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Short Contibution

A reversible block at the G_1/S border during cell cycle progression of mouse embryos

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ABSTRACT Late 2-cell stage mouse embryos were cultured in M-199 plus 100 μg/ml Na pyruvate 25 μg/ml gentamycin and 0.3% BSA with or without mimosine (200 μM, 150 μM, 100 μM and 50 μM) for a short (4-5 h) or long (18-20 h) culture period; after drug removal subsequent embryo development was evaluated. Late 2-cell stage mouse embryos treated with mimosine were blocked at the 4-cell stage. Autoradiographic studies show that mimosine inhibits cell cycle progression in mouse embryos at the G_1/S boundary. The onset of DNA replication occurs within 15 min of releasing the embryos from mimosine block. Embryos pretreated with mimosine at 200 μM and 150 μM for 4-5 h progress after 3-4 days in culture to hatched blastocyst (71% and 79%, respectively) compared with control (90%). However a longer pretreatment (18-20 h) with mimosine at 200 μM was significantly detrimental to the subsequent developmental progression to hatched blastocyst (2% vs 81%, $p \leq 0.05$); the proportion of degenerated embryos was significantly increased with mimosine at 200 μM and 150 μM compared with control (57% and 28% vs 4%, $p \leq 0.05$) after 3-4 days in culture. Preliminary studies with mimosine treatment at 100 μM and 50 μM for 18-20 h show that 70% and 37% of the embryos were blocked at 4-cell stage, respectively. These results indicate that mimosine inhibits cell cycle progress in mouse embryos at the G_1/S border and thus induces a reversible arrest in a dose- and time-dependent manner.

KEY WORDS: *mimosine, cell cycle, inhibitor, DNA synthesis, embryo, mouse*

Mimosine, a non proteinogen occurring amino acid, is found in the seeds and foliage of the legume genera Mimosa and leucaena (Thompson *et al.*, 1969). Recently mimosine has been shown to arrest reversibly cell cycle progression at the G_1/S boundary prior to the onset of DNA replication in mammalian cells (Lalande, 1990; Hoffman *et al.*, 1991; Mosca *et al.*, 1992). The data suggest that mimosine may be useful for the synchronization of cell cycles. In mammals there are no reliable methods for synchronizing cells at specific stages of the cell cycle in embryos without reducing their subsequent ability to develop *in vitro*. Moreover, the G_1 phase in early embryos is extremely short (Pratt, 1987). Among the S-phase-specific inhibitors widely used, aphidicolin has been shown to block cell cycle progression in S-phase and prevent the entry of cells into S-phase at the G_1/S border. However the toxicity of aphidicolin has been described in mouse embryos (Howlett, 1986).

In mouse, rabbit, bovine and sheep, the synchronization of cell cycles between a donor nucleus and a recipient cytoplast has been shown to be crucial for the developmental progression of the reconstructed embryos (Collas *et al.*, 1992b; Barnes *et al.*, 1993; Cheong *et al.*, 1993; Loi *et al.*, 1993). Previously the methods used for synchronizing the cell cycle in G_1/early S-phase have been neither reliable nor simple.

We initiated studies with mimosine in the hope that it could be used to synchronize cell cycles in mouse embryos. The present study was performed in order to determine the effects of mimosine, including dose response, duration of treatment, reversibility of the drug and release from the drug, and to examine *in vitro* the developmental ability of the late 2-cell stage mouse embryos in which cell division was inhibited.

This is the first report demonstrating that the cell cycle of mouse embryos can be synchronized in G_1/early S-phase by novel drug treatment without reducing subsequent developmental ability *in vitro*.

