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Cell Cycle Duration at the Time of Maternal Zygotic Transition for In Vitro Produced Bovine Embryos: Effect of Oxygen Tension and Transcription Inhibition[1]

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ABSTRACT

Early embryonic cleavages are mostly regulated by maternal components then control of development progressively depends on newly synthesized zygotic products. The timing of the first cleavages is a way to assess embryo quality. The goal of this study was to evaluate the duration of the fourth cell cycle, at the time of maternal-to-zygotic transition (MZT) in in vitro-produced bovine embryos by means of cinematographic analysis. We found that 75% of the embryos displayed a long fourth cycle (43.5 ± 5.4 h) whereas the remaining embryos had a very short fourth cell cycle (8.9 ± 2.9 h). Both groups did not differ in cleavage rhythm up to the eight-cell stage and timing of cavitation and blastocyst expansion was identical. However, embryos with a short fourth cell cycle had a better blastocyst rate than embryos with a long cycle (59% versus 38%, $P < 0.01$). Total cell number, inner cell mass (ICM):total cell ratio, and hatching rate were identical for blastocysts produced from embryos with either a long or a short fourth cell cycle. In a second experiment, we showed that increasing the oxygen tension, from 5% to 20%, decreased the percentage of embryos with a short fourth cell cycle, from 25% to 11% ($P < 0.01$), indicating that suboptimal culture conditions can influence the length of this cycle. Finally, we investigated whether fourth cell cycle duration could be influenced by transcription inhibition. With alpha-amanitin added at 18 h postinsemination (HPI), cleavage was reduced (66% versus 79%) and, at 70 HPI, the 9- to 16-cell rate increased (50% versus 25%) concomitantly with a 5- to 8-cell rate decrease (16% versus 47%). A similar pattern was observed when the drug was added at 6 HPI or 42 HPI but not at 0 HPI. Cinematographic analysis revealed that alpha-amanitin increased the first cell cycle duration whereas the second and third cell cycles were not affected. With the drug, one third of the embryos could develop up to the 9- to 16-cell stage and they all had a short fourth cell cycle (11.2 ± 3.7 h) with a good synchrony of cleavage between blastomeres. These results suggest that duration of the fourth cell cycle of bovine embryo, during the MZT, is under a zygotic transcriptional control that can be affected by oxidative conditions.

early development, embryo

INTRODUCTION

Control of preimplantation development first depends on maternal components accumulated by the oocyte during its growth and maturation. As development proceeds, maternally inherited molecules decay and embryogenesis becomes dependent on the expression of genetic information derived from the embryonic genome. In bovine embryo, although some transcription can already be detected before the first cleavage of the zygote [1] or at the two-cell stage [2], ribosomal transcription only occurs at the four-cell stage [3]. A high transcriptional activity in the embryo is detected from the 8-cell stage [4–7] or even later at the 16-cell stage [8] concomitantly with marked changes in protein pattern synthesis [9]. The functional organization of the nucleolus is not completely gained before the 8-cell or even the 16-cell stage [10]. Moreover, the immunoreactive histone H1, absent from chromatin of oocytes and transcriptionally inactive embryos, is not detectable before the fourth cell cycle in cattle [11]. In fact, embryonic genome activation (EGA) in mammals occurs in a stepwise manner, with progressive changes in nuclear and chromatin structure regulating the process [12].

Nevertheless, under transcription inhibition, several bovine embryos are able to reach the 16-cell stage [13–15]. This implies that products inherited from the maternal genome can be sufficient to support development until this stage. The transition from maternal to zygotic control of development (MZT) in cattle occurs between the 8- and 16-cell stages, during the fourth embryonic cell cycle [16]. In most species, MZT is a critical step characterized by a developmental block or a slowing down in cleavages under in vitro culture conditions [16, 17]. Thus, bovine embryos produced in vitro under suboptimal conditions showed a developmental arrest between the 8- to 16-cell stages [18, 19]. Under permissive in vitro culture conditions, the second and third cell cycles are quite short, 8 to 12 h, but the fourth cell cycle is very long, around 40 h [20–23], and has been called the lag phase [21]. In pig embryos, MZT occurs at the four-cell stage [24, 25]; it is also a very sensitive step under in vitro conditions [26]. If pig embryos develop further, the cycle from four to eight cells is quite long too [24, 25]. For some strains of mouse, in vitro conditions may induce a developmental block during the MZT, at the two-cell stage [27]. In vitro culture of embryos is associated with the generation of potentially damaging reactive oxygen species (ROSs) [28, 29]. In mouse, these ROSs have been directly implicated in the two-cell block [30, 31]. ROSs are unlikely to be the sole components for block or retardation of early mammalian development, but evidence for implicating them is compelling [17, 32]. The mechanisms by which ROSs affect development are not completely elucidated. ROSs exert a powerful oxidizing po-

