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The Demethylating Agent 5-Aza-2′-Deoxycytidine (5-AZA-CdR) Inhibits The Development of Preimplantation Mouse Embryos*

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Abstract DNA methylation is crucial for mammalian development, and DNA methylation is always in the dynamic status during preimplantation mouse embryos development. The effects of 5-AZA-CdR on the development of preimplantation mouse embryos were evaluated. Preimplantation mouse embryos created by *in vitro* fertilization were cultured continuously in 5-AZA-CdR (0.2, 1.0, or 5.0 μmol/L). Fertilized oocytes exposed to CZB containing 5-AZA-CdR at the pronuclear stage were unable to form morulae (0.2 and 1.0 μmol/L) or 4-cell embryos (5.0 μmol/L), while 2-cell stage embryos exposed to 5-AZA-CdR developed into uncompacted 8-cell (0.2 and 1.0 μmol/L) or 3/4-cell (5.0 μmol/L) stage embryos. The rate of morula formation was significantly lower in 4-cell embryos cultured in 5-AZA-CdR (1.0 or 5.0 μmol/L) than that in control embryos ($P < 0.05$). These data indicate that 5-AZA-CdR inhibits the development of mouse preimplantation embryos. Apoptosis, DNA methylation, and transcriptional activity were analyzed to determine the reason for these developmental defects. An annexin V-PI assay revealed that high doses of 5-AZA-CdR led to apoptosis. Compared to the controls, DNA methylation was significantly reduced in uncompacted 8-cell embryos and morulae ($P < 0.05$) in a dose-dependent manner, whereas no significant change was detected in 2- or 4-cell embryos ($P > 0.05$). The observed changes in transcriptional activity, determined by measuring the incorporation of BrUTP, were similar to the observed alterations in DNA methylation. Therefore, the developmental defects induced by 5-AZA-CdR appear to be mediated by alterations in DNA methylation and transcriptional activity in preimplantation mouse embryos.

Key words 5-AZA-CdR, apoptosis, development, DNA methylation, transcriptional activity
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The methylation of cytosine residues in the CpG dinucleotides of nuclear DNA is crucial for mammalian development[1~4] and several key biological functions, including gene expression[5], cell differentiation[6], genomic imprinting[7], X chromosome inactivation[8], chromatin modification[2], cancer[9] and aging[10,11].

Genome-wide demethylation, which characterizes preimplantation embryonic development in mice, occurs by both active and passive mechanisms. The paternal genome undergoes active and rapid genome-wide demethylation prior to DNA replication[12~14], and paternal active demethylation may be related to the activation of zygotic transcription in mice[15]. In comparison, during passive demethylation, a stepwise drop in methylation occurs up to the morula stage following the completion of the first cell cycle due to the absence of Dnmt1, a maintenance methylase[16~19]. At present, the relationship between passive demethylation and early embryonic development in mice is unknown, although it may be associated with two major morphological transitions that occur during preimplantation development, namely, compaction and cavitation.

The discovery of aberrant hypermethylation of CpG islands in cancer and leukemia cells[20~24] has sparked a renewed interest in DNA methylation inhibitors such as the cytosine analogs 5-azacytidine and 5-aza-2′-deoxycytidine (5-AZA-CdR). 5-AZA-CdR has not only been used as an inhibitor of DNA methylation[25,26] and to induce gene expression and

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cellular differentiation [27～29], but also as a potent antineoplastic[30] and antileukemic agent[31～33]. 5-AZA-CdR incorporates into DNA, forming stable adducts with DNA methyltransferases [28]. Such adducts can inhibit DNA synthesis, transcription, and repair[29, 34, 35]. Therefore, 5-AZA-CdR is a possible mutagen with demonstrated negative effects on treated cells. Moreover, 5-AZA-CdR has been shown to induce apoptosis in human cancer cells[36, 37] and developmental defects in mouse and rat embryos [38, 39]; however, the effects of 5-AZA-CdR have only been shown for implantation embryos and not for preimplantation embryos. Thus, in this study, the development of 5-AZA-CdR-treated mouse preimplantation embryos was analyzed to determine the relationship between DNA methylation and early mouse embryonic development. Our data may lead to the future clinical use of 5-AZA-CdR.

1 Materials and methods

1.1 *In vitro* fertilization and embryo culture

Female 21 ～28-day-old Kunming mice were superovulated with 5 IU of pregnant mares' serum gonadotropin (PMSG) and then treated 48 h later with 5 IU of human chorionic gonadotropin (hCG). Unfertilized metaphase II-arrested oocytes were collected 14～15 h later in Whitten's medium[40] from the ampullae of the oviducts. Sperm were collected from the caudal epididymides of adult Kunming males and preincubated in Whitten's medium for 2 h in an atmosphere of 5% CO_2 and 95% air at 37.5℃. The oocytes were then inseminated with the capacitated sperm in Whitten's medium. Six hours after insemination, the fertilized oocytes were washed with CZB medium[41] and cultured in a humidified atmosphere of 5% CO_2 and 95% air at 37.5℃.