Effect of mimosine on developmental progression after drug removal

In the present study we first determine the minimal drug concentration and duration of treatment required to maximally block the embryos just after the second cleavage. Low doses of mimosine 100 μM and 50 μM over a prolonged period (18-20 h) appear to arrest partially the developmental process: only 70% and 37% of the embryos were blocked respectively at the 4-cell stage (Table 1). Higher doses of mimosine (200 μM and 150 μM) induce total developmental arrest of embryos (99-100%) at the 4-cell stage

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TABLE 1

EFFECT OF MIMOSINE 100 μM AND 50 μM ON THE DEVELOPMENTAL PROGRESSION OF LATE 2-CELL STAGE MOUSE EMBRYOS AFTER 18-20 h OF TREATMENT

Treatment	Total number[b] of embryos	Embryo stages (%)			
		Dg	2-cell	4-cell	8-cell
Control	33	—	—	4 (18%)	29 (88%)
MIM 100 μM	67	—	—	47 (70%)	20 (30%)
MIM 50 μM	43	—	—	16 (37%)	25 (58%)

[b]Total of 2 replicates; Dg, degenerated; MIM, mimosine.

(Table 2). In all cases, we observed that cleavage occurred at the beginning of the culture period. Arrested embryos exhibited regular and well formed blastomeres (Fig. 1A and C, pretreatment with mimosine 150 μM for 4-5 h and 18-20 h respectively).

In order to examine the reversibility of this inhibition we next tested the ability of mouse embryos to cleave *in vitro* after drug removal. Post-mimosine development was monitored by examining the ability of embryos to develop during a 3-4 day culture period. The proportion of embryos pretreated with mimosine at 200 μM or 150 μM (Table 2) for 4-5 h (Fig. 1B, embryos pretreated with mimosine 150 μM) that developed to hatched blastocyst (71% and 79%, respectively) after 3-4 days in culture was not significantly different from controls (90%). No significant difference was observed in the proportion of degenerated embryos (5% and 4%, respectively) when compared with control (0.7%). The observations show that the embryos develop to the blastocyst stage in culture after drug release, suggesting that mimosine reversibly inhibits the developmental progression of mouse embryos. These findings are in agreement with those observed in human lymphoblastoid cells indicating that mimosine identifies a previously undefined reversible cell cycle arrest point (Lalande, 1990). However, both the duration of treatment and concentration of mimosine used affect post-mimosine development from the 4-cell to the hatched blastocyst stage. A longer pretreatment with mimosine 200 μM resulted in poor developmental progression to the blastocyst stage and more degenerated embryos. Thus, the proportion of embryos pretreated with mimosine 200 μM for 18-20 h that progressed to hatched blastocyst was significantly lower (2%, $p \leq 0.05$) than the control (81%). Conversely, after 18 h of mimosine treatment the proportion of degenerated embryos observed after 3-4 days in culture was significantly higher (57%, $p \leq 0.05$) than the controls (4%). Exposure to mimosine for 18-20 h, but at the lower concentration of 150 μM, decreased to 44% (controls 81%) the proportion of embryos progressing to hatched blastocysts (Fig. 1D) and increased the proportion of degenerated embryos (28%) relative to controls (4% degenerated embryos); these differences did not, however, reach statistical significance at the 5% level. Thus the 4-cell stage embryos that developed after 3-4 days in culture from embryos treated with mimosine 150 μM for 18-20 h showed small blastomeres and cytoplasmic fragments in the perivitelline space (Fig. 1D).

The statistical analysis showed significant differences ($p \leq 0.01$) in the proportion of degenerated embryos and hatched blastocyst pretreated with mimosine 200 μM when comparing 4-5 h and 18-20 h of treatment. Similarly, significant differences ($p \leq 0.01$) were observed in the proportion of hatched blastocyst pretreated with mimosine 150 μM when comparing 4-5 h and 18-20 h of treatment. By contrast, mimosine 150 μM for a short period (4-5 h) of treatment can be used without impairing the developmental ability of mouse embryos for further studies. These observations indicate that mimosine induces a reversible arrest in a dose- and time-dependent manner.

Autoradiographic study

The autoradiographic study was used to assess the effect of mimosine on cell cycle progress in mouse embryos. Fig. 2 shows that in control embryos strong labeling is invariably observed over the nuclei of 4-cell stage mouse blastomeres. By contrast, embryos treated at the late 2-cell stage with mimosine 150 μM for 5 h show only few grains over the nuclei of 4-cell stage mouse blastomeres. These results suggest that mimosine blocks cell cycle progression

TABLE 2

EFFECT OF MIMOSINE 200 μM AND 150 μM ON THE DEVELOPMENT OF LATE 2-CELL STAGE MOUSE EMBRYOS PRETREATED 4-5 h AND 18-20 h AND CULTURED 3-4 DAYS *IN VITRO*