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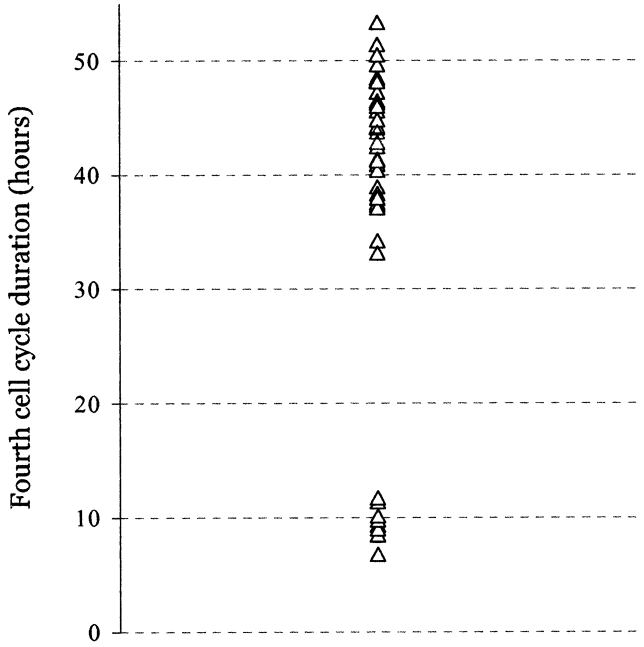

FIG. 1. Bimodal distribution of the fourth cell duration in in vitro-produced bovine blastocysts. Each point represents the fourth cell cycle duration (hours) for one embryo.

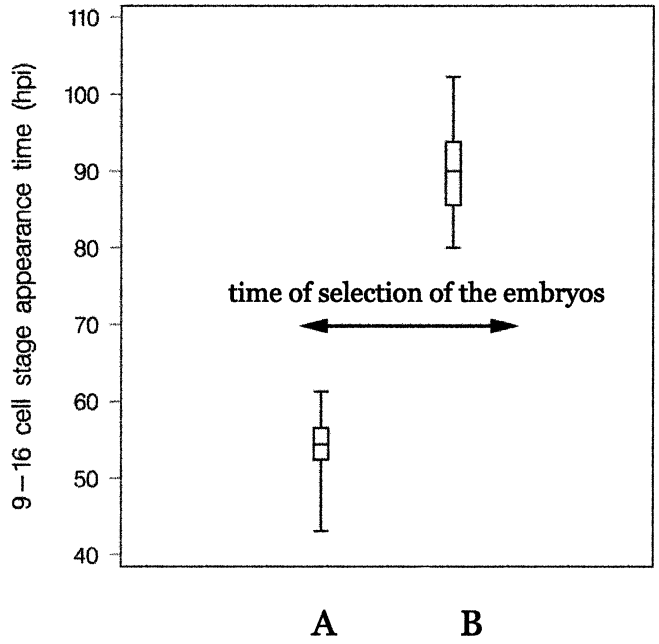

FIG. 2. Time of appearance of the 9- to 16-cell embryos with a short (**A**) or with a long (**B**) fourth cell cycle. The boxes give the median with the 25th and 75th percentiles and bars extending to the extreme values. The arrow at 70 HPI represents the time for embryo fixation or selection and allows a clear separation of the two populations.

tential on cellular molecules such as proteins, lipids, and nucleic acids and can be a major cause of cell damage [33]. Culture of bovine embryos under 20% oxygen tension already induces DNA damage after 3 days [34]. The reduction of oxygen tension during bovine embryo culture with a defined medium yields a higher blastocyst rate although cleavage remains unaffected [35–37]. Consequently, the beneficial effect of a lower tension of oxygen is detected after the first cleavage at variable times according to the studies [38–40]. Under 5% oxygen in our culture conditions, some bovine embryos were able to develop quickly, reaching the 9- to 16-cell stage already at 70 h postinsemination (HPI). The goal of this study was to analyze the overall developmental kinetics of these rapidly cleaving embryos and to better define the influence of oxygen on their appearance and cleavage rhythm. As described above, the transition between 8-cell and 16-cell stages corresponds with the major outburst of embryonic transcription; however, the four first cleavages of a bovine embryo can occur without zygotic transcription. Several authors have reported a developmental acceleration of early bovine development when transcription was inhibited [13, 15]. By means of cinematographic analysis, we have studied the effect of transcription inhibition on the duration of the first cell cycles by adding alpha-amanitin, a potent inhibitor of the RNA polymerase II, to the embryo culture medium.

