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Determination and synchronisation of G1-phase of the cell cycle in 2- and 4-cell mouse embryos

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Summary

Incorporation of [^{3}H]thymidine at different concentrations into mouse embryos at early developmental stages was determined by autoradiography. Methods to synchronise the G1-phase of mouse 2- and 4-cell embryos were also investigated. The results showed that the ability of embryos to incorporate [^{3}H]thymidine increased with development. Embryos at the 4-cell stage were not labelled when the concentration of [^{3}H]thymidine was lower than 5 μCi/ml, whereas the nuclei of embryos at morula and blastocyst stages began to show silver grains at a concentration of 0.1 μCi/ml of [^{3}H]thymidine. After 2- and 4-cell mouse embryos were synchronised at the onset of G1-phase by treatment with low temperature or nocodazole, and DNA synthesis was detected by autoradiography, the duration of G1-phase was estimated. The result showed that 43% of the 2-cell embryos had a G1-phase of ≤ 1 h, 22% had a G1-phase of ≤ 2 h, 22% had a G1-phase of ≤ 3 h and 13% had a G1-phase of ≤ 4 h. The G1-phase in 85% of the 4-cell embryos was ≤ 3 h, that in 8% of embryos was ≤ 4 h and that in 7% of embryos was ≤ 5 h. The toxicity of nocodazole on mouse embryo development was assessed based on both blastocyst formation and the number of blastomeres, and the results indicated that the effect of nocodazole on embryo development and cell cycle block was dose-dependent. The minimum concentration of nocodazole for metaphase block of mouse late 2-cell embryos was 0.05 μM, and the appropriate concentrations which did not impair development were 0.05–0.5 μM.

Keywords: DNA synthesis, Embryos, G1-phase, Mouse, Nocodazole

Introduction

Study of the cell cycle is important in both cell biology and developmental biology. Studies have shown that cell cycles of embryos are different from those of somatic cells; while the length of cell cycle in somatic cells depends on the G1-phase, that in embryos depends primarily on the S-phase, because G1-phase of embryos is very short and some cannot even be detected. However, the G1-phase in mouse and pig embryos was determined by detecting the DNA content of Feulgen-stained nuclei with microdensitometry in embryos where cell cycles were not synchronised (Smith & Johnson, 1986; Chisholm, 1988; Schoenbeck *et al*., 1992). Therefore, further studies are needed in this field.

Studies on nuclear transplantation showed that when G1-phase nuclei were transferred into metaphase II (MII) oocytes, premature chromosome condensation (PCC) and nuclear remodelling would occur. Since DNA replication did not occur in this case, the damage to chromosomes by condensation was avoided. Using G0-phase cultured cells as donors, live lambs were obtained by nuclear transfer (Campbell *et al*., 1996). Wakayama *et al*. (1998) succeeded in mouse cloning using cumulus cells as nuclear donors, which was thought to be at G0-phase. In summary, when G1-phase nuclei were transferred into MII oocytes, they were successfully reprogrammed and the reconstructed

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embryos were more developmentally competent. Double blocking was used to synchronise embryos at G1-phase (Campbell *et al*., 1994) and serum starvation was used to induce cultured somatic cells to exit the cell cycle and enter G0-phase (Iyer *et al*., 1999).

In the present study, we investigated the length of the G1-phase of mouse 2- and 4-cell embryos by autoradiography after cycle synchronisation by low-temperature storage or nocodazole treatment, in order to gain more knowledge on the mammalian embryo cell cycle and to improve the efficiency of animal cloning.

Materials and methods

Collection of embryos

Mice of the Kun-ming breed (outbred) were kept in a room with 14 h/10 h light–dark cycles, the dark starting at 2000 hours. Female mice, 8–12 weeks after birth, were induced to superovulate with PMSG (10 IU, i.p.) followed by hCG (10 IU, i.p.) 48 h later. Zygotes and late 2-cell embryos were recovered from the mated females 32 h and 49 h after hCG injection, respectively, and middle 4- and 8-cell embryos, morulae and blastocysts (cavity = 1/2) were collected 57 h, 67 h, 80 h and 91 h after hCG injection, respectively. The collected embryos were washed three times with M2 (Whittingham, 1971) before culture.

