Synchronous division of mouse two-cell embryos with nocodazole *in vitro*

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Summary. Mouse two-cell embryos were cultured in a medium supplemented with nocodazole or colcemid for 12.5–14.5 h *in vitro*, and development after elimination of these drugs was examined. All embryos cultured with nocodazole stopped at the meta-phase of the second cell cycle. When nocodazole was removed, almost all embryos divided to the normal four-cell stage within 1 h and then developed into blastocysts (98%). The proportion of embryos that developed into young after transfer to recipients was not significantly different from the control (35 versus 36%), but the developmental ability of the embryos treated with colcemid was reduced, especially after transfer to recipients.

Keywords: blastocysts; nocodazole; colcemid; mouse

Introduction

Synchronization of cell cycles between donor nucleus and recipient cytoplasm may be important for the development of reconstituted eggs receiving nuclei from early embryos (Smith *et al.*, 1988, 1990) and fetal germ cells (Kato & Tsunoda, *in press*). In mammals, there are no reliable methods for synchronizing cell divisions of early embryos without reducing their ability to develop into fetuses. One way is to treat them with cytochalasin B or D (Siracusa *et al.*, 1980), but this induces polyploidy in most mouse embryos (Snow, 1973; Tarkowski *et al.*, 1977). Another way is to inhibit nuclear division with colcemid, colchicine or vinblastin to prevent the polymerization of tubulin. Although Siracusa *et al.* (1980) found the effect of colcemid to be far more reversible than that of colchicine or vinblastin, the ability of treated embryos to develop into fetuses was not clear. Nocodazole has also been used to inhibit the polymerization of tubulin in nuclear transplantation (Surani *et al.*, 1984) and to examine cytocortical organization (Johnson *et al.*, 1988), but its effect on developmental potential is unknown.

The present study was undertaken to examine *in vitro* and *in vivo* the developmental ability of mouse two-cell embryos in which cell division was inhibited by colcemid or nocodazole.

Materials and Methods

Collection and preservation of two-cell embryos

Two-cell embryos were obtained from superovulated F₁ (C57BL/6 × CBA) female mice mated with F₁ males 40–46 h after injection with human chorionic gonadotrophin. For experimental convenience, the embryos were stored at 4°C for 7–8 h in M2 medium (Fulton & Whittingham, 1978) under liquid paraffin, before treatment. Low-temperature preservation of the embryos for this period had no adverse effect on their development into blastocysts *in vitro*.

Preparation of nocodazole and colcemid

A concentrated stock solution of nocodazole (Aldrich Chem. Co.) (Hoebeke *et al.*, 1976; Maro & Bornens, 1980; Maro *et al.*, 1986; Johnson *et al.*, 1988; Tacke & Grunz, 1990) was dissolved in dimethyl sulfoxide at 3 mg ml⁻¹ (10 mmol l⁻¹). Colcemid (Gibco) was prepared in redistilled water at 1 mg ml⁻¹ and kept at 4°C.

Treatment with nocodazole and colcemid

Two-cell embryos were cultured in M16 medium (Whittingham, 1971) supplemented with nocodazole ($3 \mu\text{g ml}^{-1}$) or colcemid (0.02 and $0.1 \mu\text{g ml}^{-1}$) for 12.5–14.5 h under liquid paraffin at 37°C in 5% CO_2 and 95% air atmosphere. (In a preliminary study, all two-cell embryos preserved at a low temperature and cultured for 12.5–14.5 h developed to the four-cell stage.) The treated embryos were washed several times with M2 and M16 media and cultured in M16 medium for 3 days. Embryos that had blastocoels were regarded as blastocysts and were transferred to uteri of day 3 or 4, or oviducts of day 1, pseudopregnant recipient females (day of plug = day 1). They were killed just before parturition to examine the live young.

Fig. 1. Control mouse embryos: (a) eight four-cell and two six-cell embryos and (b) ten blastocysts were in culture for the same duration as those shown in Figs 2(a) and 3(a) and in Figs 2(b) and 3(b), respectively.

Fig. 2. Mouse embryos treated with $3 \mu\text{g nocodazole ml}^{-1}$: (a) 2 h after drug removal and (b) blastocyst formation.

Results

All embryos treated with nocodazole were at the two-cell stage when 99% of the control embryos had cleaved to the four-cell stage (Table 1, Fig. 1a). After being washed, 98% of the treated embryos cleaved to the four-cell stage (Fig. 2a) within 1 h and all developed into blastocysts (Fig. 2b). The proportion of young obtained after transfer to recipients of blastocysts that developed from embryos treated with nocodazole (23/65, 35%) was not significantly different from that

Table 1. Effects of nocodazole and colcemid on development of mouse two-cell embryos *in vitro* and *in vivo*

Treatment	Two-cell embryos used	Number of embryos stopped at two-cell stage (%)	Number of embryos developed to		Number of embryos transferred	Number pregnant*/ number of recipients (%)	Number of young (%)
			Four-cell stage (%)	Blastocysts (%)			
Control	224	—	222 (99)	221 (99)	154	13/15 (87)	55 (36)
Nocodazole (3.0 µg ml ⁻¹)	66	66 (100)	65 (98)	65 (98)	65	5/7 (71)	23 (35)
Colcemid (0.02 µg ml ⁻¹)		109 (87)	108 (99)†	108 (99)†	57	3/5 (60)	11 (19)
Colcemid (0.1 µg ml ⁻¹)		125 (100)	118 (94)†	102 (82)†	52	4/4 (100)	2 (4)

*Females with implantation sites were classed as pregnant, even if the uterus contained no young.