MATERIALS AND METHODS

All chemicals were purchased from Sigma-Aldrich (Steinheim, Germany) unless otherwise indicated.

In Vitro Production of Embryos

Oocytes were collected by puncturing follicles from ovaries of slaughtered cows. Only intact cumulus oocyte complexes (COCs) were matured in tissue culture medium 199 (TCM-199) supplemented with 10% fetal calf serum (FCS) (ICN Biochemicals, Irvine, CA) and 10 ng/ml Epidermal Growth Factor (EGF) at 39°C and under 5% CO_2 in humidified air. Twenty-four hours later, COCs were transferred to 500 μl fertilization medium (TALP-low bicarbonate [2 mg/ml] Tyrode medium). Motile spermatozoa separated on a Percoll discontinuous density gradient were added at a final concentration of 2×10^6/ml. Oocytes and sperm were coincubated for 18 h at 39°C under a humidified atmosphere of 5% CO_2 in air. Presumptive zygotes were vortexed for 2 min to remove cumulus cells. Denuded zygotes were cultured in groups of 20–25 in 25 μl droplets of Synthetic Oviduct Fluid (SOF) modified according to Holm et al. [41] and containing 2.8 mM myo-inositol, 0.3 mM Na citrate, and 5% FCS.

Cinematography Analysis

To perform time-lapse cinematography, embryos were incubated in a culture dish placed in a small chamber on the plate of an inverted microscope (Zeiss, Oberkochen, Germany). The chamber was regularly flushed with a humidified and warmed gas mixture consisting of 5% CO_2, 5% O_2, 90% N_2. To maintain a constant temperature of 39°C, a Plexiglas box was adapted to fit onto the microscope and connected to a heating system controlled by a temperature probe. The recording equipment consisted of a color video camera and two computers. The first computer synchronized the lighting of the lamp and the shooting. The second digitized and recorded the frames with the program Perception Video Recorder (Alpha M, Grez Doiceau, Belgium). One image was recorded every 180 sec.

The development of the embryos was filmed from the zygote stage at 18 HPI for 8 days under 40× magnification. Only embryos visible in the camera field throughout the culture period were examined. Only zygotes that developed to the blastocyst stage within 7 days after insemination were designated viable and only those were analyzed for cleavage patterns. Embryos of the same origin that were simultaneously cultured in a classic incubator served as a control. Movies were analyzed by detecting, for each viable embryo, the time of appearance of two-cell, three-cell, five-cell, nine-cell, blastocyst, and hatched blastocyst stages. Thus, the first cell cycle lasted from the onset of the fertilization process up to the first cleavage. The second cycle lasted from the two-cell stage up to cleavage of one of the two blastomeres. The third cell cycle lasted from the three-cell stage up to cleavage into at least five blastomeres. The fourth cell cycle lasted from the five-cell stage up to cleavage into at least nine blastomeres. After the fourth cell cycle, not every blastomere could be observed directly due to the dark appearance of the cells and cleavage asynchrony. Compaction was a rather continuous process and was not quite pronounced in the presence of serum, so it was not recorded in this study. Cavitation was estimated by the first appearance of a stable confluent blastocoel. Blastocyst expansion was defined by the increase in diameter of the zona pellucida (ZP).

TABLE 1. Duration of first, second, third, fourth cell cycle for viable embryos and time of blastocyst formation and expansion, all blastocysts together or separated according to fourth cell-cycle duration.[a]

Cell cycle duration (hours ± SD)	All blastocysts, n = 43	Blastocysts displaying a long fourth cell cycle, n = 34	Blastocysts displaying a short fourth cell cycle, n = 9
First	28.64 ± 3.28	28.98 ± 2.96	27.34 ± 4.24
Second	8.93 ± 1.33	8.81 ± 1.36	9.39 ± 1.20
Third	8.47 ± 1.63	8.57 ± 1.58	8.08 ± 1.86
Fourth	36.25[b] ± 15.05	43.48[b] ± 5.38	8.93[c] ± 2.90
Embryonic stages (HPI[a] ± SD)			
Blastocyst	150.22 ± 12.85	151.24 ± 11.46	146.23 ± 17.62
Expansion	165.86 ± 10.07	165.89 ± 9.77	165.74 ± 11.77

[a] HPI, Hours postinsemination.
[bc] GLM procedure statistical analysis between columns; b ≠ c; $P < 0.0001$.