Treatment	Duration of Treatment	Total number[b] of embryos	Developmental stage (%)						
			Dg	2-cell	4-cell	8-cell	M	B	HB
Control	4-5 h	148	1 (0.7)	—	—	—	2 (1)	12 (8)	133 (90)
MIM 200 μM	4-5 h	145	8 (5)	1	1	—	6 (4)	25 (17)	103 (71)
MIM 150 μM	4-5 h	154	7 (4)	—	—	—	7 (4)	19 (12)	121 (79)
Control	18-20 h	129	5 (4)	3	1	—	9 (7)	7 (5)	104 (81)
MIM 200 μM	18-20 h	120	67* (57)	1	22 (18)	14 (12)	—	14 (12)	2* (2)
MIM 150 μM	18-20 h	128	32 (28)	2 (1)	2 (1)	2 (1)	2 (1)	24 (19)	57[a] (44)

[b]Total of 5 replicates; *$p \leq 0.05$; [a]$p \leq 0.01$ when compared 4-5 h to 18-20 h; Dg, degenerated; M, morula; B, blastocyst; HB, hatched blastocysts; MIM, mimosine.

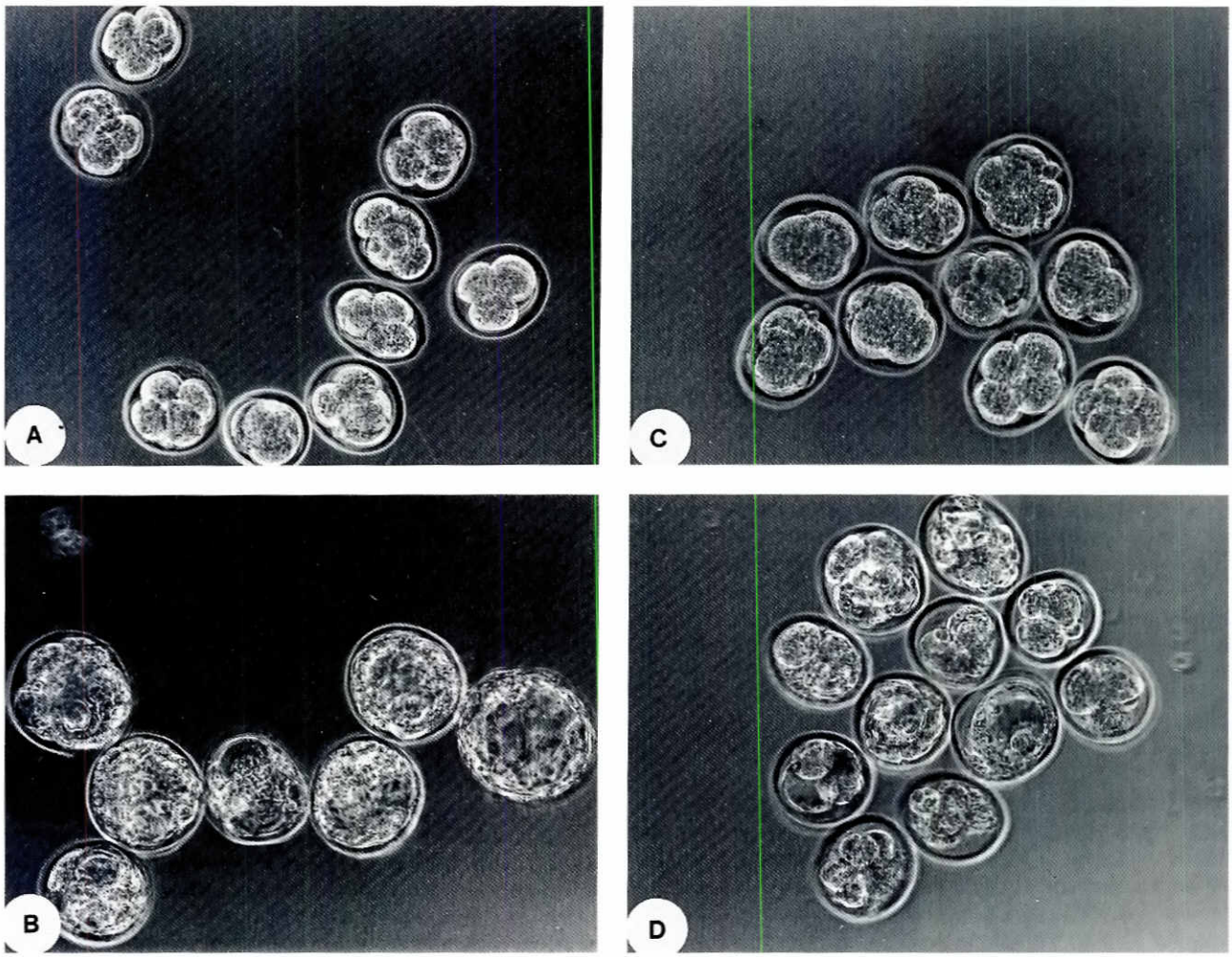


Fig. 1. Mouse embryos treated with 150 μM mimosine for (A) 4-5 h and (C) 18-20 h. *At the end of the treatment period (4-5 h and 18-20 h) none of these 4-cell stage embryos exhibited signs of degeneration or fragmentation. Blastocyst formation after drug removal;* **(B)** *embryos pretreated 4-5 h and* **(D)** *embryos pretreated 18-20 h with 150 μM mimosine (x200).*

of 4-cell stage mouse embryos at the G_1/S boundary prior to the period of intensive DNA synthesis. This is consistent with recent findings demonstrating that mimosine reversibly arrests human lymphoblastoid cells before the onset of active DNA replication as measured by flow cytometry analysis of DNA content and Northern blot of selected mRNAs such as p53 mRNA (Lalande, 1990; Watson *et al.*, 1991).

Immunocytochemistry

Indirect immunofluorescence detection of BrDU was used to measure levels of DNA synthesis in mimosine-treated embryos (Fig. 3). Observations revealed that 4 cell stage mouse embryos in the control group exhibited intensive labeling over the nuclei (Fig. 3B). At the zero time point of release from