CHAPTER III

RESULTS

Experiment I : Effects of PMSG and hCG concentration for the stimulation of immature female mice on *in vitro* oocyte maturation, fertilization, and preimplantation embryo development.

After culturing in maturation medium for 24 hours the percentage of denuded immature oocytes from mice primed with either 5, 7.5, or 10 IU PMSG developed to the MII stage were 67.0 ± 1.4, 69.5 ± 2.6, and 62.1 ± 1.8, while in cumulus cell-enclosed immature oocytes were 78.2 ± 3.1, 87.4 ± 2.4, and 74.6 ± 2.6, respectively (Table 3.1). The present study show that the highest percentage of the MII stage oocytes derived from 7.5 IU PMSG-primed mice ($P<0.05$). Therefore, 7.5 IU should be the suitable concentration of PMSG for the stimulation of immature female mice to obtain immature oocytes for the IVM/IVF study in the mouse model. In addition, in all PMSG-primed mice groups of this experiment the GV stage of cumulus cell-enclosed oocytes proceeded more readily to the MII stage in maturation medium than the GV stage of denuded oocytes.

A. 2-cell stage

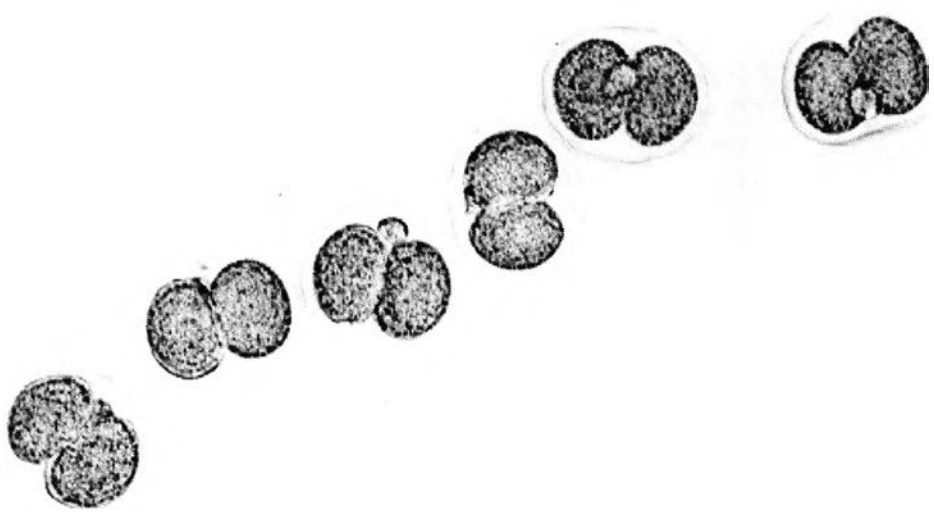
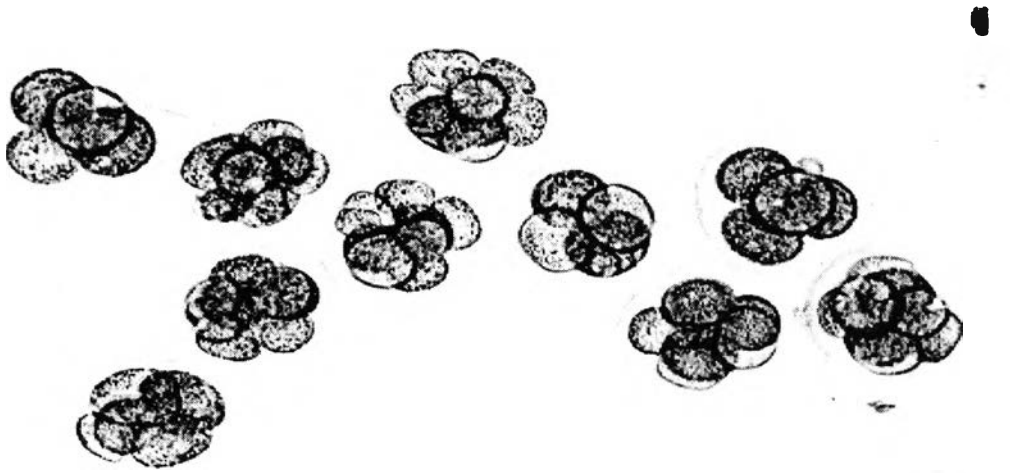


Figure 3.1 *In vitro* preimplantation mouse embryo development (x 200)

A. 2-cell stage ; B. 4- to 8-cell stage ; C. morula stage ;

D. early blastocyst stage ; and E. hatched blastocyst stage

B. 4- to 8-cell stage



C. morula stage

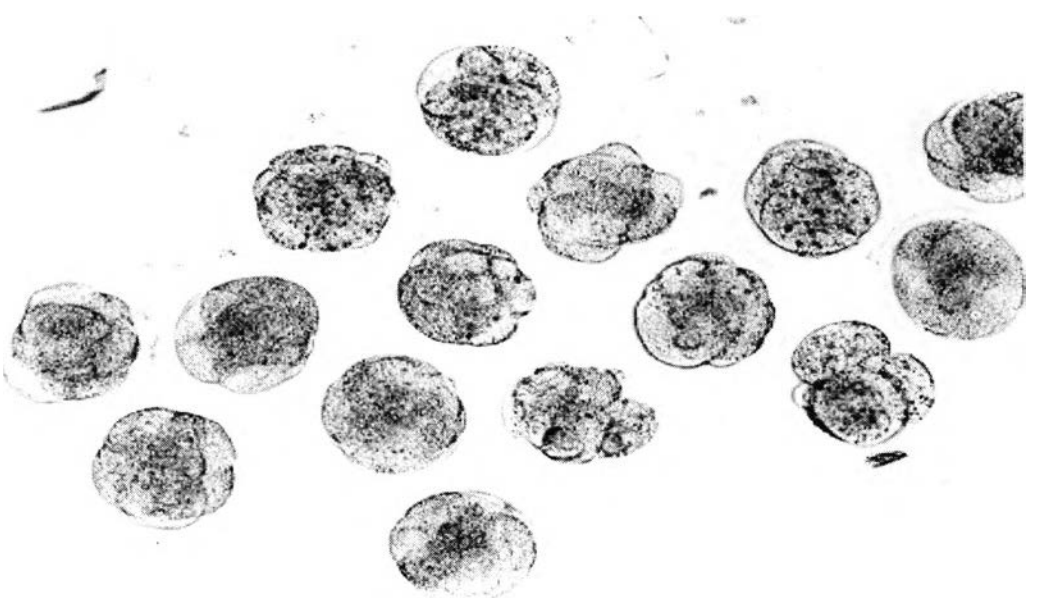
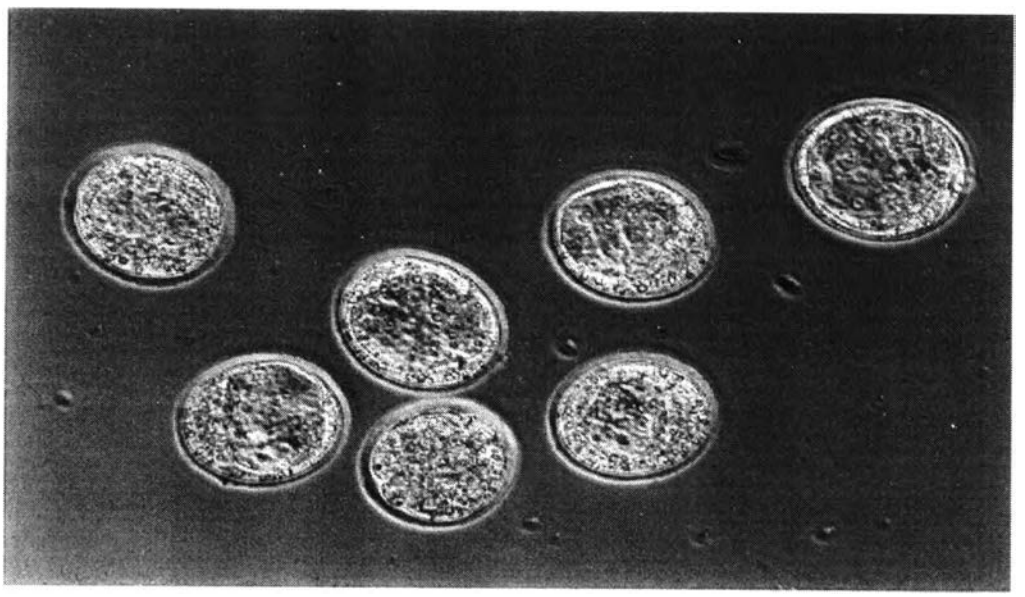