Effect of Oxygen on Nuclei Number at 70 HPI

According to cinematographic data (Figs. 1 and 2), the fourth cell cycle could be short or long and the five- to eight-cell embryos were cleaving into 9–16 cells either around 50 HPI (short fourth cell cycle) or around 90 HPI (long fourth cell cycle). No cleavage of the blastomeres was observed between 60 and 80 HPI. Consequently, 70 HPI was chosen as the best time for a clear discrimination between embryos with a short or a long fourth cell cycle.

To test the influence of oxygen on the proportion of embryos with a short or a long fourth cell cycle, 60–70 embryos were cultured simultaneously under 5% or 20% oxygen tension. At 70 HPI, all embryos were fixed with ethanol for 24 h. They were stained with bis-benzimide (Hoechst 33342-Calbiochem, Dormstadt, Germany) at 10 μg/ml in 2.3%(w/v) sodium citrate and visualized with an epifluorescence microscope in order to count the total number of nuclei. This experiment was repeated three times.

Developmental Potential According to Fourth Cell-Cycle Duration

As described, in order to separate embryos according to fourth cell-cycle duration, five- to eight-cell embryos were selected at 70 HPI and carefully examined under a stereomicroscope. Embryos were classified as 9–16 cells (embryos with a short fourth cell cycle) or as 5–8 cells (embryos with a long fourth cell cycle). The two types of embryos were then further cultured in separate culture drops for 4 more days. At Day 7 postinsemination, developmental rate was evaluated in each group. This experiment was repeated four times with 120–180 zygotes each time. In the first two replicates, blastocysts were fixed and stained with Hoechst 33342 for total nuclei counting. In the last two replicates, a differential staining method was used to discriminate between trophectoderm and inner cell mass (ICM) cells [42]. Briefly, Day 7 blastocysts were transferred for 1 min in TCM199 containing 0.5% Triton (Roche Molecular Biochemical, Mannheim, Germany) and 50 μg/ml propidium iodide, a nucleic marker excluded from intact cells. Then they were transferred immediately in iced ethanol with 25 μg/ml of Hoechst 33342, a nucleic marker penetrating all cells. Under a fluorescent light, peripheral trophectoderm cells were stained in red with IP while ICM cells remained intact and stained in blue with Hoechst. For each staining method, a similar number of blastocysts emerging from embryos with a short or with a long fourth cell cycle was analyzed for statistical purpose.

Effect of Transcription Inhibition on Developmental Kinetics

Alpha-amanitin was added to the culture medium (18 HPI) at a final concentration of 100 μg/ml, as described in previous studies [13–14]. Sixty to 70 embryos were cultured with or without the drug. All embryos were collected at 70 HPI, fixed, and stained with Hoechst 33342 for nuclei counting. This experiment was replicated four times.

For cinematographic analysis, embryos were cultured in medium containing 100 μg/ml of alpha-amanitin and picture frames were recorded every 3 min from 18 HPI up to 90 HPI (there was no more development after this time). Eight movies were realized with a total of 167 embryos recorded.

To test the effect of alpha-amanitin on fertilization, the drug was directly added to the fertilization well at 0 HPI and zygotes were collected at 18 HPI in order to check the fertilization status.

Finally, the effect of adding alpha-amanitin at different times during the very early development was tested. The drug was added to the fertilization well at 0 or 6 HPI or to the culture medium at 18 or 42 HPI, always at a final concentration of 100 μg/ml. All embryos, (133–194 for each condition) were collected, fixed, and stained at 70 HPI.

Statistical Analysis

Time-lapse data were analyzed with a mixed linear model using the duration of the fourth cell cycle (long or short) or alpha-amanitin (added or not) as fixed factors and experiment (movies) as a random factor. The effect of oxygen on developmental stages reached by embryos at 70 HPI was evaluated using a chi-square test. Blastocyst percentages according to the duration of the fourth cell cycle were first transformed using an arcsine transformation before being analyzed by a chi-square test. Cell numbers of these blastocysts were analyzed by ANOVA II with replicate and lag-phase (presence or not) as fixed factors.

Statistical analysis of cleavage, 5- to 8-cell and 9- to 16-cell rates in the presence or not of alpha-amanitin added at different times was performed using ANOVA II with drug addition and experiments as fixed factors. Separate analyses were carried out for each observation time.

