and placental development: [*Igf2* (*insulin-like growth factor 2*, MGI:96434), *Peg3* (*paternally expressed 3*, MGI:104748), *Snrpn* (*small nuclear ribonucleoprotein N*, MGI: 98347) and *Kcnq1ot1*(*Kcnq1 overlapping transcript 1*, MGI:1926855)]. *Gapdh* (*glyceraldehyde-3-phosphate dehydrogenase*, MGI: 95640) served as a control gene. The two culture media (KSOM/AA and M16) were used under either a gas phase similar to the atmosphere (37°C under 5% CO_2, 20% O_2 and 75% N_2) or a gas phase similar to the fallopian tube (37°C under 5% CO_2, 5% O_2 and 90% N_2) [15] in order to analyze the expression of each imprinted gene. For statistical analysis, Mann-Whitney's U test was used.

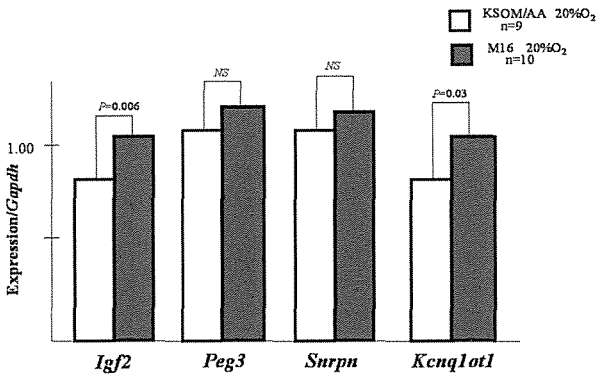

Fig. 3. Comparison of two *in vitro* culture media (KSOM/AA and M16 20% O_2). Expression of *Igf2* and *Kcnqlot1* by M16-treated embryos was significantly higher than that by KSOM/AA-treated embryos.

Results

When compared with *in vivo* embryos, no significant differences were seen in the expression of the study genes (*Igf2*, *Peg3*, *Snrpn* and *Kcnq1ot1*) in KSOM/AA 20% O_2-treated *in vitro* embryos (Fig. 1). The expression of *Igf2* was significantly elevated ($P < 0.05$) compared to *in vivo* embryos incubated in M16, but no significant differences were seen in the expressions of *Snrpn*, *Peg3* or *Kcnq1ot1* (Fig. 2). Between the two *in vitro* embryo cultures, expressions of *Igf2* and *Kcnq1ot1* were significantly higher for M16-cultured embryos ($P < 0.05$), but no significant differences were seen in the expressions of *Snrpn* and *Peg3* (Fig. 3).

Between the two gas phases (20% O_2 and 5% O_2), no significant differences were seen in the expression of any gene (*Igf2*, *Peg3*, *Snrpn* and *Kcnq1ot1*) in cultures

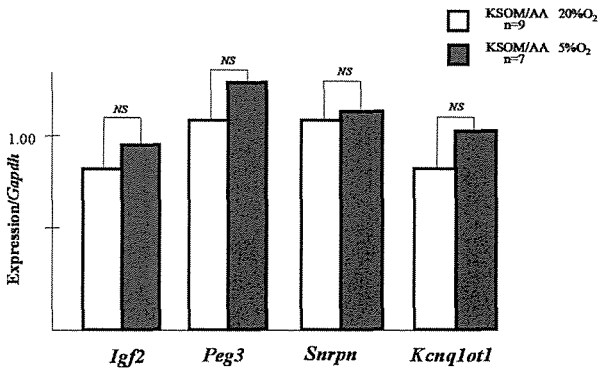

Fig. 4. Comparison between two *in vitro* gas phases (KSOM/AA). No significant differences were seen in the expressions of *Igf2*, *Peg3*, *Snrpn* and *Kcnq1ot1*.

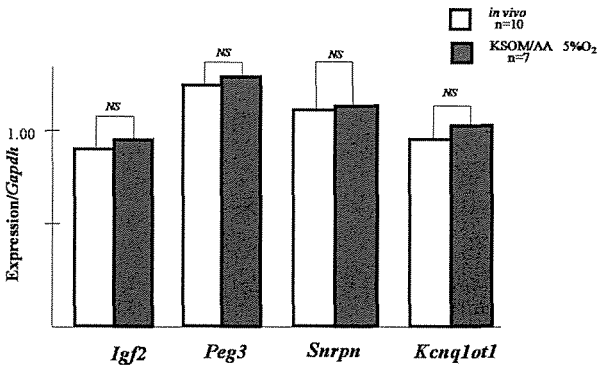

Fig. 5. *In vivo* and *in vitro* embryos (KSOM/AA 5% O_2). No significant differences were seen in the expression of *Igf2*, *Peg3*, Snrpn and *Kcnq1ot1*.