Figure 3.1 (continue) *In vitro* preimplantation mouse embryo development

D. early blastocyst stage



E. hatched blastocyst stage

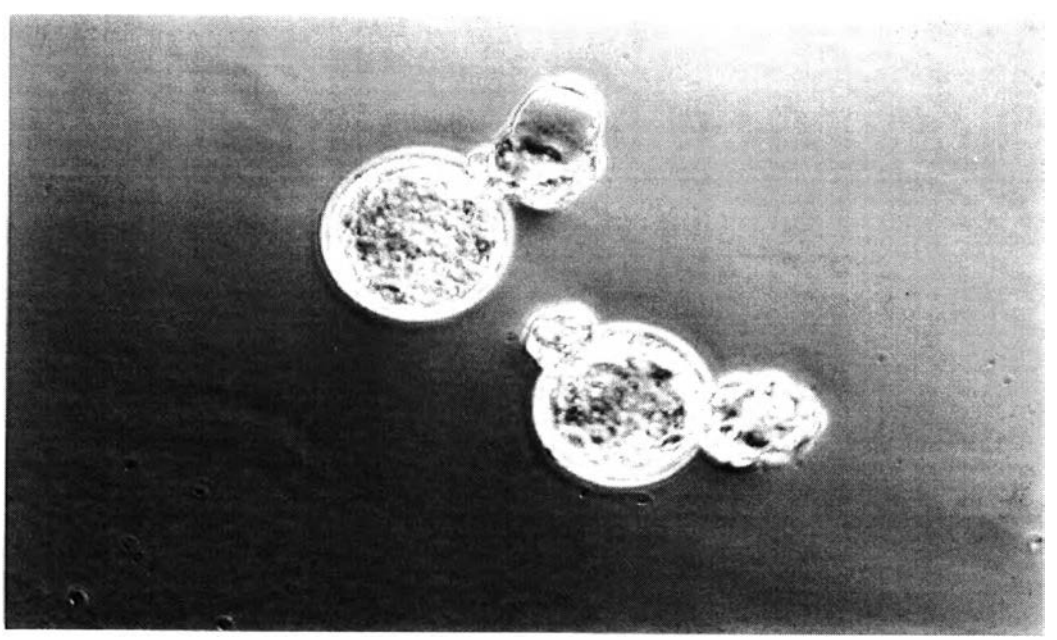


Figure 3.1 (continue) *In vitro* preimplantation mouse embryo development

The percentage of *in vitro* maturation of denuded immature oocytes from either 5, 7.5 or 10 IU hCG-primed mice upon culturing in maturation medium for 24 hours were 71.6 ± 2.1, 68.4 ± 4.4, and 69.3 ± 4.3, while in cumulus cell-enclosed immature oocytes were 77.6 ± 4.1, 86.3 ± 3.2, and 83.1 ± 2.7, respectively (Table 3.2). The present study shown that the highest percentage of *in vitro* maturation of oocytes obtained from 7.5 IU hCG-primed mice. Therefore, 7.5 IU should be the suitable concentration of hCG for the stimulation of immature female mice to obtain immature oocytes for the IVM/IVF study in the mouse model in this experiment, similar to the effect described for PMSG. In addition, the GV stage of cumulus cell-enclosed oocytes more readily proceeds to the MII stage in maturation medium than the GV stage of denuded oocytes, as has been observed with PMSG-primed mice. Comparison of the effect of various concentrations of PMSG and hCG on *in vitro* maturation of denuded and cumulus cell-enclosed oocytes has been presented in Figures 3.2 and 3.3, respectively.

However, to demonstrate the effect of PMSG on *in vivo* maturation of oocytes, immature female mice were injected with 7.5 IU PMSG (the suitable concentration in this study) and recorded the *in vivo* development and maturation of oocytes 12 - 60 hours after PMSG injection. The results from this experiment shown that PMSG has only a minimum effect on nuclear maturation of oocytes. Almost all of the oocytes obtained were identified as the GV stage not undergo to the GVBD or MII stage (Table 3.3).

Table 3.1 Percentage of the MII stage oocyte derived from *in vitro* maturation of denuded and cumulus cell-enclosed immature oocytes from either 5, 7.5, or 10 IU PMSG stimulated immature female mice. They were cultured in maturation medium at 37°C in a humidified atmosphere of 5%CO_2 in air for 24 hours.

Dose of PMSG	percent of MII stage oocytes (No. of oocytes)	
	denuded oocytes	cumulus cell-enclosed oocytes
5 IU	67.0 ± 1.4 (100/150)	78.2 ± 3.1 (117/150) [c]
7.5 IU	69.5 ± 2.6 (107/150) [a]	87.4 ± 2.4 (131/150) [b,c]
10 IU	62.1 ± 1.8 (93/150)	74.6 ± 2.6 (112/150) [c]

Data derived from fifteen replicate experiments and expressed as mean ± SD. A total of ten oocytes was screened per replicate experiment.

(a) significantly different ($P<0.05$) from 10 IU PMSG group : denuded oocytes
(b) significantly different ($P<0.05$) from 10 IU PMSG group : cumulus cell-enclosed oocytes
(c) significantly different ($P<0.05$) from denuded oocytes at the same PMSG concentration

Table 3.2 Percentage of the MII stage oocyte derived from *in vitro* maturation of denuded and cumulus cell-enclosed immature oocytes from either 5, 7.5, or 10 IU hCG stimulated immature female mice. They were cultured in maturation medium at 37°C in a humidified atmosphere of 5%CO_2 in air for 24 hours.

Dose of hCG	percent of MII stage oocytes (No. of oocytes)	
	denuded oocytes	cumulus cell-enclosed oocytes
5 IU	71.6 ± 2.1 (107/150) [a]	77.6 ± 4.1 (116/150)
7.5 IU	68.4 ± 4.4 (102/150)	86.3 ± 3.2 (129/150) [b,c]
10 IU	69.3 ± 4.3 (104/150)	83.1 ± 2.7 (125/150) [c]

Data derived from fifteen replicate experiments and expressed as mean ± SD. A total of ten oocytes was screened per replicate experiment.

(a) no significantly different ($P>0.05$) in all groups : denuded oocytes
(b) significantly different ($P<0.05$) from 5 IU hCG group : cumulus cell-enclosed oocytes
(c) significantly different ($P<0.05$) from denuded oocytes at the same hCG concentration

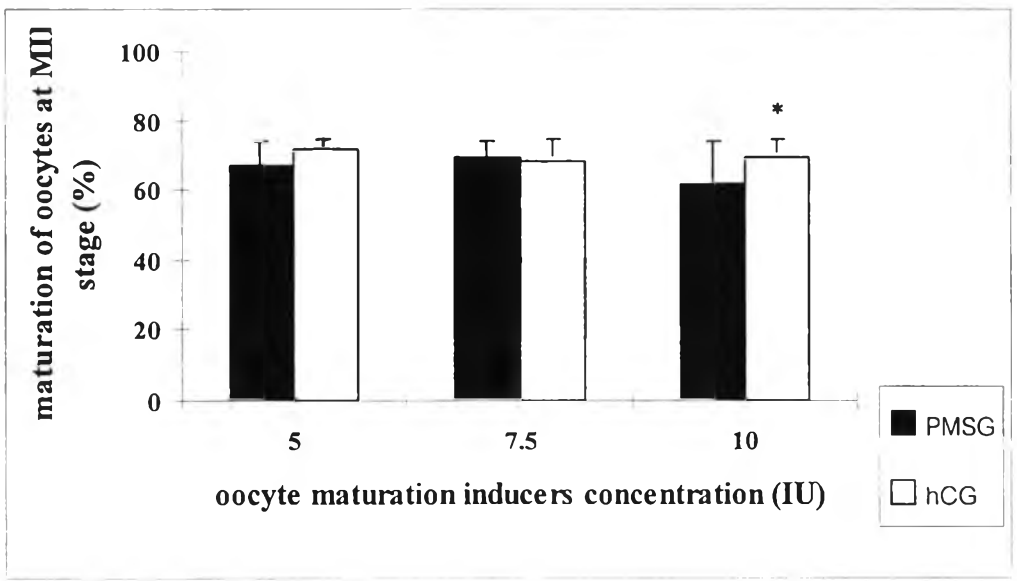


Figure 3.2 Comparison of the effect of either 5, 7.5, or 10 IU PMSG or hCG on *in vitro* maturation at the MII stage of denuded oocytes. (Data from Table 3.1 and 3.2)

(*) significantly different ($P<0.05$) from PMSG-primed mice.

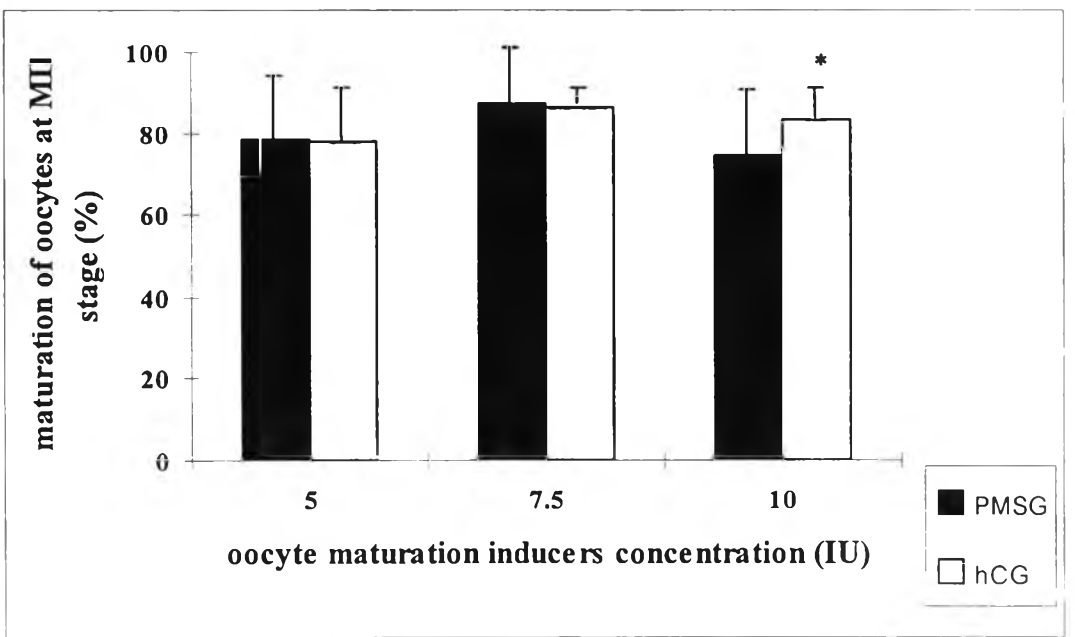


Figure 3.3 Comparison of the effect of either 5, 7.5, or 10 IU PMSG or hCG on *in vitro* maturation at the MII stage of cumulus cell enclosed oocytes. (Data from Table 3.1 and 3.2)

(*) significantly different ($P<0.05$) from PMSG-primed mice.

To demonstrate the effect of hCG on *in vivo* maturation of oocytes, immature female mice were injected with 7.5 IU hCG (the most suitable concentration in this study) and recorded the *in vivo* development and maturation of oocytes 12 - 60 hours after hCG injection. The results of this experiment show that *in vivo* development and maturation of oocytes 12-60 hours after hCG injection can undergo to the GVBD and MII stage (Table 3.4). These results indicated that hCG plays a more crucial role as to nuclear maturation of *in vivo* matured oocytes than PMSG.