1.2 Treatment with 5-AZA-CdR

5-AZA-CdR was purchased from Sigma. Pronuclear, 2-cell, and 4-cell embryos were incubated with 5-AZA-CdR (diluted in CZB) at 0.2, 1.0 and 5.0 μmol/L and allowed to develop *in vitro*. The medium was replaced with CZB containing fresh 5-AZA-CdR every 24 h.

1.3 Evaluation of apoptosis using annexin V and propidium iodide

Annexin Ⅴ and propidium iodide (PI) staining (BIPEC Biopharma, Cambridge, MA) of living embryos was used to identify apoptotic cells. Soon after initiating apoptosis, most cells translocate phosphatidylserine (PS) from the inner face of the plasma membrane to the cell surface. Once on the cell surface, PS can be easily detected by staining with FITC-annexin Ⅴ. Cells that have lost membrane integrity will have red (PI-stained) nuclei. The embryos were washed twice for 5 min each with cold PBS, then placed in annexin binding buffer and incubated for 15 min in the presence of FITC-annexin Ⅴ at 4 to 8℃ in the dark. Next, 10 μl of PI solution was added and the samples were incubated for another 5 min at 4 to 8℃ in the dark. The embryos were then transferred to a drop of PBS containing Vectashield (Vector Laboratories, Burlingame, CA) on a glass slide and examined under fluorescence microscopy. Positive staining for annexin Ⅴ on the outer membrane surface was observed as a bright green signal.

1.4 Immunochemical analysis

For the detection of 5-methyl-cytosine (5-MeC), the embryos were washed in PBS containing 3 g/L polyvinylpyrrolidone (PBS/PVP), fixed for 1 h in 3.7% paraformaldehyde in PBS, and permeabilized with 0.5% Triton X-100 in PBS for 20 min at room temperature. The embryos were then treated with 2 mol/L HCl at room temperature for 30 min then neutralized with 100 mmol/L Tris-HCl buffer (pH 8.5) for 10 min. After extensive washing with 0.05% Tween 20 in PBS, the embryos were incubated with anti-5-MeC antibodies (Abcam plc., Cambridge, UK), followed by incubation with a Cy3-conjugated anti-mouse IgG secondary antibody (Jackson ImmunoResearch, West Grove, PA). The DNA was stained by treatment with 3 g/L DAPI for 20 min, and the cells were mounted on a glass slide in Vectashield. Fluorescence was detected under a fluorescence microscope, and the signal intensity was quantified using NIH Image software (Bethesda, MD).

1.5 *In vitro* transcription assay

The transcriptional activities of the embryos were determined by measuring the incorporation of 5-bromouridine-5-triphosphate (BrUTP; Sigma) into membrane-permeabilized embryos[42]. Briefly, the plasma membranes of the embryos were permeabilized by treatment with 0.05% Triton X-100 for 1 to 2 min. The embryos were then subjected to *in vitro* transcription reactions in which UTP was replaced by BrUTP. After incubation for 15 min at 33℃, the embryos were fixed overnight in 3.7% paraformaldehyde. Permeabilization of the nuclear membrane was performed with 0.2% Triton X-100 for 3 min. The incorporated BrU was

detected by immunostaining with anti-BrdU antibodies, together with a Cy3-conjugated anti-mouse IgG secondary antibody (Jackson ImmunoResearch). Fluorescence was detected by fluorescence microscopy, and the signal intensity was quantified using NIH Image software.

1.6 Quantification of fluorescence intensity

The fluorescence intensity was quantified using ImageJ 1.35s as previously described [42]. In brief, the pixel value within a constant area from five different nuclear regions and five different cytoplasmic regions was measured, and the average cytoplasmic value was subtracted from the average nuclear value.

1.7 Data analysis

Each experiment included controls, and three trials were conducted for each treatment. The data were analyzed by one-way analysis of variance (ANOVA) after being transformed *via* LSD (Least-Significant Difference) using SPSS software (SPSS Inc., Chicago, IL; $P<0.05$ was considered significant).

2 Results

2.1 5-AZA-CdR decreases the developmental competence of preimplantation embryos

Pronuclear embryos were collected at 6 h post-fertilization and cultured in medium containing various concentrations of 5-AZA-CdR. The developmental rates were registered 24, 48, 72 and 96 h after insemination, corresponding to 2-cell embryos, 4-cell embryos, morulae, and blastocysts, respectively (Table 1). These pronuclear embryos were unable to develop to 8-cell embryos at all concentrations of 5-AZA-CdR (0.2, 1.0 and 5.0 μmol/L). They developed to the 5 ～7 cell stage when cultured in 0.2 and 1.0 μmol/L 5-AZA-CdR, whereas only developed to 2-cell embryos at 5.0 μmol/L 5-AZA-CdR. Thus, the toxicity of 5-AZA-CdR toward preimplantation embryos increases with the concentration.