Difference in mean nuclei number of the 9- to 16-cell embryos was tested by ANOVA II with the fixed factors experiment and alpha-amanitin. *P* values less than 0.05 were considered significant.

RESULTS

Cinematographic Analysis Under 5% Oxygen

Eight movies were analyzed with a total of 160 embryos. The developmental potential in the cinematography chamber (27% of blastocysts) was not different from the one observed in the control drops kept in a classical incubator (29% of blastocysts, n = 152). Mean duration for the four first cell cycles (hours) for these blastocysts and the average time of cavitation and expansion (hours postinsemination, HPI) are shown in the first column of Table 1. The duration of the fourth cell cycle, from the 5- to 8-cell to the 9- to 16-cell stages, had a mean value of 36.25 h with a very high standard deviation (±15.05 h) compared with the first three cell cycles. Indeed, the embryonic distribution according to the duration of this fourth cell cycle does not follow a normal distribution (normality test of Shapiro and Wilk, $P < 0.001$). As represented in Figure 1, the distribution is bimodal (statistical analysis by QQ test) and there are no values between 12 and 30 h.

The embryos were classified a posteriori into two categories according to the duration of the fourth cell cycle: a short fourth cell cycle <12 h (n = 9) or a long fourth cell cycle >30 h (n = 34). For these two categories, there was no difference for the duration of the first three cell cycles or for the moment of cavitation and expansion of the corresponding blastocysts (Table 1). Hatching rate was similar with 4/9 (44%) for embryos with a short fourth cell cycle versus 18/34 (53%) for those with a long fourth cell cycle.

TABLE 2. Effect of oxygen tension on developmental stages reached at 70 HPI.[a]

Oxygen tension	Total zygotes	Number of cleaved embryos (%)	Number of embryos 5–8 nuclei (%)	Number of embryos 9–16 nuclei (%)
20%	192	139 (72)	94 (49)	22 (11)[b]
5%	198	148 (75)	112 (56)	47 (24)[c]

[a] HPI, hours postinsemination.
[bc] χ^2 analysis b ≠ c; $P < 0.01$.

TABLE 3. Total cell number, ICM cell number and ICM : total cell ratio (mean ± SEM) of Day 7 blastocysts according to the duration of their fourth cell cycle.[a]

Fourth cell cycle	Number of blasto-cysts	Total cell number	ICM cell number	ICM : total cell ratio
Long	21	127.57 ± 7.8	40.86 ± 2.97	0.32 ± 0.01
Short	23	114.83 ± 7.85	35.57 ± 2.01	0.32 ± 0.01

[a] ICM, inner cell mass.

As cleavage rhythm was very similar for all viable embryos up to the 8-cell stage, 5- to 8-cell embryos cleaved to the 9- to 16-cell stage around 50 HPI (short fourth cell cycle) or around 90 HPI (long fourth cell cycle). No cleavage was observed between 60 and 80 HPI (Fig. 2). Consequently, the number of blastomeres at 70 HPI allowed a clear cut between embryos with a short or with a long fourth cell cycle. In order to study important numbers of embryos in the following experiments, the selection of embryos according to the developmental stage reached by 70 HPI was used.

Effect of Oxygen Tension on Embryo Nuclei Number at 70 HPI

The rate of cleavage and the rate of embryos reaching at least the five- to eight-cell stage were not different under 5% or under 20% oxygen. However, under 5% oxygen, a higher percentage of the embryos ≥5–8 cells had already reached the 9- to 16-cell stage at 70 HPI (Table 2).

Potential of Development According to the Fourth Cell Cycle Duration

Among 619 embryos cultured in vitro, 363 (57%) reached at least the five- to eight-cell stage by 70 HPI. Among these, 26% had more than eight blastomeres, which implies they have had a short fourth cell cycle. These embryos had a higher blastocyst rate than embryos with a long fourth cell cycle (59% versus 38%, $P < 0.01$). However, blastocysts obtained from embryos with a short fourth cell cycle had the same total cell numbers (141.7 ± 8.2; n = 27) as blastocysts derived from embryos with a long fourth cell cycle (132.5 ± 7.5; n = 34). Using the differential staining method, the total cell number and cell allocation to ICM and trophectoderm were not different either (Table 3).