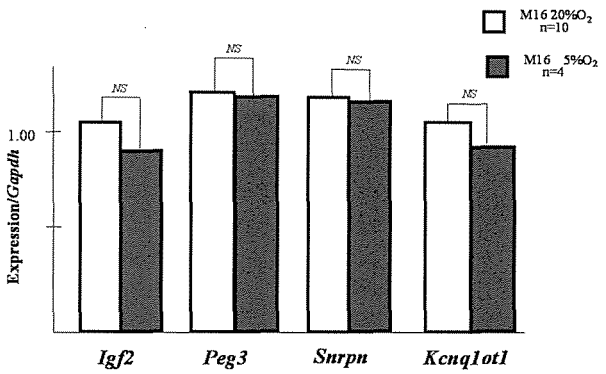

Fig. 6. Comparison between two *in vitro* gas phases (M16). No significant differences were seen in the expressions of *Igf2*, *Peg3*, Snrpn and *Kcnq1ot1*.

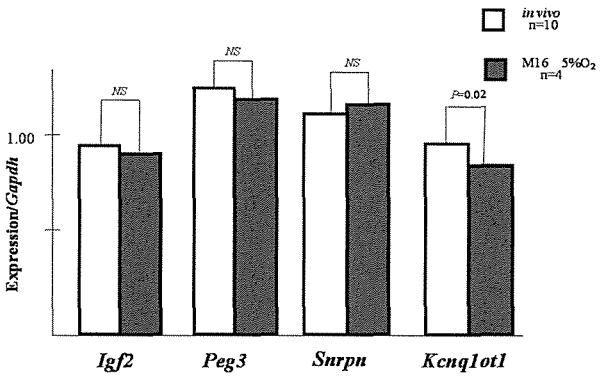

Fig. 7. *In vivo* and *in vitro* embryos (M16 5% O_2). *Kcnq1ot1* expression by *in vitro* embryos was lower than that by *in vivo* embryos.

with KSOM/AA (Fig. 4). In addition, no significant differences were seen in the expression of any gene (*Igf2*, *Peg3*, *Snrpn* and *Kcnq1ot1*) between *in vivo* embryos and KSOM/AA 5% O_2- cultured embryos (Fig. 5). Between 20% O_2 and 5% O_2, there was no significant differences in the expression of any gene (*Igf2*, *Peg3*, *Snrpn* and *Kcnq1ot1*) in cultures using M16 (Fig. 6). However, between *in vivo* embryos and M16 5% O_2-treated embryos, expression of *Kcnq1ot1* by the *in vitro* embryos was significantly lower ($P < 0.05$) (Fig. 7).

Compared with M16 which is an amino acid-free culture medium, changes in imprinted gene expression were smaller for the amino acid-enriched culture medium; KSOM/AA. When incubated in M16, changes in *Igf2* were confirmed in gas phases similar to atmosphere conditions (37°C, 5% CO_2, 20% O_2 and 75% N_2), and by incubating under gas phases similar to the fallopian tube (37°C, 5% CO_2, 5% O_2 and 90% N_2), changes in *Igf2* improved, but changes in *Kcnq1ot1* were observed.

Discussion

Many imprinted genes play an important role in the process of fetal development, placental development and neonatal development [4], and the number of reported imprinted genes is 119 in mice and 133 in humans [16]. In humans, it has been shown that changes in the expression of several imprinted genes are related to not only cardiovascular disease and glucose metabolism disorder, but also diseases closely related to vital prognosis, as represented by malignancy [8]. Furthermore, it has been reported that when compared with natural born children, the incidence of diseases caused by abnormal imprinted gene

expression is higher in ART children [3]. Furthermore, ART may increase the risk for abnormal imprinted gene expression, increasing the risk of Angelman syndrome or Beckwith-Wiedemann syndrome [2, 3, 5–7].

ART processes include ovarian stimulation, oocyte retrieval, sperm collection, fertilization, *in vitro* culture, embryo transfer and freezing/thawing, and these processes may affect epigenetics. However, as it is not clear when the expression of imprinted genes is affected during embryonic development, the present study investigated the expression of imprinted genes (*Igf2*, *Peg3*, *Snrpn* and *Kcnq1ot1*) that are expressed in early mouse embryos and are known to be involved in fetal and placental development using single mouse blastocysts collected from *in vivo* and *in vitro* embryos.