Table 1 Development of mouse embryos cultured in CZB medium containing various concentrations of 5-AZA-CdR beginning at the pronuclear stage

c(5-AZA-CdR)/(μmol•L^{-1})	No. of fertilized oocytes	No. ($\bar{x}\pm s$) of embryos developing into/%			
		2-cell embryos	3/4-cell embryos	Morulae	Blastocysts
0.0	71	57(81.2±5.8)[1)]	50(70.7±2.1)[1)]	31(44.1±3.6)[1)]	23(33.1±5.5)[1)]
0.2	76	64(75.1±2.1)[1)]	60(70.7±3.8)[1)]	0(0.0±0.0)[2)]	0(0.0±0.0)[2)]
1.0	98	86(86.9±6.0)[1)]	76(76.9±4.6)[1)]	0(0.0±0.0)[2)]	0(0.0±0.0)[2)]
5.0	75	59(79.3±2.5)[1)]	0(0.0±0.0)[2)]	0(0.0±0.0)[2)]	0(0.0±0.0)[2)]

[1,2)] Values with a common superscript in the same column do not differ significantly ($P>0.05$).

Because 5-AZA-CdR had no effect on the rate of 2-cell formation, we tried adding 5-AZA-CdR to CZB between the 2-cell and blastula stages. The percentage of embryos that developed to each stage is shown in Table 2. Following treatment with 5-AZA-CdR (0.2, 1.0 and 5.0 μmol/L), the rate of 3/4-cell formation did not differ significantly from that in the control group ($P > 0.05$). In comparison, for the development of 8-cell, the rate in control was significantly higher than 0.2 and 1.0 μmol/L 5-AZA-CdR($P<0.05$). Interestingly, 8-cell embryos in 0.2 and 1.0 μmol/L 5-AZA-CdR treatments could not compact as the control and they were still blocked at 8-cell stage while the control developed to blastocysts. In 5.0 μmol/L 5-AZA-CdR, although the embryos developed to the 4-cell stage, none developed to the 8-cell stage, showing that the toxicity of the compound was greater at 5.0 μmol/L than at 0.2 or 1.0 μmol/L.

Table 2 Changes in developmental rate in 2-cell embryos exposed to 5-AZA-CdR until the blastocyst stage

c(5-AZA-CdR)/(μmol•L^{-1})	No. of 2-cell embryos	No. ($\bar{x}\pm s$) of 2-cell embryos developing into/%		
		3/4-cell embryos	8-cell (compacted) embryos	Blastocysts
0.0	85	75(88.4±0.5)[1)]	44(50.0±4.2)[1)](+)	36(40.7±4.6)[1)]
0.2	78	63(80.3±2.8)[1)]	15(18.6±4.9)[2)](–)	0(0.0±0.0)[2)]
1.0	73	60(82.4±3.0)[1)]	9(12.1±3.6)[2)](–)	0(0.0±0.0)[2)]
5.0	83	70(84.1±3.0)[1)]	0(0.0±0.0)[3)](–)	0(0.0±0.0)[2)]

[1,2,3)] Values with a common superscript in the same column do not differ significantly ($P>0.05$).

Since the percentage of embryos at the 3/4-cell stage was stable regardless of treatment (Table 2), 5-AZA-CdR was added to the culture medium between the 4-cell and blastocyst stages. At 0.2 μmol/L, the rate of morula formation was similar between the control and 5-AZA-CdR-treated embryos ($P > 0.05$, Table 3); however, at 1.0 and 5.0 μmol/L, the rate of morula formation was significantly lower among the 5-AZA-CdR-treated embryos than the controls ($P < 0.05$), and at 5.0 μmol/L, the rate dropped to its lowest value (19.9%, Table 3). After 24 h, the cytoplasmic membranes of the morulae had been destroyed and the cytoplasm appeared black. And none of them developed to blastocyst stage.

Table 3 Changes in developmental rate in 4-cell embryos exposed to 5-AZA-CdR until the formation of blastulae

c(5-AZA-CdR)/ (μmol•L^{-1})	No. of 4-cell embryos	No. ($\bar{x} \pm s$) of 4-cell embryos developing into/%	
		Morulae	Blastocysts
0.0	84	46(54.8±0.7)[1)]	36(42.7±1.5)[1)]
0.2	70	38(54.2±2.3)[1)]	0(0.0±0.0)[2)]
1.0	84	37(43.9±1.4)[2)]	0(0.0±0.0)[2)]
5.0	77	15(19.9±4.4)[3)]	0(0.0±0.0)[2)]

[1, 2, 3)] Values with a common superscript in the same column do not differ significantly ($P > 0.05$).

Taken together, these data indicate that 5-AZA-CdR has adverse effects on the development of preimplantation embryos, especially the development of morulae and blastocysts.