Preparation of nocodazole

A stock solution of nocodazole was prepared by dissolving nocodazole (Sigma, St Louis, MO) in dimethyl sulfoxide at concentrations of 3 mg/ml (10 mmol/l) and kept at –20°C.

Autoradiography

Embryos were cultured in 50 µl drops of M16 medium (Whittingham, 1971) containing 10% fetal calf serum (FCS) and [^{3}H]thymidine (activity 3296 GBq/mmol; Sigma, St Louis, MO) at 37 °C in an atmosphere of 5% CO_2 and 95% air for 1 h. At the end of culture, embryos were washed three times in M2 and incubated in 1% sodium citrate for 1 min. The embryos were then transferred into fixation medium I (methanol:acetic acid, 1:1), and were immediately aspirated with a pipette and expelled onto an acid-washed slide. When fixation medium I dried out, a drop of 90% acetic acid was added to further disperse the blastomeres. After air-drying, the slides were kept in fixation medium II (ethanol:acetic acid, 3:1) for more than 1 h at 4 °C and were stored at the same temperature after fixation. The double-fixed slides were covered with a thin layer (less than 5 µm) of nuclear IV emulsion (Beijing Atomic Energy Institution, Beijing, China), air-dried and exposed for 7 days at 4 °C. The incorporation of [^{3}H]thymidine was assessed under a bright-field microscope by the development of silver gains.

Experiment 1: Dose determination of [^{3}H]thymidine incorporation into 4- and 8-cell embryos, morulae and blastocysts

Embryos at middle 4-cell, 8-cell, morula and blastocyst stages were cultured in M16 containing 10, 5, 1, 0.5 and 0.1 µCi/ml of [^{3}H]thymidine for 1 h. At the end of culture, embryos were processed and examined by autoradiography.

Experiment 2: Time course of G1-phase in 2-cell embryos

Late zygotes were cultured in CZB medium (Chatot *et al*., 1989) and observed every 15 min. Those embryos which had just completed first cleavage were selected and stored in M2 medium at 4 °C to inhibit development. After storage for 8–9 h, the 'onset 2-cell' embryos were labelled with 40 µCi/ml [^{3}H]thymidine in CZB medium for 1, 2, 3 and 4 h, respectively. After autoradiography, the initiation of DNA synthesis was examined under a microscope.

Experiment 3: Effect of nocodazole on early embryo development

Late 2-cell embryos were cultured in M16 supplemented with different concentrations (10, 0.5, 0.1, 0.05 and 0.01 µM) of nocodazole for 12–14 h. The treated embryos were then washed several times with M2 and cultured in normal M16 for 3 days. At the end of culture, the ratio of blastocysts formed was recorded and the number of blastomeres per blastocyst was counted.

Experiment 4: Time course of G1-phase in 4-cell embryos

Late 2-cell embryos were cultured in M16 containing 0.05 µM nocodazole for 12–14 h to block them at metaphase of the second cleavage. After removing the zona pellucida by acid Tyrode solution (Betancourt *et al*., 1998), the G2/M embryos were dissociated into single blastomere and examined for division (elongation) every 15 min. Immediately after the second cleavage, the blastomere pairs at the 4-cell stage (2/4 pairs) were selected and arrested by cooling at 4 °C for 30–60 min in M2 containing 20% FCS. The blastomere pairs were then labelled by incubation in M16 containing 10 µCi/ml [^{3}H]thymidine for 1, 2, 3, 4 and 5 h before autoradiography.

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Statistical analysis

Data in this study were analysed by Student's *t*-test.

