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DEVELOPMENT OF HAPLOID AND DIPLOID MOUSE PARTHENOGENONES: EFFECT OF OOCYTE AGING *IN VIVO*

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ABSTRACT

Mouse oocytes were collected 13.5–29.5 hr after hCG injection at 4 hr intervals, then activated with 7 % ethanol for 1 min. The oocytes collected 13.5 hr after hCG injection did not respond to ethanol activation. High activation rates (90.2–98.3 %) were obtained in oocytes activated 17.5–29.5 hr after injection with hCG. Haploid parthenogenones were dominant though the number decreased as the age of oocytes advanced (89.5 % to 42.0 %). The highest number of diploid parthenogenones (20.6 %) was obtained in oocytes activated 21.5 hr after hCG injection. The number of immediate cleavage and morphologically abnormal oocytes increased when the oocyte age progressed (0.7 % to 34.7 % and 1.1 % to 23.2 %, respectively). The percentage of parthenogenones developing to blastocysts decreased with the increase in oocytes age: haploid 51.9 % to 1.4 % and diploid 100.0 % to 83.3 %. The present study demonstrates that the developmental potentials of haploid and diploid parthenogenones derived from oocytes at 17.5 and 21.5 hr are higher than those derived from oocytes at 25.5 and 29.5 hr after hCG injection.

Key words: Parthenogenesis, oocyte aging, development, mouse

INTRODUCTION

Parthenogenones may be useful not only for the study of embryogenesis[15,18], but also in animal reproduction through the multiplication of embryos using the enucleated haploid or diploid parthenogenones as recipients in the nuclear transfer technique[4,19] or by using diploid parthenogenones aggregated with a single blastomere(s) from a normal embryo[14,20]. Thus, obtaining parthenogenones with high developmental potential may be advantageous for these embryo multiplication techniques.

The postovulatory aging of mouse oocytes affects the activation rate and the type

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of parthenogenone[6,21]. However, the developmental potential of parthenogenones derived from oocytes of various ages has not yet been described. The present study was carried out to determine the age at which the haploid or diploid parthenogenones with high developmental potential could be obtained from oocytes after activation with ethanol[1,8].

MATERIALS AND METHODS

Collection of Oocytes and Embryos

F1 (C57BL/6×CBA) female mice were superovulated by intraperitoneal injection of 5 IU PMSG (Serotropin, Teikoku Zoki, Japan) followed 48 hr later by 5 IU hCG (Gonatropin, Teikoku Zoki, Japan). The unfertilized oocytes surrounded by cumulus cells were collected by piercing the swollen ampullae of the oviducts in Whitten's medium[22] with 0.11 mM Na_2EDTA (WM+EDTA) under paraffin oil at 13.5, 17.5, 21.5, 25.5 and 29.5 hr after hCG injection. Since obtaining fertilized embryos with the same age at activation as that of parthenogenones was complicated, fertilized 1-cell embryos obtained by mating with F1 male mice were collected 21.5–25.5 hr after hCG injection to serve as a control by using the same procedure as described above.

Parthenogenetic Activation and Culture

The unfertilized oocytes were treated with 7 % ethanol (Kanto Chemical Co., Inc., Japan) in WM+EDTA for 1 min, washed several times in WM+EDTA and then cultured in WM+EDTA for 5–7 hr at 37 °C in 5 % CO_2 in air. The cumulus cells were removed by hyaluronidase (150 IU / ml, Type I-S, Sigma, USA). The rate of activation and the types of parthenogenones (haploid, diploid and immediate cleavage) were determined according to Kaufman[7]. The haploid and diploid parthenogenones were further cultured in drops of WM+EDTA covered with paraffin oil, in an atmosphere of 5 % CO_2 in air at 37 °C for 96–120 hr (diploid) or 120–144 hr (haploid).

After removal of the cumulus cells, fertilized 1-cell embryos were cultured in the same atmosphere as that of parthenogenones for 96 hr.