2.2 5-AZA-CdR induces apoptosis in preimplantation embryos at high doses

We next sought to uncover whether the reason for the developmental arrest among the embryos was apoptosis and/or 5-AZA-CdR-induced demethylation. We tested for apoptotic cells using an annexin V-FITC detection kit. Few apoptotic cells were present in 2- and 4-cell embryos treated with 0.2, 1.0, or 5.0 μmol/L 5-AZA-CdR. No obvious cases of apoptosis were identified among 8-cell embryos derived from 2- or 4-cell embryos exposed to 0.2 or 1.0 μmol/L 5-AZA-CdR. However, when 5.0 μmol/L 5-AZA-CdR was added beginning at the 4-cell stage, apoptosis was identified among 8-cell embryos and morula at a rate of 13/64 and 15/56, respectively. The apoptotic cells bore typical features of early apoptotic cells, with the PS from the inner face of the plasma membrane exposed on the cell surface and no PI signal (Figure 1).

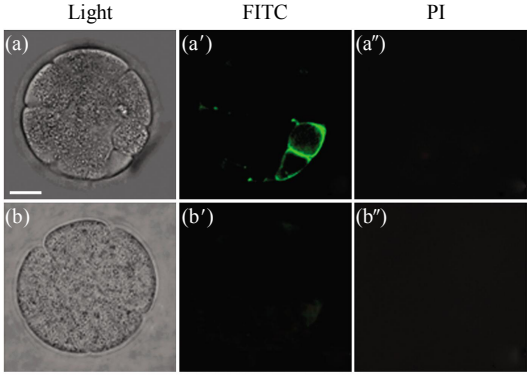

Fig. 1 Detection of apoptosis by annexin V-PI assay
Eight-cell embryos incubated (a) with and (b) without 5.0 μmol/L 5-AZA-CdR. (a′) Two cells are annexin V-FITC -positive. (a″) All eight cells are PI-negative. (b′) Annexin V- and (b″) PI-negative control 8-cell embryos. Scale bar, 10 μm.

2.3 DNA methylation in embryos at the 8-cell and morula stages is decreased by 5-AZA-CdR

Since 5.0 μmol/L 5-AZA-CdR led to embryonic apoptosis, we next addressed whether demethylation could explain why the embryos were also arrested by 0.2 and 1.0 μmol/L 5-AZA-CdR. Global DNA methylation was detected in preimplantation embryos exposed to 5-AZA-CdR beginning at the pronuclear, 2-cell, and 4-cell stages (Figure 2).

When pronuclear embryos were exposed to 5-AZA-CdR, the extent of methylation in the 2- and 4-cell embryos did not differ from that in the controls (Figure 2a-*A* ～*H*, Figure 2b-*A*). However, the degree of methylation at the 5～7 cell stage was significantly lower than that in the 8-cell control embryos ($P < 0.05$; Figure 2b-*A*).

Next, we detected the DNA methylation of embryos which derived from 2-cell embryos treated with 5-AZA-CdR. And the data were shown in Figure 2a-*I* ～*L* and Figure 2b-*B*. Although the relative fluorescence in the 5-AZA-CdR-treated 4-cell embryos was nearly the same as that in the control embryos， the extent of methylation in the 8-cell embryos treated with 0.2 and 1.0 μmol/L 5-AZA-CdR was significantly lower than that in the controls ($P < 0.05$; Figure 2a-*I*～*L*; Figure 2b-*B*). Following an additional 12 h of culture, the 8-cell embryos remained

uncompacted while the control embryos developed into compacted 8-cell embryos. The relative intensity of the arrested 8-cell embryos was significantly weaker than that of the control morulae ($P<0.05$, Figure 2b-*B*).

Figure 2a-*M*～*P* and Figure 2b-*C* showed the DNA methylation of embryos which came from 4-cell embryos exposed to 5-AZA-CdR. No difference occurred between the treated (0.2 and 1.0 μmol/L 5-AZA-CdR) and control uncompacted 8-cell embryos. However, significantly less methylation was detected in the treated morulae than in the controls ($P < 0.05$, Figure 2a-*M*～*P*).