The effect of PMSG on the *in vitro* fertilization rate of *in vitro* matured oocytes obtained from denuded and cumulus cell-enclosed oocytes is shown in Table 3.5. The *in vitro* fertilization rate of *in vitro* matured denuded oocytes obtained from mice primed with either 5, 7.5, or 10 IU PMSG amounted to 35.5 ± 4.6%, 37.0 ± 3.3%, and 38.0 ± 5.1%, respectively. In contrast, the *in vitro* fertilization rate of *in vitro* matured cumulus cell-enclosed oocytes obtained from mice primed with either 5, 7.5, or 10 IU PMSG were 42.5 ± 4.7%, 47.0 ± 3.4%, and 44.5 ± 4.3%, respectively (Table 3.5). In addition, the percentage of *in vitro* fertilization in PMSG-primed mice groups was significantly higher ($P < 0.05$) in cumulus cell-enclosed oocytes than in denuded oocytes.

The *in vitro* fertilization rate of *in vitro* matured denuded oocytes obtained from either 5, 7.5, or 10 IU hCG-primed mice were 39.0 ± 3.9%, 39.5 ± 2.0%, and 41.0 ± 4.4%, respectively (Table 3.6). In contrast, the *in vitro*

Table 3.3 Percentage of *in vivo* maturation of oocytes obtained 1 hour before and 12, 24, 36, 48, 50, 60, and 65 hours after 7.5 IU PMSG stimulation (n = 12)

Time (h)	*in vivo* maturation of oocytes at MII stage (%)		
	GV stage	GVBD stage	MII stage
1	80.3 ± 3.1	7.6 ± 6.2	-----
12	72.6 ± 5.4	11.5 ± 2.7	5.2 ± 3.8
24	78.0 ± 3.7	14.6 ± 5.1	-----
36	62.9 ± 6.2	23.5 ± 1.3	-----
48	79.6 ± 4.8	10.4 ± 3.7	1.3 ± 2.4
50	71.4 ± 1.9	16.3 ± 1.4	4.0 ± 2.9
60	66.6 ± 1.5	18.7 ± 2.7	-----
65	78.2 ± 2.6	17.3 ± 4.9	-----

Data derived from four replicate experiments and expressed as mean ± SD. A total of three mice was screened per replicate experiment.

Table 3.4 Percentage of *in vivo* maturation of oocytes obtained 1 hour before and 12, 24, 36, 48, 50, 60, and 65 hours after 7.5 IU hCG stimulation (n = 12)

Time (h)	*in vivo* maturation of oocytes at MII stage (%)		
	GV stage	GVBD stage	MII stage
1	72.3 ± 4.9	13.8 ± 7.3	2.1 ± 4.7
12	32.3 ± 5.7	51.5 ± 5.6	6.0 ± 4.1
24	36.0 ± 3.8	36.0 ± 2.9	12.0 ± 5.0
36	18.1 ± 6.4	67.8 ± 4.8	10.6 ± 2.8
48	4.5 ± 2.9	72.2 ± 3.7	18.1 ± 6.3
50	-----	70.5 ± 5.9	23.5 ± 9.2
60	-----	92.3 ± 6.1	-----
65	2.5 ± 1.1	88.4 ± 3.8	8.0 ± 5.6

Data derived from four replicate experiments and expressed as mean ± SD. A total of three mice was screened per replicate experiment.

Table 3.5 Percentage of *in vitro* fertilization of *in vitro* matured denuded and cumulus cell-enclosed oocytes obtained from either 5, 7.5, or 10 IU PMSG stimulated immature female mice after culturing in maturation medium for 24 hours.

Dose of PMSG	*in vitro* fertilization rate (No. of embryos)	
	denuded oocytes	cumulus cell-enclosed oocytes
5 IU	35.5 ± 4.6 (36/100) [a]	42.5 ± 4.7 (43/100) [c]
7.5 IU	37.0 ± 3.3 (37/100)	47.0 ± 3.4 (56/120) [b,c]
10 IU	38.0 ± 5.1 (30/80)	44.5 ± 4.3 (45/100) [c]

Data derived from four-five replicate experiments and expressed as mean ± SD.

A total of twenty oocytes was screened per replicate experiment.

(a) no significantly different ($P>0.05$) in all groups : denuded oocytes
(b) significantly different ($P<0.05$) from 5 IU PMSG group : cumulus cell-enclosed oocytes
(c) significantly different ($P<0.05$) from denuded oocytes at the same PMSG concentration

Table 3.6 Percentage of *in vitro* fertilization of *in vitro* matured denuded and cumulus cell-enclosed oocytes obtained from either 5, 7.5, or 10 IU hCG stimulated immature female mice after culturing in maturation medium for 24 hours.

Dose of hCG	*in vitro* fertilization rate (No. of embryos)	
	denuded oocytes	cumulus cell-enclosed oocytes
5 IU	39.0 ± 3.9 (31/80) [a]	43.0 ± 3.4 (43/100) [b]
7.5 IU	39.5 ± 2.0 (32/80)	45.5 ± 1.7 (46/100) [c]
10 IU	41.0 ± 4.4 (49/120)	38.5 ± 2.7 (31/80)

Data derived from at least four replicate experiments and expressed as mean ± SD. A total of twenty oocytes was screened per replicate experiment.

(a) no significantly different ($P>0.05$) in all groups : denuded oocytes
(b) no significantly different ($P>0.05$) in all groups : cumulus cell-enclosed oocytes
(c) significantly different ($P<0.05$) from denuded oocytes at the same hCG concentration

fertilization rate of *in vitro* matured cumulus cell-enclosed oocytes obtained from either 5, 7.5, or 10 IU hCG-primed mice were 43.0 ± 3.4%, 45.5 ± 1.7%, and 38.5 ± 2.7%, respectively (Table 3.6). In addition, comparison of the effect of various concentrations of PMSG and hCG on *in vitro* fertilization rate is shown in Figures 3.4 and 3.5.

The effect of PMSG on the blastocyst development of *in vitro* matured denuded and cumulus cell-enclosed oocytes obtained from the immature female stimulated with either 5, 7.5, or 10 IU PMSG and inseminated with sperm is shown in Table 3.7. After they were cultured in maturation medium under 37°C and a humidified mixture of 5%CO_2 in air for 24 hours the blastocyst development were 38.5 ± 4.1%, 41.0 ± 2.6%, and 43.0 ± 4.8% (for denuded oocytes), and 42.5 ± 3.7%, 45.0 ± 3.0%, and 37.5 ± 3.5% (for cumulus cell-enclosed), respectively. In addition, the effect of hCG on the blastocyst development of *in vitro* matured denuded and cumulus cell-enclosed oocytes obtained from the immature female mice stimulated with either 5, 7.5, or 10 IU hCG and inseminated with sperm is shown in Table 3.8. After they were cultured in maturation medium under 37°C and a humidified mixture of 5%CO_2 in air for 24 hours the blastocyst development were 42.5 ± 2.6%, 37.0 ± 3.8%, and 41.0 ± 2.1% (for denuded oocytes), and 39.5 ± 3.7%, 43.0 ± 4.4%, and 39.5 ± 4.7% (for cumulus cell-enclosed) respectively. Comparison of the effect of

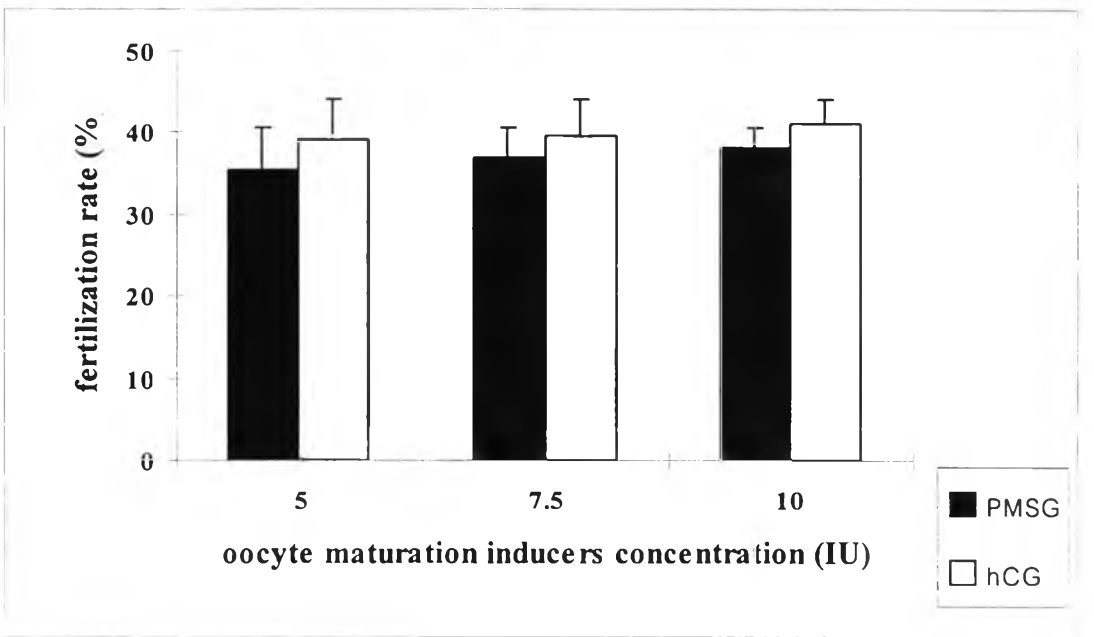


Figure 3.4 Comparison of the effects of either 5, 7.5, or 10 IU PMSG or hCG on the *in vitro* fertilization rate of *in vitro* matured denuded oocytes. (Data from Table 3.5 and 3.6)