Results

Experiment 1: Dose determination of [^{3}H]thymidine incorporation into 4- and 8-cell embryos, morulae and blastocysts

After embryos at different stages had been cultured in M16 containing different concentrations of [^{3}H]thymidine, signals appeared (Fig. 1) in most embryos when the concentration of [^{3}H]thymidine was 5 µCi/ml or above (Table 1). The minimum concentration of [^{3}H]thymidine for effective labelling differed according to the stage of the embryos. When the [^{3}H]thymidine concentration was at 0.1 µCi/ml, no signal was observed in 8-cell embryos, but the silver grains were seen clearly when the concentration was increased to 0.5 µCi/ml (Fig. 2). Morula and blastocyst nuclei began to show faint grains even at a concentration of 0.1 µCi/ml, and when the concentration reached 5 µCi/ml, many silver grains appeared in the nuclear area (Figs. 3, 4). These results indicated that the ability of mouse embryos to incorporate [^{3}H]thymidine increased with development.

Experiment 2: Time course of G1-phase in 2-cell embryos

The embryos that had just finished the first cleavage were designated as 'onset 2-cell' embryos. When the onset 2-cell embryos, which had been stored in M2 medium under 4 °C for 8–9 h, were cultured in M16 at 37 °C for 1, 2, 3 and 4 h, 43%, 66%, 88% and 100% of

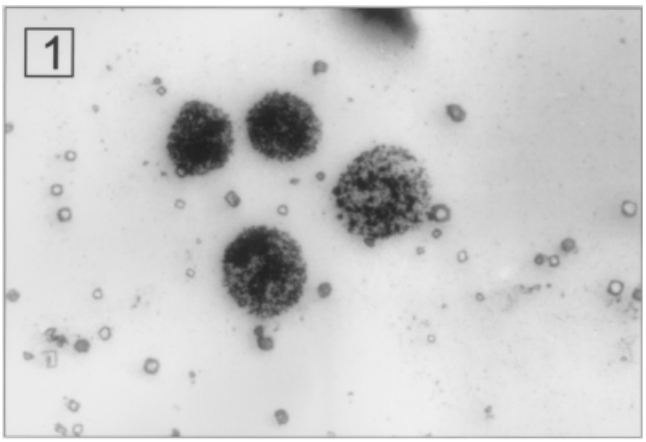

Figure 1 Four-cell embryo labelled with [^{3}H]thymidine at a concentration of 5 µCi/ml. Scale bar represents 100 µm.

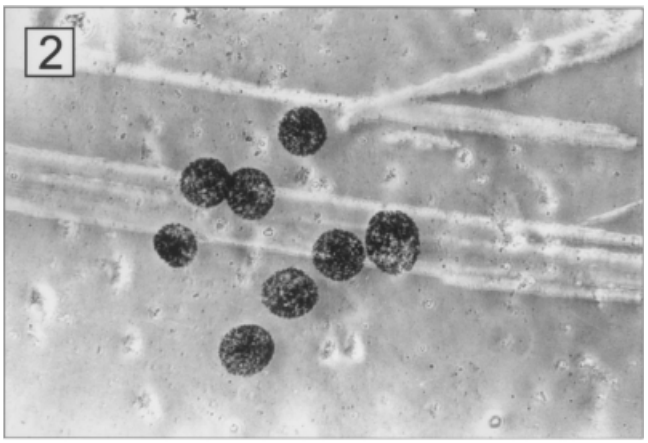

Figure 2 Eight-cell embryo labelled with [^{3}H]thymidine at a concentration of 0.5 µCi/ml. Scale bar represents 200 µm.

Table 1 Incorporation of [^{3}H]thymidine into mouse embryos at different developmental stages

	Concentration of [^{3}H]thymidine (µCi/ml)				
Embryo stage	0.1	0.5	1	5	10
Mid-4-cell	100% (19) –	100% (20) –	100% (20) –	25.6 (10) – 74.4% (29) +	7.5% (3) – 30% (12) + 62.5% (25) ++
Mid-8-cell	100% (17) –	18.2% (4) – 40.9% (9) + 40.9% (9) ++	14.8% (4) – 29.6% (8) + 55.6% (15) +	6.7% (3) + 11.1% (5) ++ 82.2% (37) +++	6.9% (2) + 24.1% (7) ++ 69.0% (20) +++
Mid-morula	100% (20) +	50% (7) – 50% (7) +	29.0% (9) + 71.0% (22) +	10% (3) + 23.3% (7) ++ 66.7% (20) +++	100% (17) +++
Mid-blastocyst	100% (15) +	40.5% (15) + 59.5% (22) +	100% (10) ++	100% (15) +++	100% (23) +++