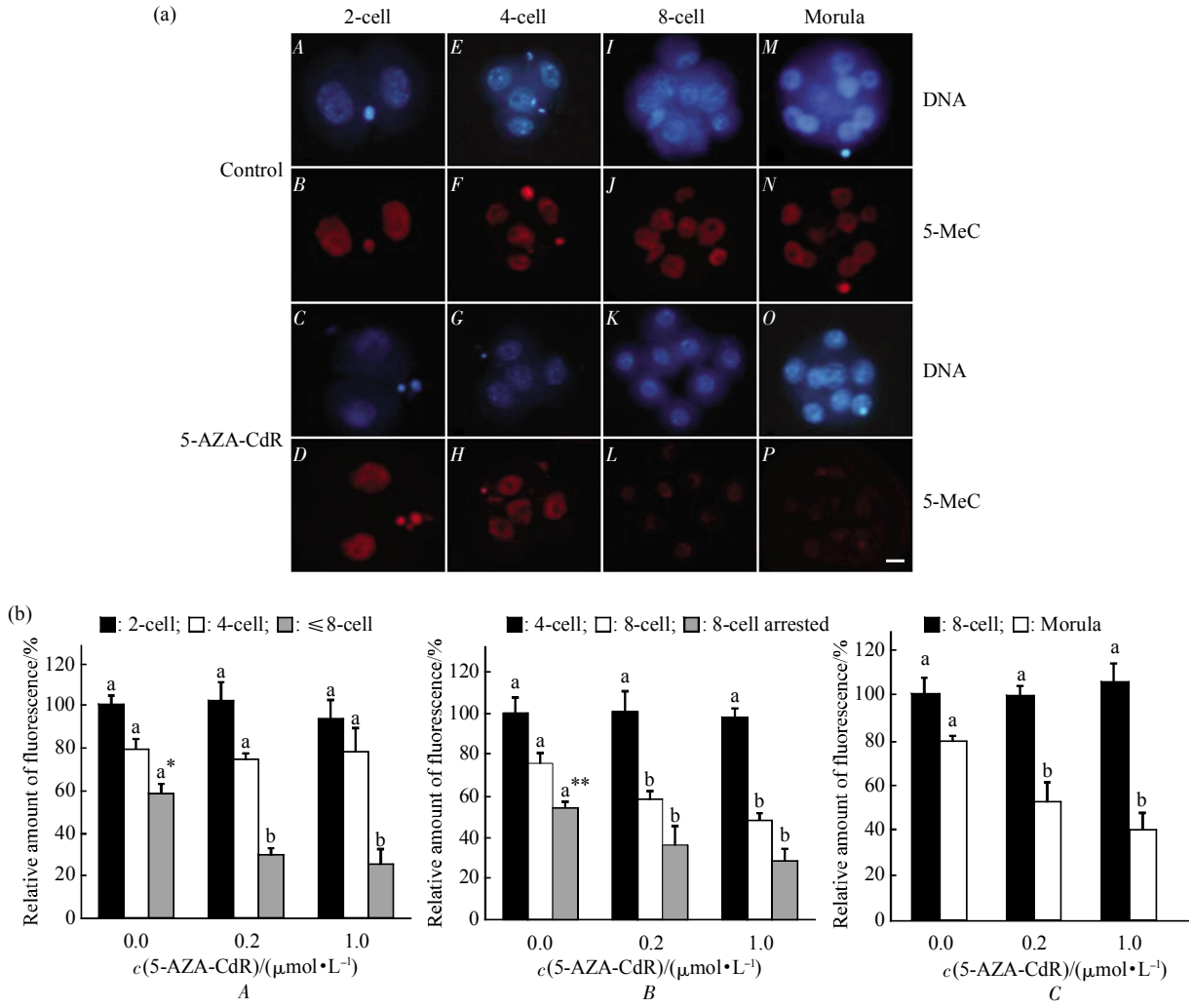

Fig. 2 5-MeC immunofluorescence and its relative amount in preimplantation embryos

(a) 5-MeC immunofluorescence in preimplantation embryos. 5-MeC was visualized using a Cy3-conjugated secondary antibody (red). The embryos were counterstained with DAPI to identify the nuclear compartment (blue). *A B*, *E F*, *I J* and *M N* are the controls for *C D*, *G H*, *K L* and *O P*, respectively. *C*, *D*: The level of DNA methylation in 2-cell embryos cultured in 0.2 μmol/L 5-AZA-CdR. *G*, *H*: Pronuclear embryos cultured in 1.0 μmol/L 5-AZA-CdR until the 4-cell stage; *K*, *L*: 0.2 μmol/L 5-AZA-CdR was added to the medium beginning at the 2-cell stage. Eight-cell embryos were collected and assayed for DNA methylation; *O*, *P*: 1.0 μmol/L 5-AZA-CdR was included in the culture medium from the 4-cell stage to the blastula stage. DNA methylation in the compacted 8-cell embryos (morulae) was measured. Scale bar, 10 μm. (b) Changes in total genomic methylation per nucleus quantified by ImageJ software. *A* ～ *C*: Show the changes in DNA methylation produced when 5-AZA-CdR was added at the pronuclear, 2-cell, and 4-cell stage, respectively. a, b: Values with a common superscript in the same column do not differ significantly ($P > 0.05$). *: The relative amount of methylation in the 8-cell embryos; **: The relative amount of methylation in the morulae.

2.4 Changes in transcriptional activity in 5-AZA-CdR-treated preimplantation embryos

As DNA methylation is associated with gene expression, we investigated whether the total level of transcriptional activity was altered in preimplantation embryos treated with 5-AZA-CdR at the pronuclear, 2-cell, and 4-cell stages (Figure 3).

When pronuclear embryos were cultured in

5-AZA-CdR, the transcriptional activity of 2- and 4-cell embryos in 0.2 and 1.0 μmol/L 5-AZA-CdR (Figure 3a-*C D*, *G H*) was similar to that in the controls (Figure 3a-*A B*, *E F*). Moreover, no difference was observed between the two treatments ($P > 0.05$, Figure 3b-*A*). However, BrUTP incorporation decreased sharply in the embryos at the 5～7 cell stage.

