Morphokinetic Analysis of Preimplantation Development of Mouse Oocytes Fertilised in Vitro Using a Non-humidifying Incubator With Time-lapse Cinematography (CCM-iBIS)

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Research Article

Keywords: cinematography, mouse oocytes, in vitro, Preimplantation development, CCM-iBIS

Posted Date: January 11th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-142391/v1

Morphokinetic analysis of preimplantation development of mouse oocytes fertilised in vitro using a non-humidifying incubator with time-lapse cinematography (CCM-iBIS)

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Abstract

Preimplantation development of mouse oocytes fertilised in vitro was assessed in a non-humidified incubator with time-lapse cinematography (CCM-iBIS). The developmental rates of embryos to the 4-cell and blastocyst stages under 5% CO_2, 5% O_2, and 90% N_2 in CCM-iBIS were significantly higher than those under 5% CO_2 in air in CCM-iBIS and a conventional CO_2 incubator (CPO_2-2301). The developmental speed of embryos was much faster in those cultured under lower oxygen tension in CCM-iBIS than in higher oxygen tension in CCM-iBIS and CPO_2-2301. Embryonic development was much faster and more synchronised under lower oxygen tension. Non-humidified culture did not affect the development of the embryos. Mouse embryos cultured at lower oxygen tension reached 2-cell at 17 h, 3-cell at 39 h, 4-cell at 40 h, initiation of compaction at 58 h, morula at 70 h, and blastocyst at 82 h after insemination on average. Although compaction partially unravelled with cell division in compacting embryos, it appeared to complete depending on the timing of observation. Observation at a conventional 24-h interval likely misjudges the developmental rate to morula. Determination of embryonic development 72 h after insemination may be more appropriate for “compacting morula” rather than “morula” in mice.

Introduction

Since the first successful in vitro fertilisation (IVF) of mice in 1968 using uterine spermatozoa collected after mating [1], components of culture medium as well as procedures of IVF have been substantially modified and improved. In 1969, successful mouse IVF with epididymal spermatozoa in the presence of bovine follicular fluid was reported by Iwamatsu and Chang [2]. In 1971, Toyoda et al. [3] established an IVF method for mouse eggs with epididymal spermatozoa in a modified Krebs-Ringer bicarbonate solution supplemented with glucose, Na-pyruvate, and bovine serum albumin (BSA) with higher fertilisation rates and reproducibility. This study demonstrated that mouse spermatozoa can be capacitated in vitro in a chemically defined medium (TYH) without the necessity of fluids from the female reproductive tract. To date, TYH medium has been extensively used as a standard medium for IVF in mice as well as other species of mammals.

Fertilised mouse eggs derived from certain inbred strains or F1 hybrids can be cultured throughout the preimplantation period in a modified Krebs-Ringer bicarbonate solution supplemented with glucose, Na-pyruvate, Na-lactate, and BSA in vitro; however, the majority derived from random bred strains, such as ICR, arrested their development at the two-cell stage [4]. This phenomenon, called in vitro two-cell block, can be overcome by the addition of EDTA-2Na to the culture medium [5-7]. In addition, the development of normal progeny has been

confirmed by the subsequent transfer of IVF embryos cultured throughout the preimplantation stage in Whitten medium [4] supplemented with EDTA-2Na [8]. Subsequently, culture media for mouse preimplantation development of embryos have been developed. Chatot et al. [9] developed Chatot, Ziomek, and Bavister (CZB) medium containing an increased lactate/pyruvate ratio, EDTA, and glutamine but lacking glucose and phosphate. Fertilised mouse eggs could develop beyond the 2-cell stage and reach the morula but not the blastocyst stage when cultured in CZB medium. However, washing embryos in the medium containing glucose after 48 h of culture is sufficient to allow development to the blastocyst stage [9]. The presence of glucose in CZB medium was only necessary from the 4-cell stage and detrimental to earlier stages of embryos in mice. Subsequently, the components of CZB were modified by sequential simplex optimisation methods to design a medium (SOM) that overcomes the two-cell block in an outbred strain of mouse [10]. A modification of the SOM medium, designated KSOM, with an increased concentration of potassium supported development beyond the 2-cell stage and provided a larger yield of blastocysts in an outbred strain of mouse [11, 12]. Moreover, KSOM was further modified to mKSOM^{AA} with an increased concentration of glucose and the addition of 19 amino acids [13].