mimosine the labeling over the nuclei of each blastomere was severely impaired (Fig. 3D). The incorporation of BrDU was evident at 15 min post-release and increased between 15 and 30 min (Fig. 3F and H). A stable labeling was observed after 60 and 120 min (data not shown). This result demonstrates that the 4-cell stage mouse embryos released from mimosine enter in S-phase subsequent to a 15 min lag that is presumably necessary for mimosine to dissociate from its substrate. This kinetic analysis confirms the hypothesis (Lalande, 1990) that mimosine identifies a previously undefined reversible cell cycle arrest point in very late G_1 phase. These observations are in agreement with other findings in human lymphoblastoid cells (Watson *et al.*, 1991).

Conclusions

Mimosine appears to be a suitable compound for affecting the synchronous release of cells from G_1 into S-phase and for analyzing the biochemical events associated with the cell cycle phase transition. However, the precise mechanism of action of mimosine is at present unknown and require further investigation. The compound is a known iron chelator (Kontoghiorghes and Evans, 1985) and the mechanism of action may involve inhibition of an iron- dependent pathway in late G_1 phase. More recently, it has been shown that mimosine apparently blocks cell cycle progress by suppressing the formation of a rare amino acid, hypusine in the eukaryotic translation initiation factor 4D (Hoffman *et al.*, 1991). The role of hypusine in mammalian cell cycle control as well as the effect of mimosine on this process are not yet well understood.

The availability of mimosine will be extremely useful for cell cycle synchronization between donor nucleus and recipient cytoplasm, which is central to the development of reconstructed