When 0.2 or 1.0 μmol/L 5-AZA-CdR was added from 2-cell stage, only at 1.0 μmol/L, the transcriptional activity of 8-cell embryos (Figure 3a-*K L*) was significantly lower than that in the control (Figure 3a-*I J*, $P < 0.05$). In addition, the relative fluorescence of the arrested 8-cell embryos decreased sharply (Figure 3b-*B*). For 4-cell embryos obtained from treatment with 5-AZA-CdR, they had nearly the same level of transcriptional activity as the controls (Figure 3b-*B*).

As for morulae developed from 4-cell embryos treated with 5-AZA-CdR, the transcriptional activity was significantly lower than that in the control (Figure 3a-*M*～*P*). However, the transcriptional activity of the uncompacted 8-cell embryos in treatment groups was roughly the same with the control, regardless of the concentration of 5-AZA-CdR (0.2 or 1.0 μmol/L; Figure 3b-*C*).

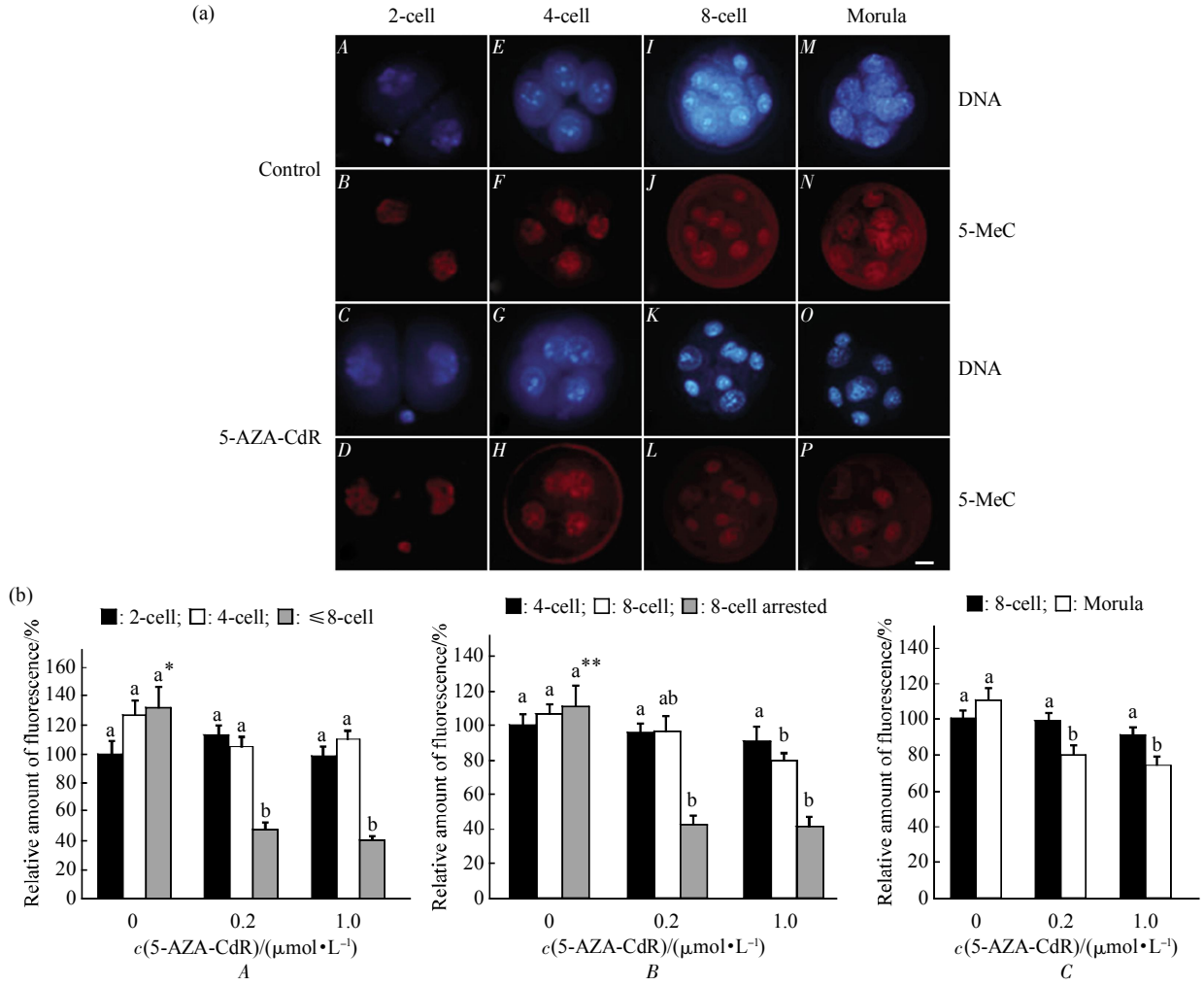

Fig. 3 BrUTP immunofluorescence and its relative amount in preimplantation embryos