Amino acid-enriched KSOM/AA and amino acid-free M16 were used as *in vitro* culture media. No significant differences were seen in the embryonic expressions of imprinted genes in KSOM/AA cultures when compared to *in vivo* embryos (Fig. 1). However, the embryonic expression of *Igf2*, which is known to be a potent growth factor in fetuses [17], was significantly elevated ($P < 0.05$) in cultures using amino acid-free M16 (Fig. 2). The expression of *Igf2* at the blastocyst stage was significantly higher in M16 cultures than that of *in vivo* embryos. Between the two *in vitro* embryo cultures, the expressions of *Igf2* and *Kcnq1ot1*, which is paternally expressed, were significantly greater in the amino acid-free M16 [18] ($P < 0.05$) (Fig. 3).

Studies have reported that *Igf2*, *Igf2r* and *H19* show abnormal expression in overgrown placentas obtained from cloned mice [19] and *Igf2*-deficient mice exhibited delayed placental development and reduced placental transfer performance [20]. Therefore, amino acid-enriched KSOM/AA may better suppress abnormal imprinted gene expression at the blastocyst stage than amino acid-free M16. This result may provide additional support for the importance of amino acids in blastocyst development [21]. At the cellular level, it has been reported that the number of cells at the blastocyst stage is higher and vacuole formation is faster for amino acid-enriched KSOM/AA than for amino acid-free media [21], suggesting the effectiveness of KSOM/AA in embryonic development.

In another report, compare the two, M16 and *in vivo*, with *H19*, *Igf2*, *Grb10*, *Grb7* and *Mest* at fetus (Day 14), all imprinted gene expressions were not significantly different from those in control fetuses [22]. However, our result showed *Igf2* expression was increased in M16 culture. The difference of *Igf2* expression was caused by the stage of development (blastocyst and fetus). This may suggest that imprinted gene expression changes during development.

With regard to the effects of amino acids on early embryos, it is thought that an increase in protein synthesis from the fourth to the fifth day after hCG administration during the process of embryonic development facilitates development to blastocysts, and that protein synthesis requires amino acid uptake. When the amino acid changes in amino acid-enriched M16 were measured, the levels of seven amino acids (aspartate, arginine, glycine, alanine, isoleucine, leucine and lysine) were low on the fourth day and the levels of nine amino acids (aspartate, glutamate, asparagine, tyrosine, methionine, valine, phenylalanine, isoleucine and leucine) were low on the fifth day [14]. This suggests that aspartate, glutamate, arginine, isoleucine and leucine are important amino acids. In addition, after addition of amino acids to amino acid-free KSOM, blastocyst development rate, hatching rate and blastocyst count all increase [21]. These findings suggest that culture medium components, in particular the presence of amino acids, affect embryonic development, and that in early embryos, amino acids affect imprinted gene expressions at the blastocyst stage, which requires amino acids, resulting in abnormal *Igf2* and *Kcnq1ot1* expression. Although these results suggest that culture media affect imprinted gene expression, important culture conditions include both culture media and incubator gas phase, particularly oxygen concentration.

Oxygen concentration inside the fallopian tubes of mammals is 35–60 mmHg, and that inside the uterus is 11–14 mmHg. In rabbits and hamsters, the concentration of oxygen inside the uterus during implantation decreases to 5.3–3.5% [23]. Studies have shown that lowering the oxygen concentration in the gas phase of the incubator to a level comparable to that of the body improves embryonic development during embryo cluture, resulting in favorable blastocyst development rates and cell count [24, 25]. Therefore, we used two culture media, M16 and KSOM/AA, and incubated embryos under two gas phases, 20% O_2 and 5% O_2, in order to investigate the expression of *Igf2*, *Peg3*, *Snrpn* and *Kcnq1ot1*. However, no significant differences were seen with respect to culture media or oxygen concentration (Figs. 4, 6).

In the comparison between *in vivo* embryos and amino acid-free M16 5% O_2-treated embryos, no significant differences was seen in *Igf2* expression, which was higher/lower under 20% O_2, but the expression of *Kcnq1ot1* was significantly lower ($P <$

0.05) (Fig. 7). Abnormal *Igf2* expression was improved by lowering the oxygen concentration from 20% to 5%, but this induced abnormal *Kcnq1ot1* expression. This shows that low oxygen concentration is not necessarily beneficial, and oxygen concentrations of less than 7% can increase blastocyst development rate and cell count. In mice, however, the blastocyst development rate at 2% oxygen concentration is not higher than that of 7% or 20% oxygen concentration, while embryonic reactions vary at ≤ 5% oxygen concentration [26–28]. In addition, when incubating mouse embryos at 2% oxygen concentration, the expressions of *Slc2a1*, *Slc2a3* and *Vegf* (oxygen regulating genes) was 3 to 4 times higher than those at 7% or 20% oxygen concentration [27], suggesting that abnormal expression can be improved or exacerbated depending on the imprinted gene type.