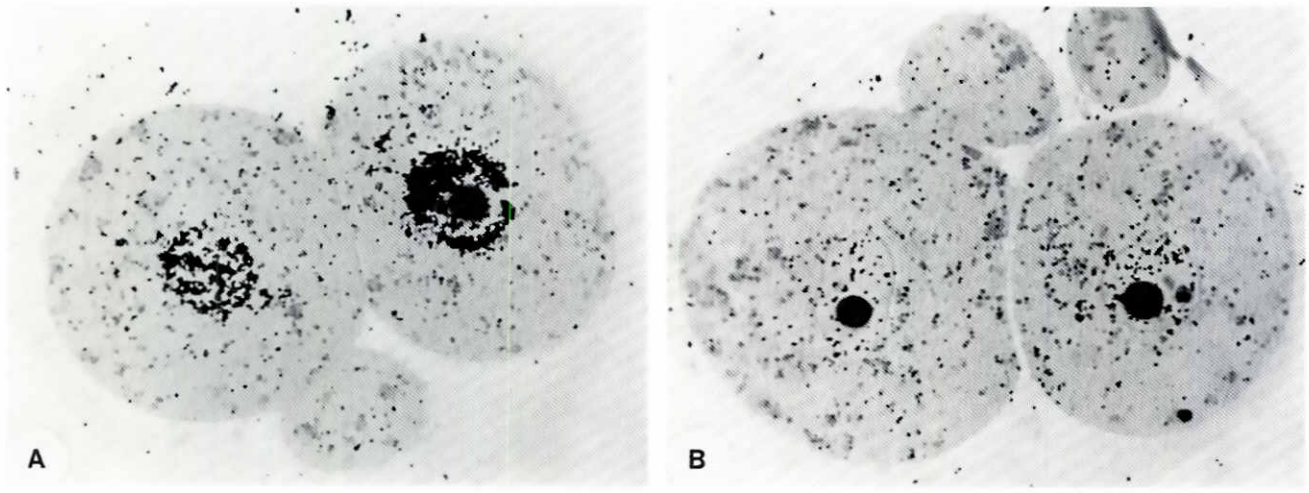


Fig. 2. Nuclei of 4-cell stage mouse embryos labeled with ^{3}H-thymidine for 60 min. *Note in* **(A)** *an intensive labeling in control embryos and* **(B)** *a very weak labeling in pretreated embryos with mimosine 150 μM for 4 h (x320).*

embryos after nuclear transplantation. Studies in rabbit and mouse using donor blastomeres at defined cell cycle stages have shown a better development to blastocyst when G_1/early S-phase nuclei are transferred to metaphase-II cytoplasts than with late S or G_2 phase nuclei (Collas *et al.*, 1992b; Cheong, 1993). Also it has been shown that reconstructed embryos with late S-phase nuclei exhibit chromosome abnormalities (Collas *et al.*, 1992a). In bovine it seems that only G_1/ early S-phase nuclei, transferred at the time of activation, undergo DNA synthesis and maintain correct ploidy in the reconstructed embryos. In contrast, no DNA synthesis was observed in the hybrid embryos when G_2 nuclei were transplanted into activated metaphase-II cytoplasts, suggesting that rereplication of G_2 nuclei was prevented (Campbell *et al.*, 1993). On the other hand, normal spindle formation was observed in the mouse when G_2 nuclei were fused to metaphase-II cytoplasts (Fulka *et al.*, 1993) and hence developmental progression to blastocyst stage occurred (Kono *et al.*, 1992). In this latter case, a longer exposure to MPF (maturation promoting factor), central in cell cycle regulation, was required. More recently, in bovine and sheep S-phase nuclei transferred into S-phase cytoplasts appeared to result in a high proportion of reconstructed embryos with normal chromosome content and therefore, high rates of development to blastocyst stage (Barnes *et al.*, 1993; Loi *et al.*, 1993).

In mammalian cells, emphasis has been placed on identifying replication inhibitors that can be used either to arrest or synchronize actively dividing cells. Among the most widely used drugs for these applications are hydroxyurea (Campbell *et al.*, 1993), 5-fluordeoxyuridine and aphidicolin (Howlett, 1986; Campbell *et al.*, 1993; Cheong *et al.*, 1993). These well defined drugs have been used in various combinations with and without the addition of colcemid or nocodazole (Collas *et al.*, 1992a,b; Kato and Tsunoda, 1992; Campbell *et al.*, 1993). Aphidicolin has become the agent of choice for synchronizing cells in S-phase and has been reported to be fully reversible. However such inhibition was found to be toxic and non-reversible in long term culture in mouse embryos (Dean and Rossant, 1984) reducing the ability of the embryos to recover and cleave after drug removal (Howlett, 1986). Recent studies on the effect of mimosine and aphidicolin upon cell cycle demonstrate that these two inhibitors do not act at the same point (Hoffman *et al.*, 1991). Although aphidicolin blocks cell cycle progression in early S-phase, it was found that mimosine acts prior to aphidicolin (Hoffman *et al.*, 1991; Watson *et al.*, 1991).