Effect of Transcription Inhibition on Developmental Kinetics

When alpha-amanitin was added to the culture medium at 18 HPI, the rate of cleavage decreased (66%, n = 248 versus 79%, n = 263 in the control drop, $P < 0.05$). Among the cleaved embryos fixed at 70 HPI, the 5- to 8-cell rate decreased also (16% versus 47% in control), but simultaneously, the 9- to 16-cell rate increased (50% versus 25% in control). Cleaved embryos reached the 9- to 16-cell stage faster. The mean number of nuclei obtained within the 9- to 16-cell category was also higher in the presence of the drug than without, with 13.4 ± 3.2 nuclei with the drug (n = 50) versus 11.4 ± 1.8 nuclei without the drug (n = 45); $P < 0.01$.

The movies were analyzed to find out when developmental acceleration occurred. Among 167 embryos, 55 reached the 9- to 16-cell stage (33%). Only those were kept for kinetic analysis. With alpha-amanitin, the timing of the first cleavage was significantly delayed (Fig. 3). The duration of the next two cycles remained unaffected but the duration of the fourth cell cycle was highly reduced (11.2 h) and was close to the short duration observed without the drug (8.9 h).

When alpha-amanitin was added at the beginning of the fertilization process (0 HPI) 81% of the zygotes (n = 70) fixed at 18 HPI had 2 pronuclei compared to 83% (n = 56) without the drug. Alpha-amanitin did not interfere with pronuclei formation.

If alpha-amanitin was added at 0, 6, or 18 HPI, cleavage was always decreased compared with control (Fig. 4A). When the drug was added at 42 HPI, cleavage had already occurred. Among cleaved embryos fixed at 70 HPI, a decrease in 5- to 8-cell rate with a simultaneous increase in 9- to 16-cell rate was observed with alpha-amanitin added at 6, 18, and 42 HPI (Fig. 4B). If alpha-amanitin was added at 0 HPI, there was a decrease of the 5- to 8-cell rate but no increase of the 9- to 16-cell rate.

DISCUSSION

In bovine embryo, the fourth cell cycle corresponds to the switch from maternal to zygotic control of development or MZT [16]; it lasts 40–50 h and was called lag phase [21]. A long cycle at the MZT was reported for pig and cat embryos too [24, 25, 43]. The molecular reasons for this developmental slowing down are largely unknown. Under our conditions, bovine embryos displayed two types of duration for this cycle: either the usual long one, around 40–45 h, or a short one of 9 h. Under 5% oxygen, more embryos displayed a short fourth cell cycle than under 20% oxygen. Reduction of oxygen tension during the culture of

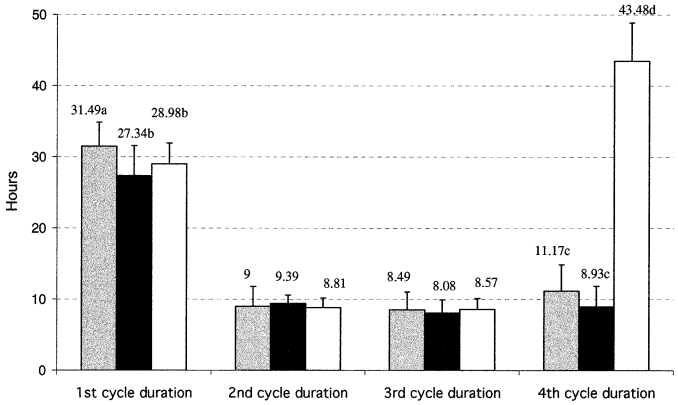

FIG. 3. Durations of first, second, third, and fourth cell cycles with alpha-amanitin added to the culture medium at 18 HPI (100 μg/ml). Grey bars represent values obtained with the drug and compared with values obtained without drug for embryos with a short fourth cell cycle (black bars) and for embryos with a long fourth cell cycle (open bars). GLM procedure: a ≠ b, $P < 0.001$; and c ≠ d, $P < 0.0001$.

bovine embryos improves the blastocyst rate but has no effect on cleavage [35–37] or on the five- to eight-cell rate [38, 39]. Once a higher rate of eight-cell embryos was observed under 20% oxygen compared with 5% but it was followed by a higher blastocyst rate under 5% oxygen [40]. So, when oxygen tension is reduced, embryonic development appears to improve between the eight-cell and blastocyst stages. Furthermore, under 20% oxygen, more embryos blocked development between the 4- and 16-cell stages than under 5% oxygen [44]. The fourth cell cycle, during the MZT, could be especially sensitive to oxidative conditions. Similarly, when bovine embryos were cultured with EDTA, they had a higher number of nuclei at 72 HPI than without EDTA (9.9 versus 8.5) [45], meaning that more embryos went already through the 5- to 8-cell to 9- to 16-cell transition with EDTA. This may also be due to a lower oxidative stress through the chelation of heavy metal ions by EDTA. For mouse and hamster embryos, a reduced oxygen tension and/or addition of antioxidant to the culture medium had also allowed overcoming the two-cell block at the MZT of these species [31, 46].