†Including embryos with small blastomeres.

Fig. 3. Mouse embryos treated with 0.1 µg colcemid ml⁻¹: (a) 2 h after drug removal and (b) blastocyst formation. The arrows show the unequal blastomere division or embryos with small blastomeres. All embryos showing a cavity were classed as blastocysts and transferred.

for the control embryos (55/154, 36%) (Table 1). The number of cells per blastocyst was 42.8 ± 7.2 (mean $\pm$ SD) in the control embryos and 28.5 ± 7.6 in the nocodazole-treated ones; but, 14 h later, the number of cells in the nocodazole-treated embryos was similar to that in the control (47.1 ± 13.6). The delay induced by the drug appeared to persist until the blastocyst stage.

Most embryos (87 and 100%) treated with colcemid (0.02 and 0.1 µg ml⁻¹, respectively) were at the two-cell stage, as also noted when nocodazole was used. The proportion of embryos that developed to the four-cell stage (99% for 0.02 µg ml⁻¹ and 94% for 0.1 µg ml⁻¹) after washing was not significantly different from the control (99%). However, most four-cell embryos that developed from embryos treated with colcemid showed several small blastomeres or cytoplasmic fragments in the perivitelline space (Fig. 3a). The proportion of embryos that developed to blastocysts was essentially the same as the control (221/224, 99%) when colcemid was used at 0.02 µg ml⁻¹ (108/109, 99%), but small fragments were again observed (Fig. 3b). The proportion of embryos that developed into blastocysts was significantly ($P < 0.001$) lower at 0.1 µg colcemid ml⁻¹ (102/125, 82%). The proportion of young that developed from embryos treated with colcemid after transfer to recipients, was significantly ($P < 0.05$, $P < 0.001$) different (11/57, 19% for 0.02 µg ml⁻¹ and 2/52, 4% for 0.1 µg ml⁻¹) from that obtained following the transfer of control embryos (55/154, 36%). No significant difference was observed in the results after transfer of the blastocysts into uteri or oviducts.

Discussion

The effect of microtubule-disruptive drugs, colcemid and nocodazole, on the developmental ability of mouse two-cell embryos and reversibility of treatment were examined. To the authors' knowledge, this is the first report demonstrating that cell division of embryos can be synchronized after treatment with such drugs without reducing the developmental ability *in vitro* and *in vivo*.

The effect of nocodazole was more reversible than that of colcemid. Most embryos treated with nocodazole ($3 \mu\text{g ml}^{-1}$, Hoebeke *et al.*, 1976; Maro & Bornens, 1980; Maro *et al.*, 1986; Johnson *et al.*, 1988; Tacke & Grunz, 1990) divided to the four-cell stage, which was morphologically normal, within 1 h of being rinsed in a medium lacking the drug, and development to the blastocyst stage was not reduced significantly compared with the control (98 versus 99%). The proportion of blastocysts that developed to young after transfer to recipients was similar to that in the control (35 versus 36%). Johnson *et al.* (1988) suggested that the arrest of blastomeres of eight-cell embryos for more than 6 h in nocodazole led to abnormalities, but they did not examine the ability of the treated embryos to develop into live young.

Colcemid disrupts microtubule assembly far more reversibly than vinblastine or colchicine (Siracusa *et al.*, 1980). However, the proportion of embryos treated with colcemid that developed to blastocysts was significantly lower at $0.1 \mu\text{g ml}^{-1}$, but was the same as the control at $0.02 \mu\text{g ml}^{-1}$. These findings are in agreement with those of Siracusa *et al.* (1980), who noted that the development of two-cell embryos to blastocysts was reduced to 75% of the control with colcemid $3 \times 10^{-7} \text{ mol l}^{-1}$ ($0.1 \mu\text{g ml}^{-1}$). Ability of the blastocysts, after colcemid treatment, to develop into young after transfer to recipients was significantly reduced, even when the embryos were treated at the lower concentration ($0.02 \mu\text{g ml}^{-1}$). Most two-cell embryos treated with colcemid were at the two-cell stage when the control embryos were at the four-cell stage. They divided into four cells within 1 h, as also noted for nocodazole, but had several small blastomeres or cytoplasmic fragments (as shown in Figs 3a and b) even at the blastocyst stage. Such an unequal cytoplasmic division is probably due to the formation of multipolar mitoses similar to that shown for other cells treated with griseofulvin (Grisham *et al.*, 1973; Adair, 1974; Weber *et al.*, 1976). This might have been one reason for the low developmental ability of these blastocysts, but we did not observe nuclear material in the fragments and did not examine the normality of the chromosomes of treated embryos.

Synchronization of the cell cycle between a donor nucleus and recipient cytoplasm is important for pronuclear and nuclear exchange (Smith *et al.*, 1988, 1990), nuclear transplantation of fetal germ cells (Kato & Tsunoda, in press) and the production of chimaeric embryos (Kono *et al.*, 1989). The observation that nocodazole can synchronize cell division of two-cell mouse embryos up to the four-cell stage may be applicable to such studies.

This investigation was supported by a grant from the Japanese Ministry of Science and Technology (Developmental Biotechnology), Ministry of Education, Science and Culture (No. 01102028), Bio Science Laboratory (Yamagata), Human Science Foundation, Inamori Foundation and the Sasakawa Scientific Research Grant, Kinki University.

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Received 14 January 1991