Embryos incubated using amino acid-enriched KSOM/AA expressed imprinted genes at levels similar to *in vivo* embryos. Therefore, embryos cultured in amino acid-enriched KSOM/AA are appropriate from the viewpoint of gene expression. With regard to oxygen concentration, by creating an environment similar to the body, some abnormal imprinted gene expression was suppressed, but at the same time, new abnormal imprinted gene expression was seen. However, as mentioned above, interspecies differences in early embryo development are well known, and further investigations are needed to determine the effects on human embryos.

Of the imprinted genes investigated in the present study, *Snrpn* and *Peg3* was not affected by culture media or gas phases [29], demonstrating that not all imprinted genes are expressed abnormally at the blastocyst stage *Peg3* mutations caused growth delays [30]. These *Snrpn* and *Peg3* are affected by mRNA processing and defects and deficiencies in these genes cause Angelman syndrome and Prader-Willi syndrome. The results suggest the possibility that improved culture conditions, such as gas phase and culture media could result in fewer abnormalities at the cell and gene expression levels, thereby decreasing the incidence of large offspring syndrome (LOS), metabolic syndrome and malignancy associated with ART.

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8種類の合成培地を用いてマウス前胞状期卵胞の体外培養を行った．α-MEM，Waymouth，D-MEM/F-12，D-MEM（L；低濃度グルコース）での卵胞生存率と腔形成率は，D-MEM（H；高濃度グルコース），M199，IMDM，RPMI1640よりも有意に高かった．体外培養10日目の卵子径は，α-MEM（74.3 ± 1.1 μm）で最も大きく，Waymouth（66.9 ± 2.1 μm）では有意に小さかった．体外発育卵子の凝集核クロマチン率は，α-MEM（93%），D-MEM（L）（87%）で高く，Waymouth（37%）で低かった．さらに，hCG/EGF添加後のMII率は，α-MEM（66%）で最も高く，WaymouthではMII卵子が得られなかった．以上より，マウス前胞状期卵胞の体外培養に用いる合成培地は，卵胞生存および卵成熟に影響を及ぼすことが明らかとなった．

キーワード：前胞状期卵胞，体外発育，培養液，マウス

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マウス第2減数分裂中期（MII）卵子を卵丘細胞除去後体外培養でAgingさせ，紡錘体形態の変化と卵子の体外受精（IVF）後の発生能を比較し，さらに得られた胚盤胞の細胞数を計測し評価した．卵子をNon-aged区（対照区）と10，15，25時間Aged区の5区に分け，その外的形態変化を観察後，MII紡錘体の形態を免疫蛍光染色により観察した．正常形態の紡錘体は25時間Aged区で有意に減少したが，染色体の配列異常については，同区でも有意な増加は認められなかった．次に体外で15，16.5，18時間Agedさせた卵子を精巣上体尾部精子で体外受精させ，第2極体放出を確認した卵子を120時間体外で発生培養した．15時間区では受精率に差がなかったが，18時間区で有意に減少した．胚盤胞形成率は全てのAged区で対照区より有意に低かった．Aged卵子由来胚盤胞の細胞数は，対照区に比べ有意に少なかった．卵丘細胞除去卵子は15時間Aged後，受精能を有するものの胚発生能は顕著に減少することが明らかとなった．

キーワード：マウス卵子，*In vitro* aging，MII期紡錘体，体外受精，胚発生

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生殖補助医療（ART）がepigeneticsの変化に関与している懸念が報告された．epigenetics異常はLarge offspring syndromeに代表される胎仔発育異常やmetabolic syndrome，malignancyといった生命予後に影響する疾患に関与していることが明らかとなり，ARTがepigenetics異常に起因していれば回避を図らなくてはならない．そこでARTがepigeneticsに及ぼす影響を検討することを目的としてマウス初期胚において発現を認め，胎仔・胎盤発育への関与が知られているimprinted gene（*Igf2*，*Peg3*，*Snrpn*，*Kcnq1ot1*）の発現をマウス*in vivo*胚と*in vitro*胚で分析し比較検討した．また*in vitro*胚では培養環境によるimprinted gene発現変化を分析する目的で2種類の培養液（KSOM/AAとM16）と2種類の気相（O_2 5%と20%）で培養した．*In vivo*胚と*in vitro*胚（KSOM/AA）ではimprinted gene発現に変化は認められなかったが，*in vivo*胚と*in vitro*胚（M16）では変化を認め，気相を変えることで同じ培養液でも発現に変化を認め，imprinted gene発現に培養液組成および酸素濃度が関与することが明らかとなった．培養環境を整えていくことがARTにとって重要であると考えられる．

キーワード：エピジェネティクス，胚盤胞，遺伝子発現，培養環境