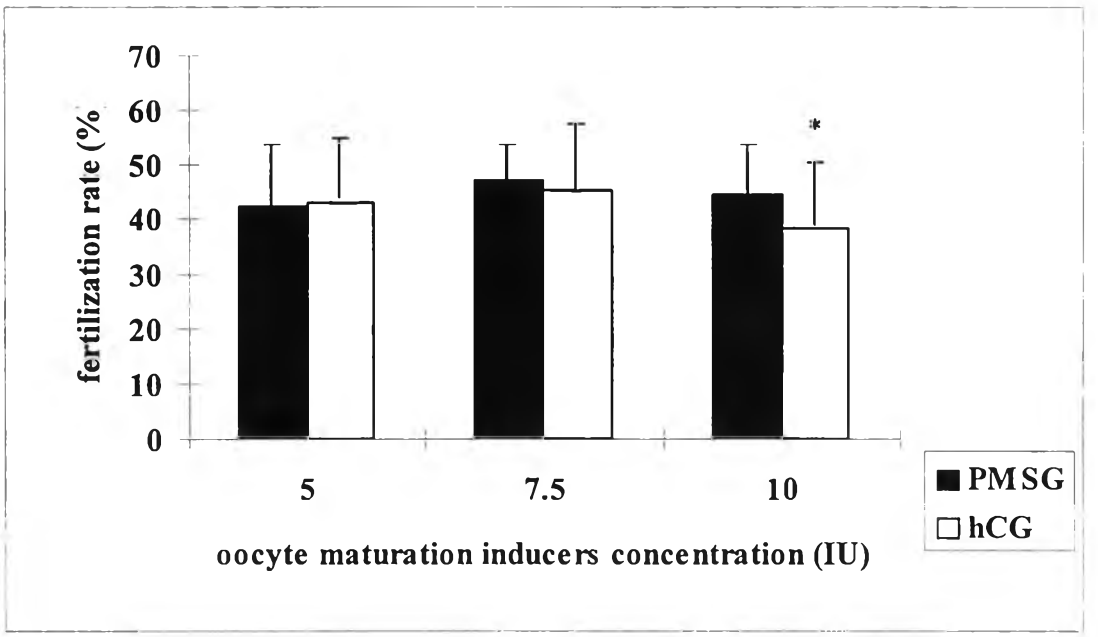


Figure 3.5 Comparison of the effects of either 5, 7.5, or 10 IU PMSG or hCG on the *in vitro* fertilization rate of *in vitro* matured cumulus cell enclosed oocytes. (Data from Table 3.5 and 3.6)

(*) significantly different ($P<0.05$) from PMSG-primed mice.

Table 3.7 Percentage of blastocyst development of *in vitro* matured denuded and cumulus cell-enclosed oocytes obtained from the immature female mice stimulated with either 5, 7.5, or 10 IU PMSG and inseminated with sperm. They were cultured in maturation medium for 24 hours.

Dose of PMSG	percent of blastocyst development (No. of embryos)	
	denuded oocytes	cumulus cell-enclosed oocytes
5 IU	38.5 ± 4.1 (39/100) [a]	42.5 ± 3.7 (51/120) [b]
7.5 IU	41.0 ± 2.6 (33/80)	45.0 ± 3.0 (36/80)
10 IU	43.0 ± 4.8 (52/120)	37.5 ± 3.5 (30/80)

Data derived from at least four replicate experiments and expressed as mean ± SD. A total of twenty oocytes was screened per replicate experiment.

(a) no significantly different (P>0.05) in all groups of denuded oocytes
(b) no significantly different (P>0.05) in all groups of cumulus cell-enclosed oocytes

Table 3.8 Percentage of blastocyst development of *in vitro* matured denuded and cumulus cell-enclosed oocytes obtained from the immature female mice stimulated with either 5, 7.5, or 10 IU hCG and inseminated with sperm. They were cultured in maturation medium for 24 hours.

Dose of hCG	percent of blastocyst development (No. of embryos)	
	denuded oocytes	cumulus cell-enclosed oocytes
5 IU	42.5 ± 2.6 (43/100) [a]	39.5 ± 3.7 (40/100) [b]
7.5 IU	37.0 ± 3.8 (30/80)	43.0 ± 4.4 (43/100)
10 IU	41.0 ± 2.1 (33/80)	39.5 ± 4.7 (40/100)

Data derived from four-five replicate experiments and expressed as mean ± SD.

A total of twenty oocytes was screened per replicate experiment.

(a) no significantly different (P>0.05) in all groups of denuded oocytes
(b) no significantly different (P>0.05) in all groups of cumulus cell-enclosed oocytes

various concentrations of PMSG and hCG on the blastocyst development is shown in Figures 3.6 and 3.7.

Experiment II : Effects of oocyte maturation duration on *in vitro* oocyte maturation, fertilization, and preimplantation embryo development in PMSG- primed and hCG-primed mice.

The results of experiments described above indicate that an oocyte maturation duration of 24 hours may not be suitable for the IVM/IVF studies in mouse model because it caused a significant decrease in the percentage of *in vitro* fertilization compared with that of *in vivo* matured oocytes from previous study (Tanphaichitr et al., 1993). Therefore, the effects of oocyte maturation duration on *in vitro* oocyte maturation, fertilization, and preimplantation embryo development in 7.5 IU PMSG-primed mice were examined in this experiment. Table 3.9 shows that *in vitro* oocyte maturation depended on the incubation time. When the *in vitro* oocyte maturation duration was extended from 3 to 24 hours, the percentage of oocyte maturation shown a significant increase ($P<0.05$) from 11.2 ± 3.7 to 78.2 ± 4.5, respectively.

The effect of oocyte maturation duration on *in vitro* oocyte maturation in 7.5 IU hCG-primed mice were examined in this experiment. Table 3.10 shows that *in vitro* oocyte maturation depended on the incubation duration as shown

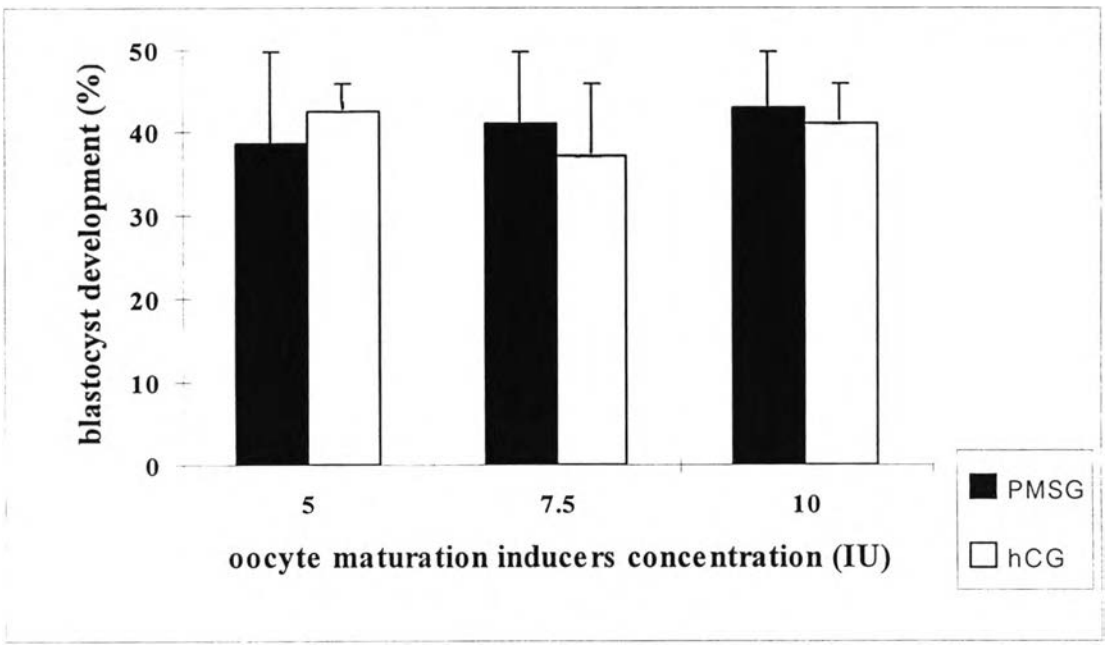


Figure 3.6 Comparison of the effects of either 5, 7.5, or 10 IU PMSG or hCG on the blastocyst development of 2-cell embryos were obtained from *in vitro* matured denuded oocytes. (Data from Table 3.7 and 3.8)

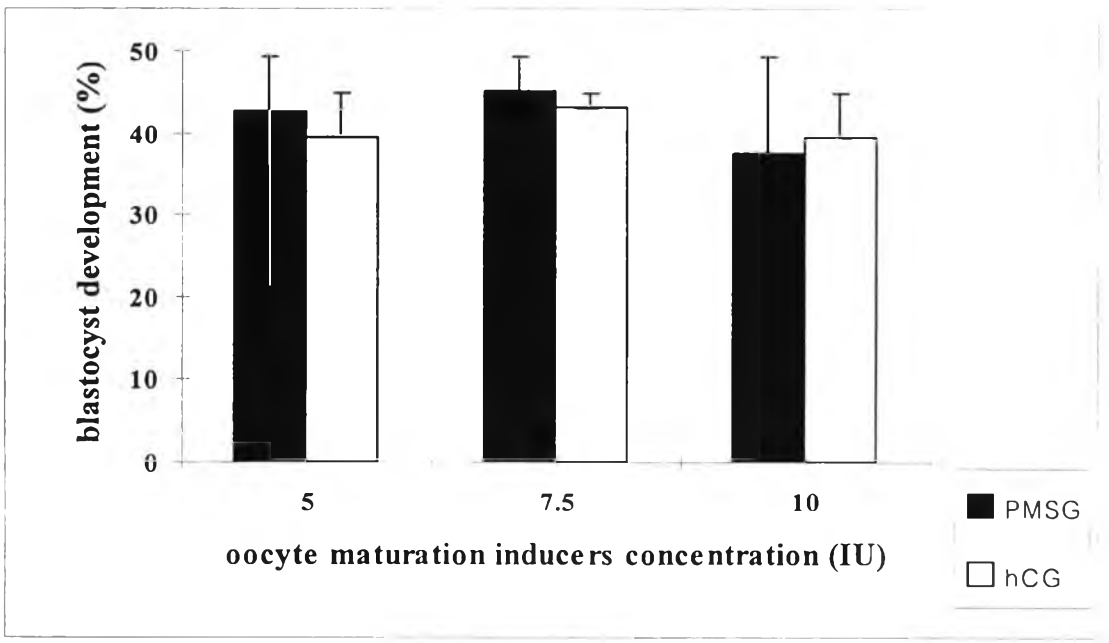


Figure 3.7 Comparison of the effects of either 5, 7.5, or 10 IU PMSG or hCG on the blastocyst development of 2-cell embryos were obtained from *in vitro* matured cumulus cell-enclosed oocytes. (Data from Table 3.7 and 3.8)

Table 3.9 Percentage of *in vitro* maturation of cumulus cell-enclosed immature oocytes at the MII stage obtained 48 hours after priming of mice with 7.5 IU PMSG and culturing in maturation medium for 3 - 24 hours.