(a) BrUTP immunofluorescence in preimplantation embryos. BrUTP was visualized using a Cy3-conjugated secondary antibody (red). The embryos were counterstained with DAPI to identify the nuclear compartment (blue). *A B*, *E F*, *I J* and *M N* are the controls for *C D*, *G H*, *K L* and *O P*, respectively. *C*, *D*: Transcriptional activity in 2-cell embryos cultured in 0.2 μmol/L 5-AZA-CdR. G, H: Pronuclear embryos cultured in 1.0 μmol/L 5-AZA-CdR until the 4-cell stage; *K*, *L*: 1.0 μmol/L 5-AZA-CdR was added to the medium beginning at the 2-cell stage. Eight-cell embryos were collected and assayed for transcriptional activity; *O*, *P*: 1.0 μmol/L 5-AZA-CdR was included in the culture medium from the 4-cell stage to the blastula stage. Transcriptional activity in the compacted 8-cell embryos (morulae) was measured. Scale bar, 10 μm. (b) BrUTP incorporation was quantified using ImageJ software. *A*～*C*: Show the changes in transcriptional activity following exposure to 5-AZA-CdR beginning at the pronuclear, 2-cell, and 4-cell stage, respectively. a, b: Values with a common superscript in the same column do not differ significantly ($P > 0.05$). *: The relative amount of methylation in the 8-cell embryos; **: The relative amount of methylation in the morulae.

3 Discussion

3.1 5-AZA-CdR affects the development of competence in preimplantation embryos

DNA methylation regulates gene expression during the development of mammals[5, 26, 43]. It also plays a crucial role in cellular differentiation[6], genomic imprinting[7], X chromosome inactivation[8], and chromatin modification[2]. Therefore, aberrant methylation may have adverse effects on embryonic development. In a previous study, pregnant mice were treated with 0.05 to 3 mg/kg 5-AZA-CdR on day 10 of gestation and then killed 4, 8 or 28 h later. Subsequent analysis showed that 5-AZA-CdR led to cell death and cell cycle perturbation in the embryos[1]. Another experiment revealed that exposure to 1 mg/kg 5-AZA-CdR on day 10 of gestation led to axial and appendicular skeletal defects (mainly limb defects) in developing mice[38, 44]. Similar teratogenic effects have also been reported in rats[39].

In this study, 5-AZA-CdR had little effect on the developmental rate of 2- and 4-cell embryos, but it did influence the development of 8-cell embryos, morulae, and blastocysts. So 5-AZA-CdR had effects on compaction and cavitation in preimplantation embryos. According to Hamatani *et al.*[45], preimplantation embryos pass through three developmental phases: Ⅰ (fertilized oocytes), Ⅱ (2- and 4-cell stage), and Ⅲ (8-cell stage, morula, and blastocyst). Phase Ⅲ development was the most strongly influenced by 5-AZA-CdR in our experiments. Roughly 3 300 genes are involved in the transition from phase Ⅱ to Ⅲ[45]. As 5-AZA-CdR is a demethylating agent, the observed defects in embryonic development may be due to alterations in DNA methylation and gene expression. Notably, the cytotoxic effects of 5-AZA-CdR were dose- and time-dependent, which is consistent with previous results[46, 47].

3.2 A strong dose of 5-AZA-CdR induces apoptosis in early mouse embryos

As 5-AZA-CdR induces apoptosis in human cancer cells, it is widely used as an antitumor agent[36, 37]. 5-AZA-CdR is a deoxycytidine analog that incorporates into DNA by forming irreversible covalent bonds with Dnmt1[30], which halts DNA synthesis and causes the eventual degradation of Dnmt1. Thus, 5-AZA-CdR potentially has dual effects on treated cells[48]. High doses of 5-AZA-CdR trigger apoptosis due to the formation of DNA adducts and the arrest of DNA synthesis. In contrast, cells exposed to low doses of 5-AZA-CdR survive but exhibit altered gene expression, reduced proliferation, and/or increased apoptosis. Based on these results, we questioned whether these effects apply to early preimplantation embryos, and whether apoptosis leads to developmental failure.

Our data show that at 5.0 μmol/L, 5-AZA-CdR did induce embryonic apoptosis, especially at the 8-cell stage and in morulae, whereas almost no apoptosis was observed at doses of 0.2 and 1.0 μmol/L. Notably, regardless of the dose, apoptosis was not observed in 2- or 4-cell embryos, possibly due to the reduced level of Dnmt1 in their nuclei[49]. When exposed to 5-AZA-CdR, stem cells with reduced levels of Dnmt1 were significantly more resistant to the toxic effects of the drug than wild-type cells[28]. Thus, Jüttermann *et al*[28]. thought that the toxicity of 5-AZA-CdR toward mammalian cells was mediated primarily by covalent trapping of DNA methyltransferase rather than DNA demethylation. However, a loss of genomic methylation can cause apoptosis and epigenetic deregulation[4]. In addition, Nieto *et al*[50]. suggested that 5-AZA-CdR-induced apoptosis was related to p53-deficiency. Determining the true cause of 5-AZA-CdR-induced apoptosis will require further study.