Cinematographic analysis of embryos with a long or a short fourth cell cycle did not show other differences in their developmental kinetics. The timing of the first cleavage has a major influence on the probability of an embryo developing to the blastocyst stage [21, 47,, 48]. But here, only blastocysts were analyzed and there was no correlation between fourth cell-cycle duration and the timing of first cleavage. However, embryos with a short fourth cell cycle had a better blastocyst rate as the fast-cleaving embryos reported with coculture [47]. But blastocysts emerging from embryos with a short fourth cell cycle did not have a higher number of cells. Consequently, a slowing down in the cleavage rhythm might occur during the next cycles. With cinematography, it is not possible to count the number of cells after the 16-cell stage in the dark bovine embryo. Even the eight cleavages leading from 8 cells to 16 cells cannot be detected for each embryo. The mean number of nuclei obtained for the 9- to 16-cell embryos at 70 HPI was 11.7, and it confirmed that usually four out of the eight blastomeres had cleaved while the others will not cleave before 90 HPI. This asynchrony of cleavage between blastomeres arising during the fourth cycle could be one step for cell differentiation into ICM or trophectoderm (TE). Indeed, the first signs of cell polarity in bovine embryos have already been detected at that stage [49, 50]. But cell allocation was not different for embryos with a short or with a long fourth cell cycle. It has been recently described that, with SOF medium, the ICM:total cell ratio was lower when embryos were cultured under 5% oxygen compared with 20% oxygen [51]. Consequently, oxygen could interfere with cell allocation but, from our results, it would not be due to the variable proportion of embryos with a short fourth cell cycle.

The durations of the first cell cycles were similar to the values published for viable embryos cultured with SOF medium [23, 52]. The second and third cell cycles are always very short because the gap phases (G1, G2) are reduced or absent, whereas a long fourth cell cycle would be due to the appearance of gap phases concomitantly with the major onset of zygotic transcription [19]. Here, because the short fourth cell cycle had exactly the same duration as the second and third cycles, it could be characterized by the absence of gap phases, too.

With alpha-amanitin, bovine embryos showed a developmental acceleration during the cleavage stages [13, 15],