Time (h)	percent of oocytes at the MII stage (No. of oocytes)
3	11.2 ± 3.7 (34/300)
6	22.5 ± 2.9 (68/300) [a]
9	46.2 ± 2.3 (139/300) [a,b]
12	70.0 ± 5.7 (140/200) [a,b,c]
15	76.4 ± 3.2 (153/200) [a,b,c]
18	80.0 ± 1.9 (160/200) [a,b,c,d]
21	84.1 ± 2.7 (168/200) [a,b,c,d]
24	78.2 ± 4.5 (156/200) [a,b,c,d]

Data derived from twenty-thirty replicate experiments and expressed as mean ± SD. A total of ten oocytes was screened per replicate experiment.

(a) significantly different ($P<0.05$) from the No. of oocytes cultured in medium for 3 hours.
(b) significantly different ($P<0.05$) from the No. of oocytes cultured in medium for 6 hours.
(c) significantly different ($P<0.05$) from the No. of oocytes cultured in medium for 9 hours.
(d) significantly different ($P<0.05$) from the No. of oocytes cultured in medium for 12 hours.

Table 3.10 Percentage of *in vitro* maturation of cumulus cell-enclosed immature oocytes at the MII stage obtained 48 hours after priming of mice with 7.5 IU hCG and culturing in maturation medium for 3 - 24 hours.

Time (h)	percent of oocytes at MII stage (No. of oocytes)
3	36.4 ± 2.1 (109/300)
6	57.6 ± 3.4 (173/300) [a]
9	78.1 ± 2.7 (156/200) [a,b]
12	83.1 ± 4.2 (166/200) [a,b]
15	87.6 ± 3.4 (175/200) [a,b,c]
18	76.8 ± 1.3 (154/200) [a,b]
21	81.0 ± 1.9 (162/200) [a,b]
24	84.7 ± 3.2 (169/200) [a,b]

Data derived from twenty-thirty replicate experiments and expressed as mean ± SD. A total of ten oocytes was screened per replicate experiment.

(a) significantly different ($P<0.05$) from the No. of oocytes cultured in medium for 3 hours.
(b) significantly different ($P<0.05$) from the No. of oocytes cultured in medium for 6 hours.
(c) significantly different ($P<0.05$) from the No. of oocytes cultured in medium for 9 hours.

for PMSG-primed mice. When the *in vitro* oocyte maturation duration was extended from 3 to 24 hours, the percentage of oocyte maturation shown a significant increase ($P<0.05$) from 36.4 ± 2.1 to 84.7 ± 3.2, respectively. Comparison of the effects of the oocyte maturation duration on *in vitro* oocyte maturation in hCG-primed mice and PMSG-primed mice is shown in Figure 3.8.

The percentage of *in vitro* fertilization in cumulus cell-enclosed oocytes obtained from 7.5 IU PMSG-primed mice and cultured in maturation medium for 3, 6, 9, 12, 15, 18, 21, and 24 hours was 41.0 ± 3.7, 43.5 ± 5.4, 48.0 ± 4.1, 55.5 ± 6.2, 51.0 ± 4.7, 48.0 ± 3.7, 42.5 ± 5.1, and 43.5 ± 4.4, respectively (Table 3.11). These data suggested that the optimum *in vitro* oocyte maturation duration for IVM/IVF studies in mice was around 15 hours.

The percentage of *in vitro* fertilization in cumulus cell-enclosed oocytes obtained from 7.5 IU hCG-primed mice and cultured in maturation medium for 3, 6, 9, 12, 15, 18, 21, and 24 hours was 38.0 ± 2.4, 46.5 ± 3.1, 56.5 ± 4.8, 54.0 ± 4.4, 65.5 ± 3.4, 54.5 ± 4.9, 41.0 ± 6.3, and 42.0 ± 3.7, respectively (Table 3.12). These data suggested that the optimum *in vitro* oocyte maturation duration for the IVM/IVF studies in hCG-primed mice was around 15 hours as observed with PMSG-primed mice. Comparison of the effects of oocyte maturation duration on the *in vitro* fertilization rate in hCG-primed mice and PMSG-primed mice is shown in Figure 3.9.

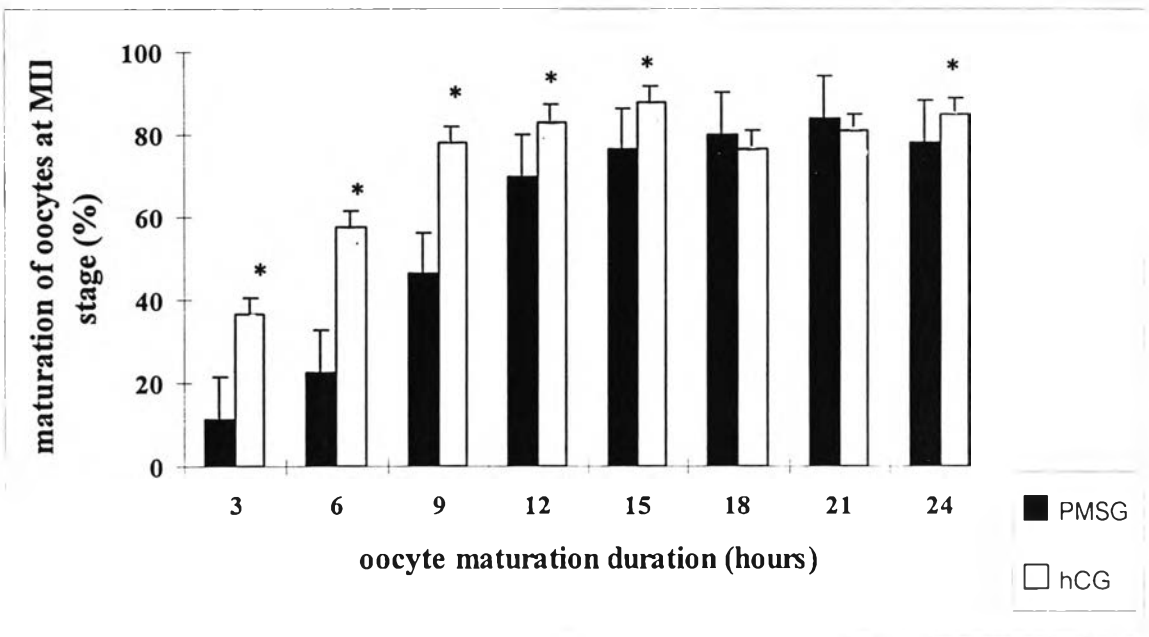


Figure 3.8 Comparison of the effect of *in vitro* oocyte maturation duration, 3-24 hours, on *in vitro* maturation of oocytes at the MII stage. The cumulus cell-enclosed immature oocytes were obtained from 7.5 IU PMSG- or 7.5 IU hCG-primed mice and cultured in maturation medium for 3 - 24 hours. (Data from Table 3.9 and 3.10)

(*) significantly different ($P<0.05$) from PMSG-prime mice.

Table 3.11 Percentage of *in vitro* fertilization rate of *in vitro* matured oocytes obtained from 7.5 IU PMSG-primed mice and cultured in maturation medium for 3 - 24 hours.

Time (h)	*in vitro* fertilization rate (No. of embryos)
3	41.0 ± 3.7 (33/80)
6	43.5 ± 5.4 (44/100)
9	48.0 ± 4.1 (58/120) [a]
12	55.5 ± 6.2 (67/120) [a,b]
15	51.0 ± 4.7 (51/100) [a,b]
18	48.0 ± 3.7 (58/120) [a]
21	42.5 ± 5.1 (43/100)
24	43.5 ± 4.4 (52/120)

Data derived from at least four replicate experiments and expressed as mean ± SD. A total of twenty oocytes was screened per replicate experiment.

(a) significantly different ($P<0.05$) from the No. of oocytes cultured in medium for 3 hours.
(b) significantly different ($P<0.05$) from the No. of oocytes cultured in medium for 6 hours.

Table 3.12 *In vitro* fertilization rate of *in vitro* matured oocytes obtained from 7.5 IU hCG-primed mice and cultured in maturation medium for 3 - 24 hours.

Time (h)	*in vitro* fertilization rate (No. of embryos)
3	38.0 ± 2.4 (38/100)
6	46.5 ± 3.1 (56/120) [a]
9	56.5 ± 4.8 (68/120) [a,b]
12	54.0 ± 4.4 (65/120) [a]
15	65.5 ± 3.4 (79/120) [a,b,c]
18	54.5 ± 4.9 (65/120) [a]
21	41.0 ± 6.3 (49/120) [c]
24	42.0 ± 3.7 (50/120) [c]

Data derived from five-six replicate experiments and expressed as mean ± SD.

A total of twenty oocytes was screened per replicate experiment.

(a) significantly different ($P<0.05$) from the No. of oocytes cultured in medium for 3 hours.
(b) significantly different ($P<0.05$) from the No. of oocytes cultured in medium for 6 hours.
(c) significantly different ($P<0.05$) from the No. of oocytes cultured in medium for 9 hours.