Values are the percentage (no.) of embryos examined.
–, no silver grains were detected; +, faint silver grains; ++, clear silver grains; +++, silver grains in high density.

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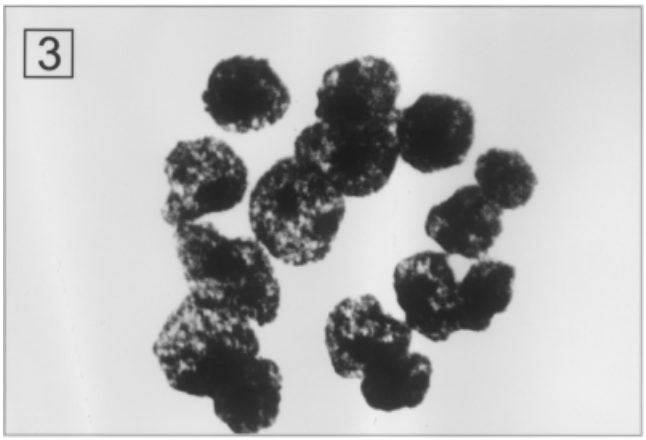

Figure 3 Morula labelled with [^{3}H]thymidine at a concentration of 5 μCi/ml. Scale bar represents 100 μm.

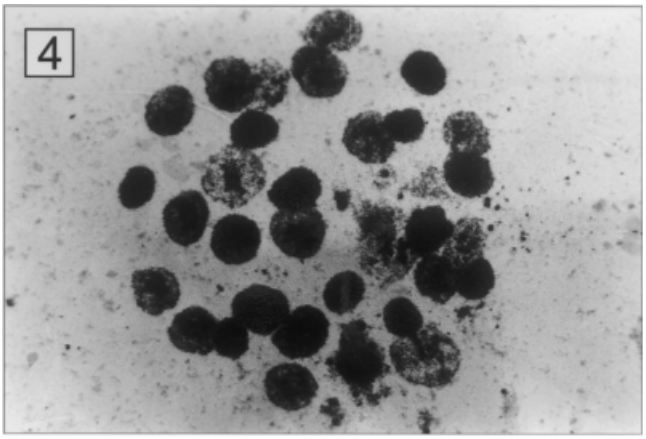

Figure 4 Blastocyst labelled with [^{3}H]thymidine at a concentration of 5 μCi/ml. Scale bar represents 200 μm.

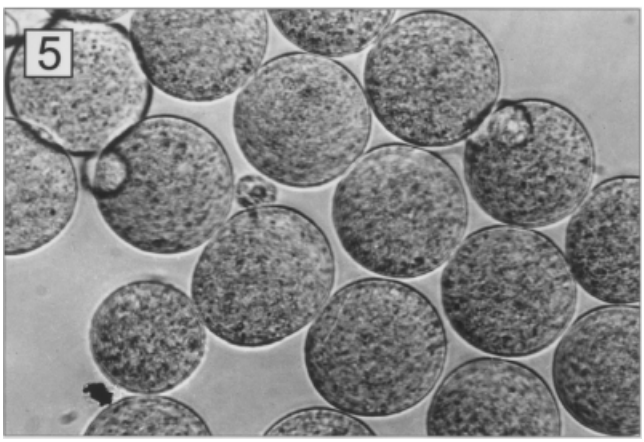

Figure 5 Isolated blastomeres of 2-cell embryos at M-phase. Scale bar represents 12 μm.