The observation in the present paper that mimosine can reversibly synchronize the cell cycle at the G_1/S border will have important applications both for nuclear transplantation and for analyzing the biochemical and regulatory events associated with cell cycle phase transition.

Experimental Procedures

Embryo collection

The embryos collected at the late 2-cell stage were obtained from 4-week-old superovulated F_1 hybrid mice (C57BL/6J x CBA). The females were injected with 5-10 IU of PMSG followed 44 h later with 5 IU of hCG, before being placed overnight in individual cages with males of proven fertility. The following morning, the effectiveness of mating was confirmed by the presence of a vaginal plug. Late 2-cell stage embryos were collected 48-50 h post-hCG in HEPES-buffered M-199. A pool of all zygotes from different females was collected before each experiment.

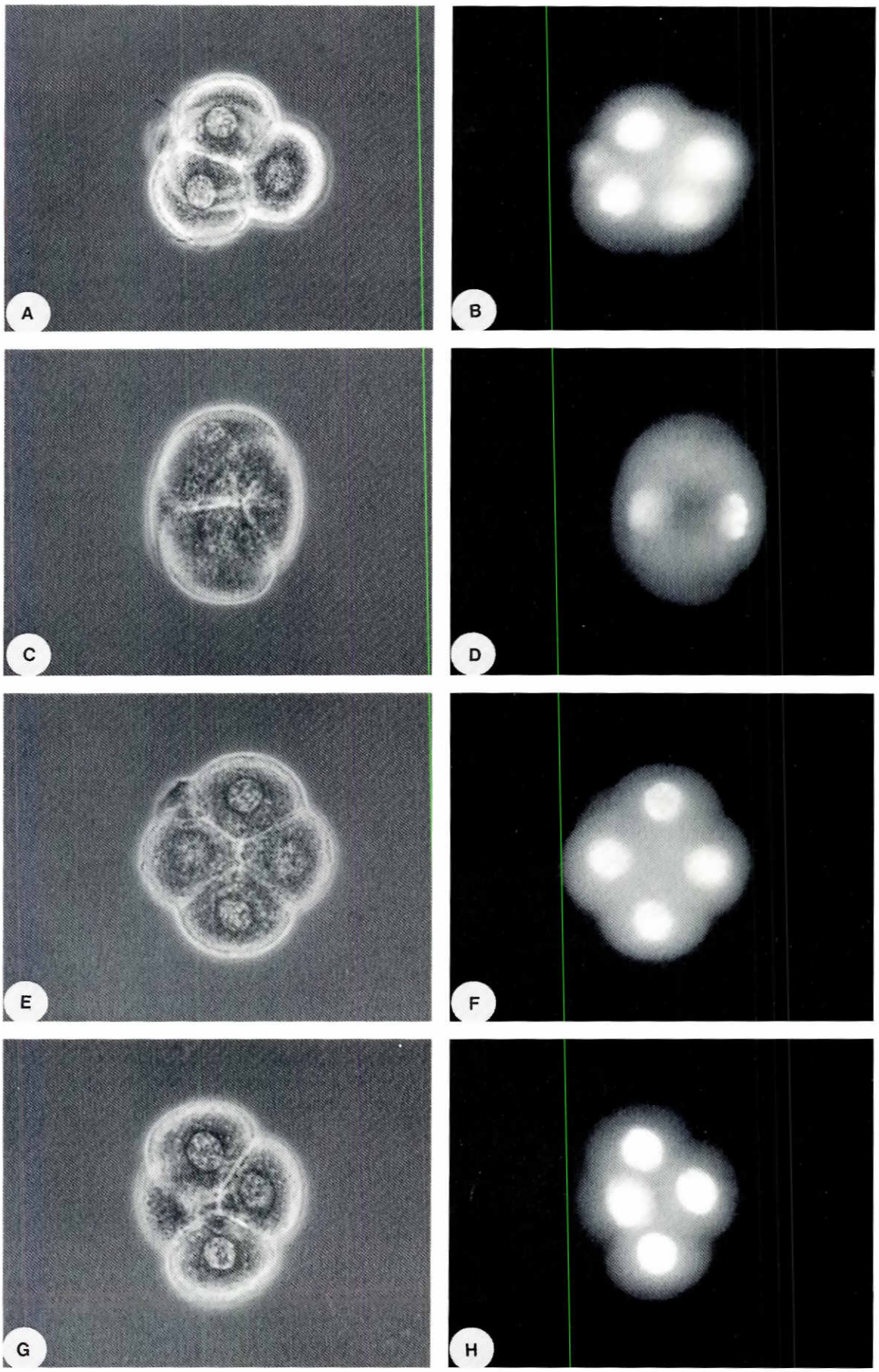
Preparation of mimosine

A stock solution of Mimosine (Sigma, St. Louis, USA) was prepared by resuspending the drug in HEPES-buffered M-199 at 10 mM.

Treatment with mimosine and cleavage assessment

Late 2-cell stage mouse embryos were cultured in 4-well tissue culture plates in M-199 supplemented with Na-pyruvate (100 μg/ml), gentamycin (25 μg/ml) and BSA (3 mg/ml) with or without mimosine for a short (4-5 h) and a long (18-20 h) culture period at 37°C in 5% CO_2 in humidified air. In order to define the limiting inhibitory effect of mimosine different concentrations were tested: 200 μM, 150 μM, 100 μM and 50 μM. Cleavage was carefully examined at the beginning of the culture period and the viability of

Fig. 3. Assessment of DNA synthesis in the nuclei of 4-cell stage mouse embryos released from mimosine. (A,C,E and G) *4-cell stage embryos in phase contrast (x400).* **(B)** *BrDU incorporation in the nuclei of control group embryos (x400).* **(D)** *DNA replication arrest 4 h after treatment with mimosine (x400).* **(F)** *Resumption of DNA replication 15 min post-release from mimosine (x400).* **(H)** *BrDU incorporation in the nuclei 30 min post-release from mimosine (x400).*