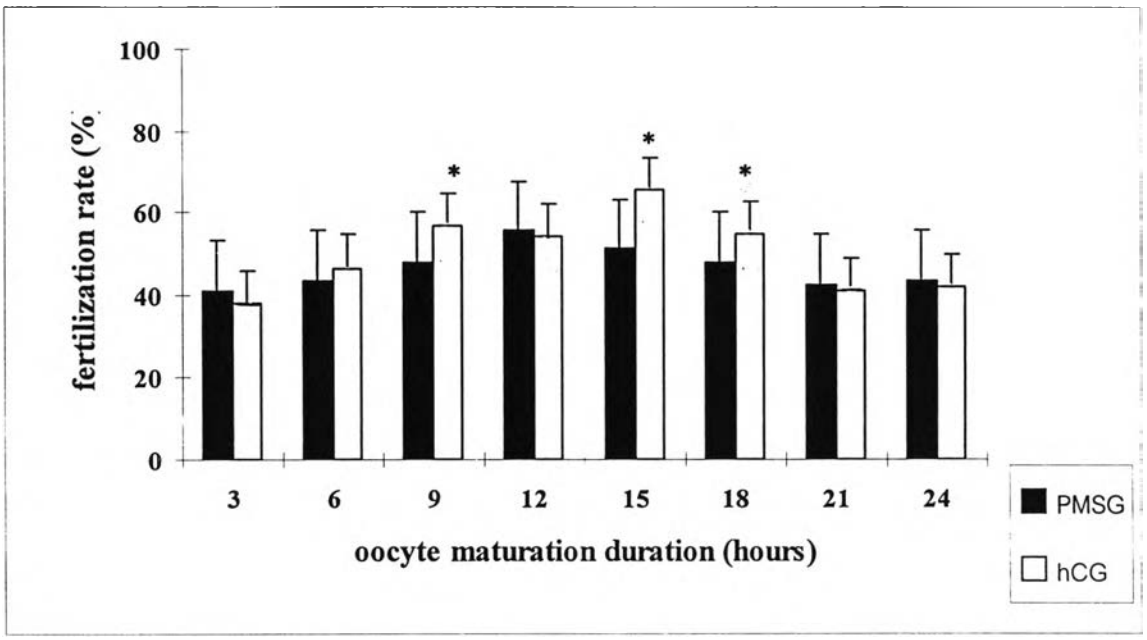


Figure 3.9 Comparison of the effects of *in vitro* oocyte maturation duration, 3-24 hours, on the *in vitro* fertilization rate of *in vitro* matured oocytes. The cumulus cell-enclosed immature oocytes were obtained from 7.5 IU PMSG- or 7.5 IU hCG-primed mice and cultured in maturation medium for 3 - 24 hours. (Data from Table 3.11 and 3.12)

(*) significantly different ($P<0.05$) from PMSG-prime mice.

The effect of oocyte maturation duration on preimplantation embryo development in 7.5 IU PMSG-primed mice is showed in Table 3.13. Two-cell embryos obtained after fertilization of oocytes matured *in vitro* in various durations (3, 6, 9, 12, 15, 18, 21, and 24 hours) and cultured in T6 medium for 120 hours show the percentage of blastocyst development as 41.3 ± 3.6, 44.6 ± 1.8, 47.1 ± 4.2, 51.4 ± 2.7, 57.6 ± 3.1, 52.3 ± 2.7, 54.6 ± 4.5, and 46.1 ± 4.4, respectively.

The effect of oocyte maturation duration on preimplantation embryo development in 7.5 IU hCG-primed mice is showed in Table 3.14. Two-cell embryos obtained after fertilization of oocytes matured *in vitro* in various durations (3, 6, 9, 12, 15, 18, 21, and 24 hours) and cultured in T6 medium for 120 hours show the percentage of blastocyst development as 43.6 ± 2.4, 47.4 ± 1.8, 53.0 ± 3.6, 58.3 ± 3.7, 64.4 ± 2.5, 59.7 ± 4.1, 46.3 ± 3.0, and 43.2 ± 4.4, respectively. In addition, the percentage of blastocyst development in hCG-primed mice was higher ($P<0.05$) than that in PMSG-primed mice after 6 hours of *in vitro* oocyte maturation as presented in Figure 3.10.

Table 3.13 Percentage of blastocyst development of 2-cell embryos obtained after each incubation time of *in vitro* matured oocytes and inseminated with PGC sperm after priming of mice with 7.5 IU PMSG. They were cultured in T6 medium for 120 hours after incubation.

Time (h)	percent of blastocyst development (No. of embryos)
3	41.3 ± 3.6 (39/94)
6	44.6 ± 1.8 (48/108)
9	47.1 ± 4.2 (53/112) [a]
12	51.4 ± 2.7 (70/136) [a]
15	57.6 ± 3.1 (63/109) [a,b,c]
18	52.3 ± 2.7 (65/124) [a,b]
21	54.6 ± 4.5 (61/112) [a,b,c]
24	46.1 ± 4.4 (64/138)

Data derived from at least four replicate experiments and expressed as mean ± SD. A total of twenty oocytes was screened per replicate experiment.

(a) significantly different ($P<0.05$) from the No. of oocytes cultured in medium for 3 hours.
(b) significantly different ($P<0.05$) from the No. of oocytes cultured in medium for 6 hours.
(c) significantly different ($P<0.05$) from the No. of oocytes cultured in medium for 9 hours.

Table 3.14 Percentage of blastocyst development of 2-cell embryos obtained after each incubation duration of *in vitro* matured oocytes and inseminated with PGC sperm after priming of mice with 7.5 IU hCG. They were cultured in T6 medium for 120 hours after incubation.

Time (h)	percent of blastocyst development (No. of embryos)
3	43.6 ± 2.4 (48/111)
6	47.4 ± 1.8 (46/97)
9	53.0 ± 3.6 (71/134) [a]
12	58.3 ± 3.7 (63/108) [a,b]
15	64.4 ± 2.5 (91/142) [a,b,c]
18	59.7 ± 4.1 (78/136) [a,b]
21	46.3± 3.0 (56/121)
24	43.2 ± 4.4 (47/109)

Data derived from at least four replicate experiments and expressed as mean ± SD. A total of twenty oocytes was screened per replicate experiment.

(a) significantly different ($P<0.05$) from the No. of oocytes cultured in medium for 3 hours.
(b) significantly different ($P<0.05$) from the No. of oocytes cultured in medium for 6 hours.
(c) significantly different ($P<0.05$) from the No. of oocytes cultured in medium for 9 hours.

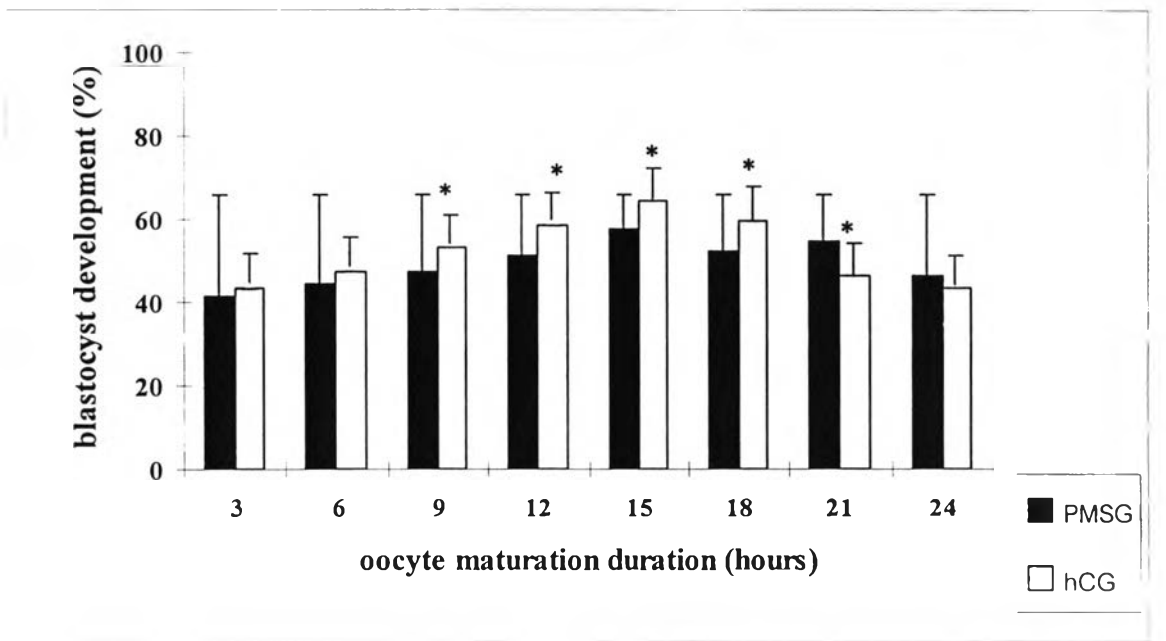


Figure 3.10 Comparison of the effect of *in vitro* oocyte maturation duration, 3-24 hours, on the development of 2-cell embryos obtained from *in vitro* matured oocytes to blastocyst. The *in vitro* matured oocytes were obtained from 7.5 IU PMSG- or 7.5 IU hCG-primed mice after culturing in maturation medium for 3 - 24 hours. (Data from Table 3.13 and 3.14)

(*) significantly different ($P<0.05$) from PMSG-prime mice.

Experiment III : Effects of oocyte maturation duration on the level of intraoocyte cAMP, zona pellucida thickness and hardness of oocytes obtained from PMSG- primed and hCG-primed mice.

The effect of *in vitro* oocyte maturation duration on cytoplasmic maturation, ZP hardening was examined in this experiment. The ZP digestion time of *in vitro* matured oocytes obtained from 7.5 IU PMSG-primed mice and cultured in maturation medium for 3 - 24 hours is shown in Table 3.15. These data indicated that *in vitro* oocyte maturation duration affects ZP hardening and may also affect *in vitro* fertilization. The time required for ZP digestion in *in vitro* matured oocytes upon culturing in maturation medium for 3 hours (1168 ± 139 seconds) was significantly shorter ($P<0.05$) than the ZP digestion time in *in vitro* matured oocytes upon culturing in maturation medium for 9 hours (1656 ± 243 seconds) to 24 hours (2509 ± 148 seconds) (Table 3.15). However, the ZP digestion time of *in vitro* matured oocytes obtained after 9 - 18 hours incubation was longer ($P<0.05$) than that of *in vitro* matured oocytes obtained after 3 - 6 hours incubation. In contrast, the percentage of *in vitro* fertilization of *in vitro* matured oocytes obtained after 12 - 15 hours incubation was higher ($P<0.05$) than that of *in vitro* matured oocytes obtained after 3 - 6 hours incubation (Table 3.11).