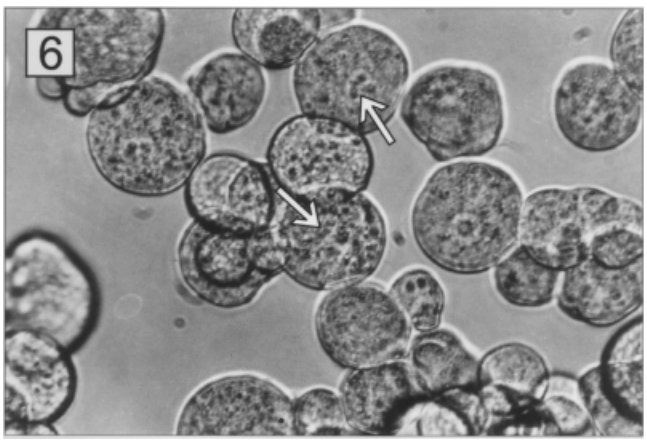

Figure 6 Isolated 2-cell blastomeres dividing into two blastomeres. Nucleus (arrows). Scale bar represents 18 μm.

them entered S-phase, respectively (Table 2). This suggested that 43% of the 2-cell embryos had a G1-phase of ≤ 1 h, 22% had a G1-phase of ≤ 2 h, 22% had a G1-phase of ≤ 3 h and 13% had a G1-phase of ≤ 4 h.

Experiment 3: Effect of nocodazole on embryo development

No 2-cell embryos treated with 0.01 μM nocodazole were blocked but all cleaved to the 4-cell stage. All the embryos were blocked at G2/M-phase when cultured with medium containing 0.05, 0.1 and 0.5 μM nocodazole. When these embryos were recultured in nocodazole-free medium, 45% of them divided into 4-cell embryos in half an hour and all the embryos divided within 1 h. The blastocyst formation rate (100%) was not significantly different from that in the control group when cultured for 72 h (Table 3). This indicated that the inhibition of development by nocodazole at these concentrations was reversible.

Although embryos treated with 10 μM nocodazole cleaved to the 4-cell stage after removal of nocodazole, the cleavage was significantly delayed (>2 h). After cultured in nocodazole-free medium for 72 h, the percentage of blastocyst formation (55%) and the average number of blastomeres (15 ± 3) were significantly lower than those in the control group (Table 3).

These results indicated that the optimum concentration of nocodazole for the metaphase block of mouse late 2-cell embryos was 0.05 μM.

Experiment 4: Time course of G1-phase in 4-cell embryos

Late 2-cell embryos were synchronised in M-phase by nocodazole treatment at a concentration of 0.05 μM for 12–14 h. When the embryos were dissociated into blastomeres and cultured in nocodazole-free M16 after treatment, most blastomeres cleaved into blastomere pairs with a distinct nuclear membrane and nucleoli (Figs. 5, 6) within 2 h. When the synchronised blastomere pairs were detected by means of autoradio-

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Table 2 Lengths of G1-phases in 2-cell and 4-cell embryos

	2-cell embryos		4-cell embryos	
Labelling time (h)	No. of embryos examined	% of blastomeres at S-phase	No. of embryos examined	% of blastomeres at S-phase
1	15	43.3% (30)	15	0 (0)
2	16	65.6% (32)	17	0 (0)
3	24	87.5% (48)	20	85.0% (80)
4	22	100% (44)	18	93.1% (72)
5			21	100% (84)

Values in parentheses are the number of embryos examined.