Cell Counts

The parthenogenones and fertilized embryos at the blastocyst stage were prepared using a modification of the method of Kamiguchi et al.[5]. The blastocysts were placed in 1 % sodium citrate for 5–15 min before being transferred to a methanol: acetic acid: distilled water (5 : 1 : 4) solution (fixative 1) for about 10 sec to remove the zona pellucida. Each zona-free blastocyst was placed on a clean glass slide with a small amount of fixative 1. Subsequently, a methanol: acetic acid (3 : 1) solution (fixative 2) was slowly added using micropipette positioned near the blastocyst-containing drop and left until the cells of the blastocyst began to loosen. At this time, a dropwise addition of fixative 2 replacing fixative 1 allowed the individul cells to spread out. A further 2–3 drops of fixative 2 were added, then the slide was air

dried and stained with 2 % Giemsa for 30 min.

Statistical Analysis

The results were obtained from 4 replicates. Statistical computations were made using ANOVA followed by Duncan's multiple range test.

Results

Activation Rate and Types of Parthenogenones

The results are summarized in Table 1. Unfertilized oocytes collected at 13.5 hr after hCG injection did not respond to ethanol activation. High activation rates were obtained in oocytes at 17.5–29.5 hr after hCG injection. Haploid parthenogenones were dominant though the number decreased as oocytes aged. The highest number of diploid parthenogenones was obtained when oocytes were activated 21.5 hr after hCG injection. At 29.5 hr after hCG injection, diploid parthenogenones were not obtained. The number of immediate cleavage parthenogenones increased as oocyte age advanced. Morphological abnormalities such as irregular immedite cleavage[9]and fragmentation increased significantly ($p<0.05$) in oocytes activated 29.5 hr after hCG injection.

Developmental Potential of Parthenogenones

The results are summarized in Table 2. The developmental rate to the blastocyst stage of haploid parthenogenones was not significantly different at 17.5 and 21.5 hr after hCG injection, then decreased significantly ($p<0.01$) at 25.5 and 29.5 hr after hCG injection. The cell number of blastocysts derived from haploid parthenogenones was not significantly different among any ages. Compared to the oocytes at 17.5 hr after hCG injection, the number of blastocysts derived from diploid parthenogenones decreased significantly ($p<0.05$) at 25.5 hr after hCG injection. The blastocyst cell number of diploid parthenogenones was observed to be higher at 21.5 hr than at 25.5 hr after hCG injection.

The developmental rate to the blastocyst stage of fertilized 1-cell embryos was 98.8 ± 0.5 % (mean$\pm$SEM, $n=98$) and the cell number was 77.8 ± 2.7 ($n=88$).

Discussion

The results demonstrated that the haploid and diploid parthenogenones derived from oocytes at 17.5 hr and 21.5 hr after hCG injection had better developmental rates to the blastocyst stage than the ones derived from oocytes 25.5 and 29.5 hr after hCG injection.

In previous studies[10,21], 22.7–45.6 % of freshly ovulated mouse oocytes (12–13 hr after hCG injection) could be activated by exposure to 7–8 % ethanol for 4.5–6.5 min. In this study, no activation occurred in oocytes collected 13.5 hr after hCG injection. This may have been because of the shorter exposure period to 7 % ethanol (1 min). However, the activation rate in oocytes from 17.5–29.5 hr after hCG injection was

high whether the exposure to ethanol was for 1 min (Table 1) or 4.5 min[21]. The length of exposure to ethanol affects the frequency of each type of parthenogenone[8,13], thus the slight difference in type frequency between this experiment and a previous study[21]may be due to the different exposure period. Nonetheless, the pattern of occurrence of each type of parthenogenone in relation to the age of oocytes was similar to that in the previous study[21].