In this experiment the effect of *in vitro* oocyte maturation duration on ZP hardening of *in vitro* matured oocytes obtained from 7.5 IU hCG-primed mice was compared with those from 7.5 IU PMSG-primed mice. The ZP digestion time of *in vitro* matured oocytes obtained from 7.5 IU hCG-primed mice and cultured in maturation medium at 37°C and in a humidified atmosphere of 5%CO_2 in air for 3 - 24 hours are shown in Table 3.16. These data indicate that *in vitro* oocyte maturation duration affects ZP hardening and may affect *in vitro* fertilization. The results of this experiment are similar to those described above for 7.5 IU PMSG-primed mice, but the time required for ZP digestion of *in vitro* matured oocytes from hCG-primed mice was shorter ($P<0.05$) than that of PMSG-primed mice for 9 - 21 hours of incubation durations shown in Figure 3.11. These results indicate that the short time required for ZP digestion caused a higher percentage of *in vitro* fertilization and preimplantation embryo development in hCG-primed mice than in PMSG-primed mice.

The effect of *in vitro* oocyte maturation duration on ZP thickness of *in vitro* matured oocytes obtained from 7.5 IU PMSG-primed mice is shown in Table 3.17. The ZP thickness of *in vitro* matured oocytes upon culturing for 3 - 24 hours was 15.3 ± 2.3 μm (at 3 hours), 13.1 ± 2.6 μm (at 6 hours), 14.2 ± 1.8 μm (at 9 hours), 12.8 ± 3.6 μm (at 12 hours), 15.7 ± 1.9 μm (at 15 hours), 16.3 ± 1.7 μm (at 18 hours), 14.4 ± 2.4 μm (at 21 hours), and 13.4 ± 2.3 μm (at 24 hours). There was no significant difference ($P>0.05$) as to ZP thickness between

Table 3.15 Zona pellucida digestion time of *in vitro* matured oocytes obtained from 7.5 IU PMSG-primed mice and cultured in maturation medium for 3 - 24 hours (n=100).

Time (h)	zona pellucida digestion time (seconds)
3	1168 ± 139
6	1346 ± 266
9	1656 ± 243 [a,b]
12	1884 ± 199 [a,b]
15	1668 ± 103 [a,b]
18	2181 ± 81 [a,b,c,d,e]
21	2310 ± 112 [a,b,c,d,e]
24	2509 ± 148 [a,b,c,d,e,f]

Data derived from five replicate experiments and expressed as mean ± SD. A total of twenty oocytes was screened per replicate experiment.

(a) significantly different ($P<0.05$) from the No. of oocytes cultured in medium for 3 hours.
(b) significantly different ($P<0.05$) from the No. of oocytes cultured in medium for 6 hours.
(c) significantly different ($P<0.05$) from the No. of oocytes cultured in medium for 9 hours.
(d) significantly different ($P<0.05$) from the No. of oocytes cultured in medium for 12 hours.
(e) significantly different ($P<0.05$) from the No. of oocytes cultured in medium for 15 hours.
(f) significantly different ($P<0.05$) from the No. of oocytes cultured in medium for 18 hours.

Table 3.16 Zona pellucida digestion time of *in vitro* matured oocytes obtained from 7.5 IU hCG-primed mice and cultured in maturation medium for 3 - 24 hours (n=100).

Time (h)	zona pellucida digestion time (seconds)
3	1013 ± 112
6	1320 ± 187
9	1458 ± 231 [a]
12	1673 ± 304 [a]
15	1882 ± 147 [a,b,c]
18	1903 ± 94 [a,b,c,d]
21	2121 ± 131 [a,b,c,d]
24	2400 ± 218 [a,b,c,d,e]

Data derived from five replicate experiments and expressed as mean ± SD. A total of twenty oocytes was screened per replicate experiment.

(a) significantly different ($P<0.05$) from the No. of oocytes cultured in medium for 3 hours.
(b) significantly different ($P<0.05$) from the No. of oocytes cultured in medium for 6 hours.
(c) significantly different ($P<0.05$) from the No. of oocytes cultured in medium for 9 hours.
(d) significantly different ($P<0.05$) from the No. of oocytes cultured in medium for 12 hours.
(e) significantly different ($P<0.05$) from the No. of oocytes cultured in medium for 15 hours.

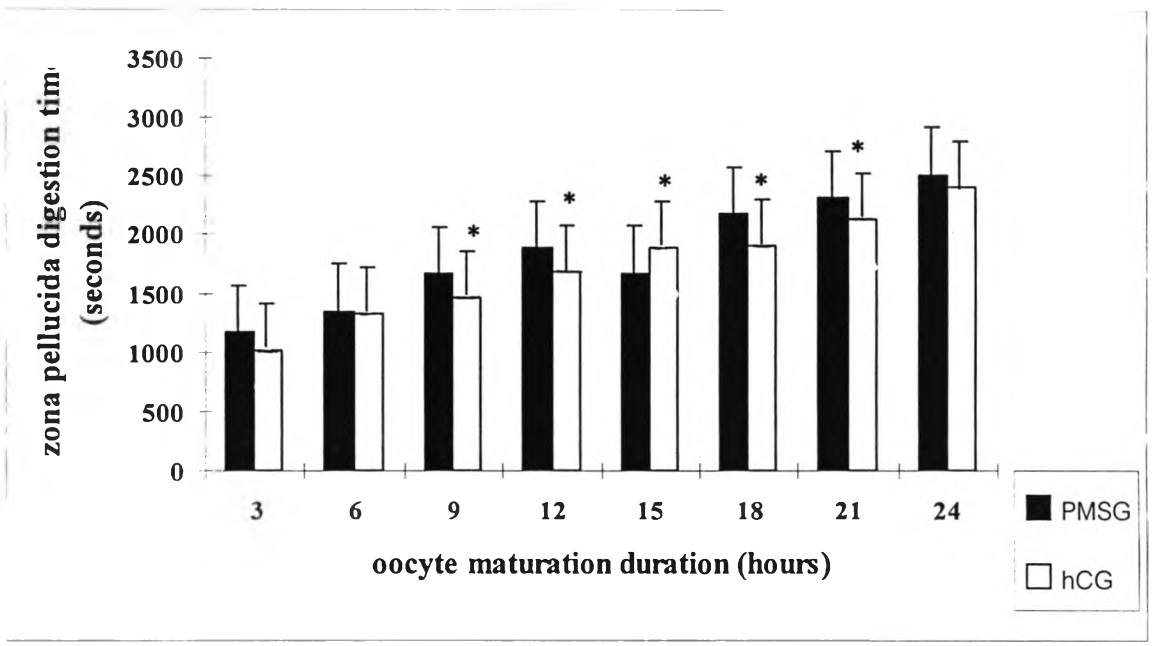


Figure 3.11 Comparison of the effect of *in vitro* oocyte maturation duration, 3-24 hours, on zona pellucida digestion time of *in vitro* matured oocytes obtained from 7.5 IU PMSG- or 7.5 IU hCG-primed mice and cultured in maturation medium for 3 - 24 hours. (Data from Table 3.15 and 3.16)

(*) significant difference ($P<0.05$) from PMSG-prime mice.

groups of different *in vitro* oocyte maturation duration indicating the ZP thickness of *in vitro* matured oocytes not to be associated with ZP hardening from previous experiment in the mouse model in this study.

The effect of *in vitro* oocyte maturation duration on ZP thickness of *in vitro* matured oocytes obtained from 7.5 IU hCG-primed mice upon culturing for 3 - 24 hours was 14.6 ± 2.3 μm (at 3 hours), 12.8 ± 2.8 μm (at 6 hours), 15.3 ± 2.3 μm (at 9 hours), 13.1 ± 2.6 μm (at 12 hours), 14.2 ± 1.8 μm (at 15 hours), 12.8 ± 3.6 μm (at 18 hours), 16.3 ± 1.7 μm (at 21 hours), and 13.4 ± 2.3 μm (at 24 hours) (Table 3.18). There was no significant difference ($P>0.05$) in ZP thickness between groups of different *in vitro* oocyte maturation time in 7.5 IU hCG-primed mice, same as that observed in 7.5 IU PMSG-primed mice except in 18 hours of maturation duration (Figure 3.12).

In this experiment the effect of *in vitro* oocyte maturation duration on intraoocyte cAMP concentration of *in vitro* matured oocytes obtained from 7.5 IU PMSG-primed mice and cultured in maturation medium at 37°C in a humidified atmosphere of 5%CO_2 in air for 3 - 24 hours was studied (Table 3.19). In addition, Table 3.19 shows that intraoocyte cAMP of mature oocytes at the MII stage was lower than intraoocyte cAMP of immature oocytes at the GV stage.

Table 3.17 Zona pellucida thickness of *in vitro* matured oocytes obtained from 7.5 IU PMSG-primed mice and cultured in maturation medium for 3 - 24 hours (n=100).

Time (h)	zona pellucida thickness (μm)
3	15.3 ± 2.3
6	13.1 ± 2.6
9	14.2 ± 1.8
12	12.8 ± 3.6
15	15.7 ± 1.9
18	16.3 ± 1.7
21	14.4 ± 2.4
24	13.4 ± 2.3

Data derived from five replicate experiments and expressed as mean SD. A total of twenty oocytes was screened per replicate experiment. There are no significant differences (P>0.05) between groups.

Table 3.18 Zona pellucida thickness of *in vitro* matured oocytes obtained from 7.5 IU hCG-primed mice and cultured in maturation medium for 3 - 24 hours (n=100).