Table 3 Nocodazole block of the cell cycle and its effect on development in mouse late 2-cell embryos

Concentration of nocodazole (μM)	No. of embryos treated	% (no.) of blastocysts	No. of blastomeres per embryo	% (no.) of embryos blocked
0	20	100% (20)[a]	38 ± 9[a]	– (0)[b]
0.01	20	95% (19)[a]	38 ± 9[a]	– (0)[b]
0.05	18	100% (18)[a]	36 ± 5[a]	100% (18)[a]
0.1	14	100% (14)[a]	34 ± 7[a]	100% (14)[a]
0.5	14	100% (14)[a]	32 ± 5[a]	100% (14)[a]
10	24	55% (13)[b]	15 ± 3[c]	100% (24)[a]

[a,b,c]Values with different superscripts in the same column were significantly different ($p < 0.01$).

graphy, no DNA synthesis was observed during the first 2 h of labelling. However, when labelled for 3, 4 and 5 h, DNA synthesis was detected in 85%, 95% and 100% blastomeres, respectively (Table 2). These results suggested that the G1-phase in 85% of the 4-cell embryos was ≤ 3 h, that in 8% of embryos was ≤ 4 h and that in 7% of embryos was ≤ 5 h.

Discussion

Detection of incorporation of radiolabelled nucleotides by autoradiography provides basic information on DNA replication and embryonic genome expression (Kopecny, 1989; Laurincik *et al*., 1998). After comparison between sectioning and spreading, Plante *et al.* (1992) found that the spreading technique is more sensitive, simpler and faster for autoradiography. By autoradiography with [^{3}H]thymidine, Kopecny *et al.* (1995) detected DNA synthesis in mouse zygotes and 4-cell embryos, and Laurincik *et al*. (1998) studied DNA synthesis in bovine zygotes. In a study on the effect of [^{3}H]thymidine on mouse embryo development, Spindle *et al.* (1982) found that the exposure time needed to reveal incorporated [^{3}H]thymidine in mouse embryos was related to the developmental stage. In the present study, we labelled mouse embryos at the 4-cell, 8-cell, morula and blastocyst stages with different concentrations of [^{3}H]thymidine and found that given the same time of labelling (1 h) and exposure (5 days), mouse embryos at different stages of development required different concentrations of [^{3}H]thymidine for labelling. Embryos at the 4-cell stage required a higher concentration (5 μCi/ml), whereas those at the morula and blastocyst stages were labelled by a concentration of [^{3}H]thymidine as low as 0.1 μCi/ml. Spindle *et al.* (1982) proposed that the low ability of early embryos to incorporate [^{3}H]thymidine may be attributed to decreased membrane penetrability or interference of the endogenous thymidine pool with incorporation of exogenous thymidine.

It has been shown that the duration of embryo cell cycles depends on the length of S-phase, because the length of G1-phase in embryo cells is very short, and some even cannot be detected. In pigs, the G1-phase of 2-cell and 4-cell embryos was shown to be less than 2 h (Schoenbeck *et al*., 1992). Smith & Johnson (1986) and Chisholm (1988) showed in mouse embryos that the G1-phase was 1 h at the 4-cell, 2 h at the 8-cell and 2 h at the 16-cell stage. However, in all the above studies the cell cycle was determined by detecting DNA content in Feulgen-stained nuclei with microdensitometry, in non-synchronised embryos. In the present study, we detected the length of the G1-phase in 2- and 4-cell embryos by autoradiography after cell cycle synchronisation with low temperature or nocodazole.

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The onset 2-cell embryos were obtained by culturing late zygotes and storing the elongated embryos in cool (4 °C) medium. The results showed that 43% of the 2-cell embryos had a G1-phase of ≤ 1 h, 22% had a G1-phase of ≤ 2 h, 22% had a G1-phase of ≤ 3 h and 13% had a G1-phase of ≤ 4 h. The onset 4-cell embryos were prepared as follows: After late 2-cell embryos had been synchronised at M-phase by nocodazole, they were dissociated into blastomeres and cultured in nocodazole-free medium. When blastomeres cleaved into blastomere pairs, they were pooled and arrested at 4 °C before labelling. Our results showed that the G1-phase in 85% of the 4-cell embryos was ≤ 3 h, that in 8% of embryos was ≤ 4 h and that in 7% of embryos was ≤ 5 h. It was found that G1-phase increased in length with development and tended to approach that of differentiated cells (Streffer *et al.*, 1980; Smith & Johnson, 1986; Chisholm, 1988). Our result also showed that the G1-phase of 4-cell embryos was slightly longer than that of 2-cell embryos.