Table 1. Effect of postovulatory aging of mouse oocytes on the activation rate and type of parthenogenone after exposure of oocytes to 7% ethanol for 1 min

Age of oocytes (hr after hCG)	No. of replicates (total no. of oocytes)	% activated (mean±SEM)	% type of parthenogenone [a] (mean±SEM)			
			H	D	IC	Ab
13.5	4 (226)	0	–	–	–	–
17.5	4 (349)	96.4±1.5[b,c]	89.5±2.5[b]	8.6±2.0[b]	0.7±0.7[b]	1.1±1.1[b]
21.5	4 (373)	96.9±0.6[b,c]	62.9±4.3[c]	20.6±3.8[c]	11.1±1.7[c]	5.3±1.7[b]
25.5	4 (411)	98.3±1.0[b]	56.9±6.6[c]	2.5±1.3[b]	33.4±5.5[d]	7.1±2.4[b]
29.5	4 (351)	90.3±4.5[c]	42.0±3.3[d]	0	34.7±3.3[d]	23.2±4.7[c]

[a] Based on the number of activated oocytes. H : haploid, D : diploid, IC : immediate cleavage, and Ab : morphologically abnormal oocytes.
[b,c,d] Values with different superscripts in the same column are significantly different ($P<0.05$).

The developmental potential of haploid parthenogenones is inferior to that of diploid parthenogenones in both cleavage rate and blastocyst development[2,3]. The presence of only one genetic component and an altered nuclear / cytoplasmic ratio may lead to a slower cleavage rate and a smaller number of blastocysts in haploid parthenogenones[3,4,11]. As for the effect oocyte age, the present results showed that aging the oocytes *in vivo* up to 21.5 hr after hCG injection (about 9.5 hr after ovulation) did not affect the developmental potential of either haploid or diploid parthenogenones (Table 2). This may be comparable with the results of Sakai and Endo[16], which suggest that the development from zygote to blastocyst seems to be unaffected by aging oocytes *in vivo* up to 12 hr after ovulation.

The effect of aging of oocytes on the number of cells at the blastocyst stage was not conclusive, because of the small number of blastocysts obtained. This was caused by the small number of parthenogenones that resulted after activation (diploid parthenogenones derived from oocytes at 17.5 and 25.5 hr after hCG injection) or developmental failure (haploid parthenogenones derived from oocytes at 25.5 and 29.5 hr after hCG injection).

In this experiment, the developmental potential of haploid and diploid parthenogenones derived from oocytes at 17.5 and 21.5 hr after hCG injection was considerably higher than in previous reports[17,19]. This may have been due to the

Table 2. Effect of postovulatory aging of mouse oocytes on the development of haploid and diploid parthenogenones

Type	Age of oocytes (hr after hCG)	No. of replicates (total no. of parthenogenones)	% blastocysts (mean ± SEM)	Cell number (mean ± SEM) (no. of blastocysts)
Haploid	17.5	4 (302)	$51.9 \pm 5.6^{a)}$	64.5 ± 1.5 (139)
	21.5	4 (226)	$36.7 \pm 8.2^{a)}$	59.5 ± 2.0 (77)
	25.5	4 (232)	$10.0 \pm 1.8^{b)}$	56.0 ± 4.1 (27)
	29.5	4 (135)	$1.4 \pm 0.9^{b)}$	63.4 ± 8.3 (7)
Diploid	17.5	4 (29)	$100.0 \pm 0.0^{c)}$	$59.1 \pm 2.7^{c,d)}$ (29)
	21.5	4 (76)	$92.8 \pm 2.7^{c,d)}$	$60.5 \pm 1.6^{d)}$ (65)
	25.5	4 (10)	$83.3 \pm 9.6^{d)}$	$51.1 \pm 6.5^{c)}$ (8)

[a,b)] Values with different superscripts are significantly different ($P<0.01$).
[c,d)] Values with different superscripts are significantly different ($P<0.05$).

shorter period of exposure to ethanol for oocyte activation, since ethanol can induce aneuploidy[13] and chromosomal damage[12]. Thus, reducing the exposure period but keeping it long for enough activation may be one factor that improves the development of parthenogenones.

The present study demonstrates that aging of oocytes up to 21.5 hr after hCG injection has no significant effect on the development of haploid and diploid parthenogenones. The study of parthenogenesis may be used to confirm the detrimental effect of aged oocytes on developmental failure.

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