3.3 5-AZA-CdR-induced alterations in DNA methylation and transcriptional activity in mouse preimplantation embryos

5-AZA-CdR decreased DNA methylation in 8-cell embryos and morulae (compacted 8-cell), but not 2- or 4-cell embryos. Why? Most Dnmt1 is concentrated in the peripheral cytoplasm, and the nuclei in 2- and 4-cell embryos do not contain detectable Dnmt1[18]. The substitution of DNA with 5-AZA-CdR led to covalent trapping of Dnmt1, thereby inhibiting Dnmt1 activity and promoting DNA demethylation[28]. Importantly, the 5-AZA-CdR-induced degradation of Dnmt1 is nuclear (i.e., 5-AZA-CdR does not degrade Dnmt1 in the cytoplasm[51]). Therefore, the amount of DNA methylation in the 5-AZA-CdR-treated 2- and 4-cell embryos was unchanged. In contrast, large amounts of Dnmt1 transiently enter into the nuclei in 8-cell embryos[18]. Thus, the observed decrease in DNA methylation in the 8-cell embryos is logical. In addition, the decrease in methylation was dose-dependent, which is consistent with previous results in sperm[47], cell lines[52], cancer

cells[51, 53], and leukemia cells[54].

Epigenetic modification of the genome by DNA methylation regulates gene expression during mammalian development[5, 26, 43]. Therefore, the aberrant DNA methylation induced by 5-AZA-CdR may lead to alterations in gene expression. The differential expression of several genes, including *Shh* and *hox*, is associated with 5-AZA-CdR-induced hind-limb defects in Swiss Webster mice[55～57]. Changes in gene expression have also been observed in 5-AZA-CdR-treated hepatoma cells[58]. Although we did not analyze these genes in this study, we did find a reduction in transcriptional activity based on the total mRNA level with a decrease in DNA methylation. *Xenopus laevis* embryos injected with 5-AZA-CdR show significant reductions in RNA synthesis[59]. Therefore, the 5-AZA-CdR-induced developmental arrest seen in this study may be the result of genetic up- and/or down-regulation.

As for the arrested cells, like the 5～7 cell and arrested 8-cell embryos, a sharp decrease took place in both DNA methylation and transcriptional activity. We assume that cell death occurred in these embryos because of a long-term developmental block. Cell death was previously observed in 5-AZA-CdR-treated embryos on day 10 of gestation[1].

Proper imprinting of mammalian genes depends on the maintenance of DNA methylation patterns during preimplantation development[49]. The transient nuclear localization of Dnmt1 in 8-cell embryos was previously shown to specifically promote methyltransferase activity at imprinted loci[60]. Thus, 5-AZA-CdR may change imprinting patterns during preimplantation development. Additional studies are needed to confirm whether imprinting was altered in our experiments.

Preimplantation embryos must maintain normal DNA methylation. Reductions in DNA methylation, alterations in transcriptional activity, and cytotoxic effects associated with 5-AZA-CdR can induce developmental defects in mouse preimplantation embryos. Given that the negative effects of 5-AZA-CdR were obvious even at low doses, further research is needed before 5-AZA-CdR can be safely used for clinical applications.

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5-脱氧杂氮胞苷抑制小鼠附植前的胚胎发育*

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摘要 DNA 甲基化在哺乳动物发育过程中有关键作用．在小鼠附植前胚胎发育过程中，DNA 甲基化一直处于动态变化过程中．通过将体外受精胚在 5-AZA-CdR 中持续培养，研究 5-AZA-CdR 对小鼠附植前胚胎发育的影响，为附植前胚胎发育机理的研究及 5-AZA-CdR 的毒副作用研究提供试验基础．从原核期加入不同浓度的 5-AZA-CdR 时，胚胎不能发育到桑椹胚(0.2 和 1.0 μmol/L)和 4- 细胞胚(5.0 μmol/L)；从 2- 细胞期加入时，胚胎阻滞于未致密化的 8- 细胞(0.2 和 1.0 μmol/L)和 3/4- 细胞期(5.0 μmol/L)；而当从 4- 细胞加入时，虽然胚胎能够发育到早期桑椹胚，但发育比例同对照相比显著降低($P < 0.05$)．进一步检测凋亡、基因组 DNA 甲基化和整体转录活性，结果显示，高浓度的 5-AZA-CdR 导致 8- 细胞和早期桑椹胚发生早期凋亡，而低浓度的 5-AZA-CdR 引起 8- 细胞和早期桑椹胚基因组 DNA 甲基化的降低和转录活性的降低，并且这种降低呈浓度依赖性．所以加入低浓度的 5-AZA-CdR 时，胚胎的 DNA 甲基化降低，引起转录活性的降低，进而导致胚胎发育的停滞．

关键词 5-AZA-CdR，凋亡，发育，DNA 甲基化，转录活性

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