To obtain synchronised onset 2-cell embryos, we stored the embryos immediately after the first cleavage at a low temperature (4 °C). It was found in rabbit embryos that metabolism was slowed down at low temperature but their development was not affected and, when the temperature returned to normal, DNA synthesis recovered rapidly (Collas *et al.*, 1992). We also found in another experiment (published in Chinese) that preservation of mouse mid 2-cell, 4-cell, 8-cell embryos, morulae and blastocysts at 4 °C for 9 h had no effect on the proportion of blastomeres incorporating [^{3}H]thymidine. This implies that preservation at 4 °C for this length of time would not delay embryo development significantly when recultured. Low-temperature storage, therefore, is a good method to synchronise embryo development.

In the present study, the late 2-cell embryos at G2/M-phase were inhibited by nocodazole to obtain synchronised onset 4-cell pairs at G1-phase. Colcemid and nocodazole are two reagents often used to inhibit cell division. Nocodazole was considered less toxic than colcemid and has usually been used to control the cell cycle in embryo micromanipulation (Kato & Tsunoda, 1992; Otaegui *et al.*, 1994; Liu *et al.*, 1997). Kato & Tsunoda (1992) assessed the toxicity of nocodazole on mouse embryo development by blastocyst formation or full-term development and found that mouse 2-cell embryos treated with nocodazole at a concentration of 10 μM for 12.5–14.5 h were able to cleave to 4-cells within 1 h after removal of the reagent; the ability to maintain full-time development was not affected. In our study, the toxicity of nocodazole on mouse embryo development was determined based on both blastocyst formation and the number of blastomeres. The results showed that nododazole at a concentration of 10 μM impaired development; the blastocyst rate (55%) and average number of blastomeres (15 ± 3) per blastocyst decreased significantly ($p < 0.01$). Otaegui *et al.* (1994) reported that the toxicity of nocodazole on development of mouse 4-, 8- and 16-cell embryos was dose-dependent and the blastocyst rate was significantly reduced at concentrations ranging from 2.5 to 10 μM nocodazole. In addition, in our experiment the time of collection of late 2-cell embryos was 49 after hCG injection. Since DNA synthesis had already been completed by this time and interaction between embryos and nocodazole was reduced, the damage by nocodazole to embryos should be less severe than that reported by others.

The block of embryo development by nocodazole was also dose-dependent. Thus, although mouse 4-cell embryos were synchronised in M-phase when treated by 0.33 μM nocodazole for 16 h, cleavage was not blocked completely and some developed to the 8-cell stage when embryos were treated with nocodazole at a concentration below 0.165 μM (Samake & Smith, 1996). Otaegui *et al.* (1994) found that with development, a higher concentration of nocodazole was needed to block mouse embryo development. When treated with 5 μM nocodazole for 12 h, development of 2-cell, 4-cell and 8-cell embryos and morulae was blocked completely, but when the concentration was reduced to 2.5 μM, development was not blocked completely except in the 2-cell embryos. In this study, cleavage of mouse 2-cell embryos was not blocked by 0.01 μM nocodazole, but it was blocked completely when the concentration of nocodazole was increased to 0.05 μM or above.

In conclusion, the ability of embryos to incorporate [^{3}H]thymidine increased with development. Embryos at the 4-cell stage were not labelled when the concentration of [H]thymidine was lower than 5 μCi/ml, whereas the nuclei of embryos at morula and blastocyst stages began to show silver grains at a concentration of 0.1 μCi/ml of [^{3}H]thymidine. G1-phase of mouse 2-cell and 4-cell embryos is short and G1-phase at the 4-cell stage is slightly longer than that at 2-cell stage. Low-temperature storage and treatment with nocodazole are two efficient methods to obtain G1-phase embryos. The effect of nocodazole on embryo development and on cell cycle blocking was dose-dependent. The optimum concentration of nocodazole for the metaphase block of mouse late 2-cell embryos was 0.05 μM.

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