A
B
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the embryos after treatment was assessed. Treated embryos were washed 4 times in 500 μl of prewarmed HEPES-buffered M-199 and then cultured in supplemented M-199 for 3-4 days at 37°C in 5% CO_2 in air. The control embryos were washed and cultured for the same period of time (as above).

Statistical analysis

Data were analyzed by an analysis of variance. The Mann-Whitney U-test was used for the comparisons between times for a given treatment.

Autoradiography and immunocytochemistry

Late 2-cell stage mouse embryos were allocated to 2 groups i) untreated (control) and ii) treated with mimosine 150 μM and cultured for 4 h at 37°C in 5% CO_2 in air. The embryos were periodically inspected and only those cleaved at the same time to the 4-cell stage were prepared for autoradiography or resuspended in fresh medium with 100 μM of 5-bromo-2-deoxyuridine 5-trisphosphate (BrDU) for release experiments.

Radiolabeling

After 4 h in culture, ^{3}H-thymidine was added in both groups to the culture medium and the embryos were incubated for 60 min. The embryos were then washed thoroughly and treated as described by Kanka *et al.* (1991) and Crozet *et al.* (1986).

Release experiment

After 1 h in culture, BrDU 100 μM was added to the medium in both groups i) untreated and ii) mimosine-treated (150 μM) embryos and cultured for 3 h at 37°C in 5% CO_2 in air. Embryos (30-40 embryos in each group) were divided into 2 groups i) untreated and ii) mimosine-treated (150 μM) and cultured for 1 h before the addition of 100 μM BrDU was added to the medium in both groups. Treated and control embryos were cultured for a further 3 h at 37°C in 5% CO_2 in air before the treated group was removed from mimosine inhibition. Both groups were cultured thereafter in fresh medium containing BrDU but no mimosine for varying lengths of time. Embryos were removed at 0, 15, 30, 60 and 120 min after release and processed for indirect immunofluorescence as described by Campbell *et al.* (1993).

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