Time (h)	zona pellucida thickness (μm)
3	14.6 ± 2.3
6	12.8 ± 2.8
9	15.3 ± 2.3
12	13.1 ± 2.6
15	14.2 ± 1.8
18	12.8 ± 3.6
21	16.3 ± 1.7
24	13.4 ± 2.3

Data derived from five replicate experiments and expressed as mean SD. A total of twenty oocytes was screened per replicate experiment. There are no significant differences ($P>0.05$) between groups.

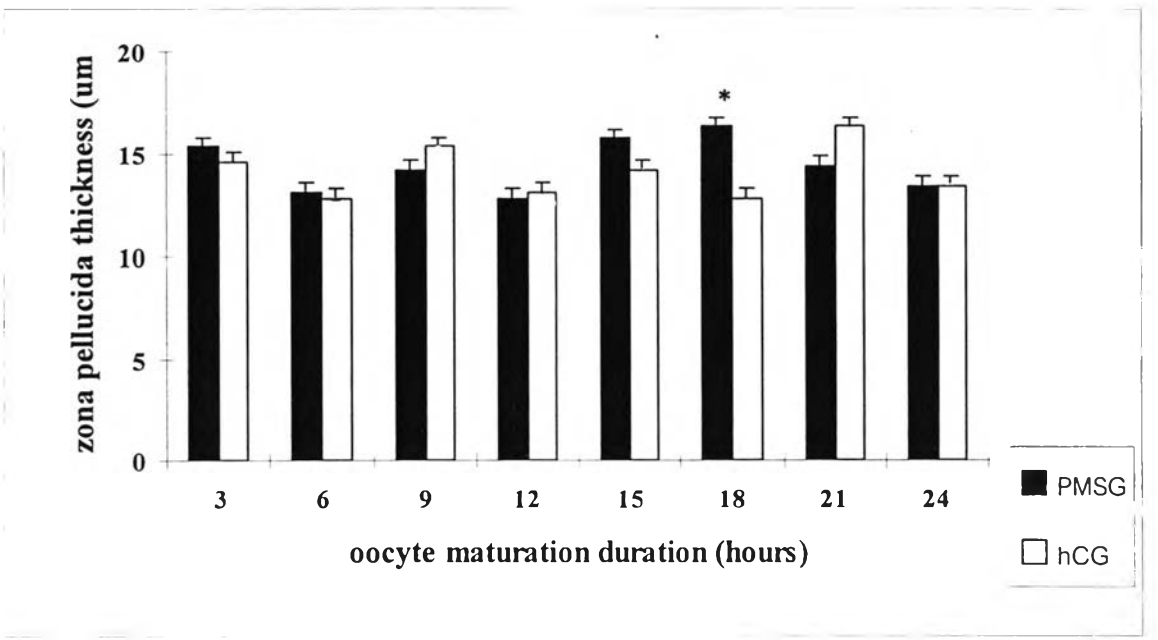


Figure 3.12 Comparison of the effect of *in vitro* oocyte maturation duration, 3-24 hours, on zona pellucida thickness of *in vitro* matured oocytes obtained from 7.5 IU PMSG- or 7.5 IU hCG-primed mice and cultured in maturation medium for 3 - 24 hours. (Data from Table 3.17 and 3.18)

(*) significantly different ($P<0.05$) from PMSG-prime mice.

A similar studied of effect of *in vitro* oocyte maturation duration on intraoocyte cAMP of *in vitro* matured oocytes obtained from 7.5 IU hCG-primed mice and cultured in maturation medium at 37°C in a humidified atmosphere of 5%CO_2 in air for 3 - 24 hours was carried out and compared with that observed in 7.5 IU PMSG-primed mice. Intraoocyte cAMP of *in vitro* matured oocytes upon culturing was 0.08 fmol/oocyte (at 3 hours incubation), 0.05 fmol/oocyte (at 6 hours), 0.04 fmol/oocyte (at 9 hours), 0.03 fmol/oocyte (at 12 hours), 0.03 fmol/oocyte (at 15 hours), 0.04 fmol/oocyte (at 18 hours), 0.07 fmol/oocyte (at 21 hours), and 0.09 fmol/oocyte (at 24 hours) (Table 3.20). In addition, the results of this experiment show the intraoocyte cAMP of *in vitro* matured oocytes upon culturing for 6 hours (0.05 fmol/oocyte), 9 hours (0.04 fmol/oocyte), 12 hours (0.03 fmol/oocyte), 15 hours (0.03 fmol/oocyte), and 18 hours (0.04 fmol/oocyte) to be significantly lower ($P<0.05$) than that of *in vitro* matured oocytes cultured for 3 hours (0.08 fmol/oocyte). Comparison of the effect of oocyte maturation duration on intraoocyte cAMP concentration of *in vitro* matured oocytes obtained from hCG-primed mice and PMSG-primed mice is showed in Figure 3.13.

Table 3.19 Intraoocyte cAMP concentration of *in vitro* matured oocytes obtained from 7.5 IU PMSG-primed mice and cultured in maturation medium for 3 - 24 hours (n=300).

Time (h)	intraoocyte cAMP of *in vitro* matured oocytes (fmol/oocyte)
3	0.09 [a]
6	0.06 [a,b]
9	0.07 [a]
12	0.06 [a,b]
15	0.08 [a]
18	0.08 [a]
21	0.10 [a,c,d,e]
24	0.13 [a,b,c,d,e,f,g,h]

Data derived from two replicate experiments. A total of one hundred-fifty oocytes was screened per replicate experiment.

(a) significantly different ($P<0.05$) from the oocytes at the GV stage : 0.16 fmol/oocyte
(b) significantly different ($P<0.05$) from the No. of oocytes cultured in medium for 3 hours.
(c) significantly different ($P<0.05$) from the No. of oocytes cultured in medium for 6 hours.
(d) significantly different ($P<0.05$) from the No. of oocytes cultured in medium for 9 hours.
(e) significantly different ($P<0.05$) from the No. of oocytes cultured in medium for 12 hours.
(f) significantly different ($P<0.05$) from the No. of oocytes cultured in medium for 15 hours.
(g) significantly different ($P<0.05$) from the No. of oocytes cultured in medium for 18 hours.
(h) significantly different ($P<0.05$) from the No. of oocytes cultured in medium for 21 hours.

Table 3.20 Intraoocyte cAMP of *in vitro* matured oocytes obtained from 7.5 IU hCG-primed mice and cultured in maturation medium for 3 - 24 hours (n=300).

Time (h)	intraoocyte cAMP of *in vitro* matured oocytes (fmol/oocyte)
3	0.08 [a]
6	0.05 [a,b]
9	0.04 [a,b]
12	0.03 [a,b]
15	0.03 [a,b]
18	0.04 [a,b]
21	0.07 [a,d,e,f,g]
24	0.09 [a,c,d,e,f,g]

Data derived from double replicate experiments. A total of one hundred-fifty oocytes was screened per replicate experiment.

(a) significantly different ($P<0.05$) from the oocytes at the GV stage : 0.15 fmol/oocyte
(b) significantly different ($P<0.05$) from the No. of oocytes cultured in medium for 3 hours.
(c) significantly different ($P<0.05$) from the No. of oocytes cultured in medium for 6 hours.
(d) significantly different ($P<0.05$) from the No. of oocytes cultured in medium for 9 hours.
(e) significantly different ($P<0.05$) from the No. of oocytes cultured in medium for 12 hours.
(f) significantly different ($P<0.05$) from the No. of oocytes cultured in medium for 15 hours.
(g) significantly different ($P<0.05$) from the No. of oocytes cultured in medium for 18 hours.

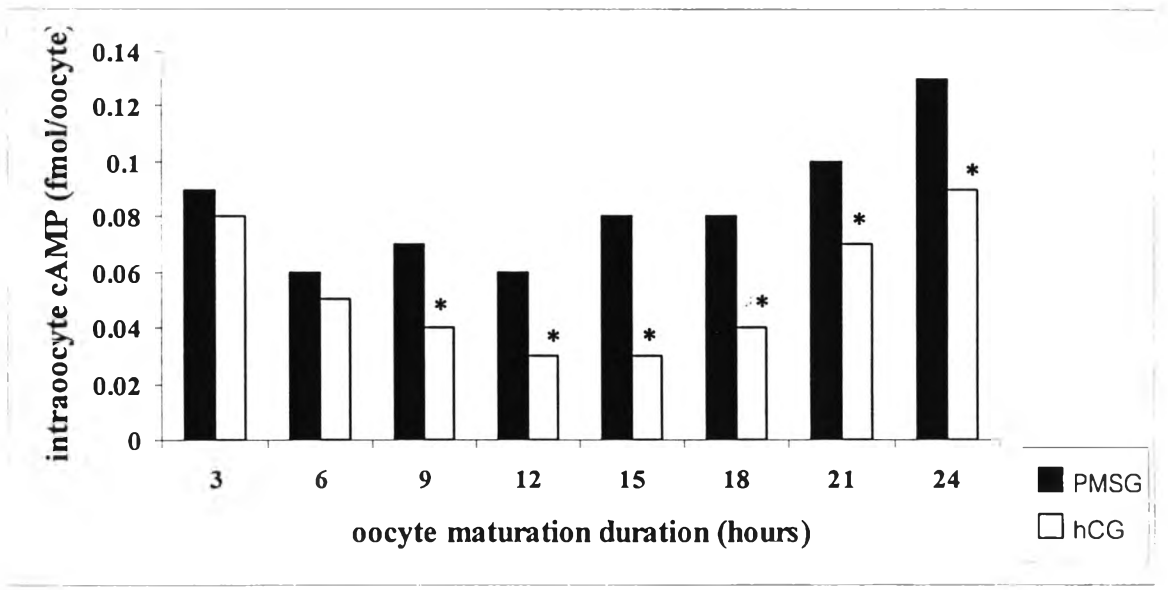


Figure 3.13 Effects of *in vitro* oocyte maturation duration, 3-24 hours, on intraoocyte cAMP of *in vitro* matured oocytes obtained from 7.5 IU PMSG- or 7.5 IU hCG-primed mice and cultured in maturation medium for 3 - 24 hours. (Data from Table 3.19 and 3.20)

(*) significantly different ($P<0.05$) from PMSG-prime mice.