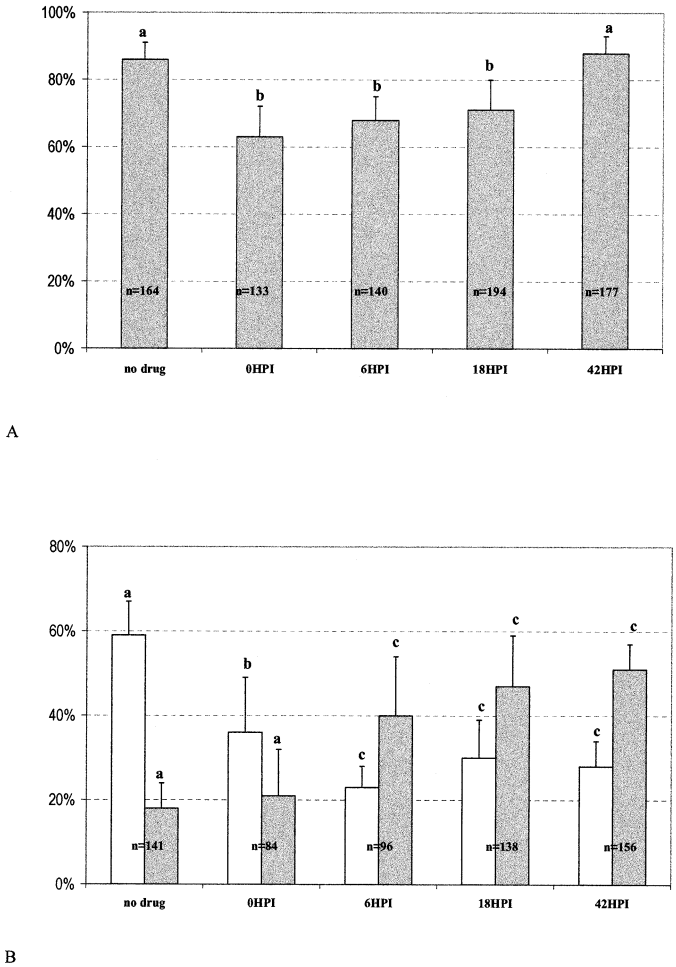

FIG. 4. Effect of alpha-amanitin added at different times during fertilization and culture. The time of drug addition is expressed in HPI (hours postinsemination). **A**) Effect on cleavage. Percentages are expressed relative to the number of zygotes in culture. **B**) Effect on developmental stages reached at 70 HPI. Percentages are expressed relative to the number of cleaved embryos. Open bars, embryos with five to eight cells; grey bars, embryos with more than eight cells. Data are presented as mean ± SEM of four replicates. Bars with the same design and different letter are significantly different; a ≠ b, $P < 0.01$; ≠ c, $P < 0.001$.

but it was not clear if it occurred during the third or the fourth cell cycle. Our data confirmed that cleavage was reduced with the drug and the first cell cycle was also slightly longer. Several proteins are already missing at the zygote stage in bovine embryos treated with alpha-amanitin [1]; maybe these proteins have a role in the first cleavage. The second and third cell cycles remained unaffected by transcription inhibition whereas the duration of the fourth cycle was always dramatically reduced to a value close to the short fourth cell cycle. This acceleration was observed when the drug was added at 6, 18, and even 42 HPI, just before the start of the fourth cell cycle. Thus, immediate transcription at the eight-cell stage appears necessary to lengthen the fourth cell cycle during the MZT. Similarly, cat and rat embryos treated with alpha-amanitin also showed a developmental acceleration during their MZT [44, 53]. As cleavages preceding the MZT should be mainly regulated at a posttranscriptional level [54], it is not surprising they were not or just slightly affected by transcription inhibition. Inhibition of transcription starting as early as 0 HPI did not interfere with pronuclei formation but appeared more deleterious for development with a cleavage

even more reduced and a 5- to 8-cell rate decrease at 70 HPI without a simultaneous increase in the 9- to 16-cell rate.

Finally, without transcription, embryos in the 9- to 16-cell category have also a higher number of nuclei; this might be due to a better cleavage synchronization of the blastomeres during the fourth cell cycle. Likewise, cleavages remain synchronous before the major onset of transcription at the midblastula transition in *Xenopus laevis* embryo [55]. If transcription is responsible for cell-cycle lengthening but does not start simultaneously in all blastomeres, it will result in asynchronous cleavage. In rat embryos treated with alpha-amanitin, some proteins of maternal origin continue to be synthesized at the four-cell stage, while these proteins disappear in control embryos [53]; maybe these proteins are key cell-cycle transcripts. Thus, zygotic transcription is required for downregulation of maternal cyclins A1 and B2 in *Xenopus laevis*, at the midblastula transition [56]. Degradation of Cdc25 also depends on embryonic transcription during the MZT of several species [57, 58]. Cdc25 triggers the cdk1 activity necessary for the G2/M transition of the cell cycle [59]. Inhibition of transcription could prevent Cdc25 degradation and maintain the cdk1 activity with a rapid G2/M transition during one more cycle. A low cdk1 activity has been shown to be responsible for mouse developmental block at the two-cell stage [60, 61].

In the presence of alpha-amanitin, mouse embryos stop development at the two-cell stage, except if oocytes are derived from a special murine strain, then development may reach the four-cell stage [62]. This might be due to genotype differences in the amount of maternal proteins and RNA stored in oocytes and made available to support early development. Here, only 30% of the bovine zygotes were able to develop up to the 16-cell stage without transcription. Bovine oocytes, collected from different follicle sizes can have very different developmental potential [63]. Maybe only 30% of them have stockpiled enough messengers and proteins necessary to achieve the first four cleavages without transcription.

In conclusion, some bovine embryos exhibit in vitro a short fourth cell cycle but, besides this cycle, their developmental kinetics are not different. The percentage of embryos with a short fourth cell cycle increases when oxygen tension is reduced. These embryos have a better developmental potential but the resulting blastocysts have neither a higher total cell number nor a different ICM:total cell ratio nor a better hatching rate compared with blastocysts emerging from embryos with a long fourth cell cycle. Without transcription, cleavage is reduced but the transition between the 5- to 8-cell and 9- to 16-cell stages is accelerated with a better synchronization of cleavage between blastomeres. These results suggest that the duration of the fourth cell cycle, during the MZT, is under a zygotic transcriptional control that can be affected by oxidative conditions.

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