Culture conditions, such as temperature and atmosphere, may also be important factors for successful preimplantation development of embryos in vitro as well as in culture media. To culture mouse embryos in vitro, an atmosphere of 5% CO_2 in air with a saturated humidity at 37 °C in an

incubator is typically used. Furthermore, a small droplet of medium containing embryos on a dish is covered with paraffin oil to avoid releasing CO_2 from the medium and evaporation and contamination of the medium for manipulation or observation of embryos outside of the CO_2 incubator [14]. Morphological observation of developing embryos seems to be essential for managing and/or improving culture conditions in experimental and clinical studies. However, frequent opening and shutting of the CO_2 incubator for microscopic observation of culturing embryos might disturb the culture conditions. Therefore, it is necessary to reduce the frequency of observation of culturing embryos for successful preimplantation development in a CO_2 incubator. Such fragmentary information may provide a limited analysis of developing embryos. Fortunately, it has become possible to consecutively observe culturing embryos in vitro via time-lapse video cinematography developed by Payne et al. [15]. A CO_2 incubator equipped with a time-lapse monitoring system is a closed system of culture and observation for embryos without exposure to the atmosphere. Therefore, it can provide stable culture conditions without stress or disturbance. Time-lapse observation has also advanced our understanding of the morphologic mechanisms of the fertilisation, development, and behaviour of early embryos during the preimplantation period. Therefore, the automatic capture of time-lapse images would provide useful information for embryo selection for subsequent embryo transfer. Previous studies have investigated pronuclear formation [15, 16], timing of cleavage [16, 17], fragmentation [18-21], compaction

[22-27], blastocoel formation [26, 28], and the shape of the inner cell mass [29] in human zygotes/embryos using a time-lapse monitoring system. Embryos that cleave early at the first cleavage have a higher potential for subsequent pre- and post-implantation development [17, 30-32]. Therefore, the timing of the first zygotic cleavage appears to be a marker of the developmental potential of mammalian embryos. Furthermore, the culture of human embryos in an incubator with a time-lapse monitoring system may improve development to the blastocyst stage and pregnancy outcomes compared to the use of a conventional CO_2 incubator [33, 34].

Initially, this system was only applied for assisted reproductive technology in human infertility treatment due to the high-priced system for introduction. Recently, a time-lapse monitoring system has been applied for the evaluation of mouse embryonic development in vitro. The first and second cleavage of mouse oocytes fertilised in vivo significantly influences the probability of reaching the blastocyst stage in culture [35]. The shorter the time of cleavage to the 2-cell stage and from the 2-cell to 3-cell stage, the higher is the chance of embryo development to the blastocyst stage [35]. When the time of cleavage and compaction of mouse 2-cell embryos fertilised in vivo was evaluated by time-lapse monitoring, it was suggested that the time of the third cleavage and compaction may be a useful morphokinetic parameter for predicting developmental potential [36]. Prior to these studies, by using time-lapse film cinematography, Sutherland et al. [37] were able, for the first time, to directly examine the division plane of 8-cell

blastomeres during the fourth cleavage of mouse embryos cultured from the late 2-cell stage.

Since these studies in mice used embryos fertilised in vivo, in the present study, preimplantation development of mouse oocytes fertilised in vitro was assessed. This was conducted in a non-humidified incubator with time-lapse cinematography, and the sperm penetration time was accurately controlled in the IVF system [3, 8, 38] to evaluate morphokinetic development with higher precision.

Results

Results of the in vitro cultivation and fertilisation of mouse embryos in a non-humidifying incubator with time-lapse cinematography (CCM-iBIS) are shown in Table 1. The developmental rates of embryos to the 4-cell stage 48 h after insemination cultured in 5% CO_2, 5% O_2, and 90% N_2, and 5% CO_2 in air in CCM-iBIS, and 5% CO_2 in air under a humidified atmosphere in a conventional CO_2 incubator (CPO_2-2301) were 97% (288/297), 89% (845/951), and 79% (79/100), respectively. The differences between the three experimental groups were significantly different at $P < 0.05$. The developmental rates of embryos to morula 72 h after insemination in lower and higher oxygen tension in CCM-iBIS were 72% (215/297) and 62% (588/951), respectively. This difference was statistically significant at $P < 0.05$. Developmental rates to the blastocyst stage at 96 h (94%; 280/297) and 120 h (97%; 288/297) after insemination under lower oxygen tension in CCM-iBIS were significantly higher ($P < 0.05$) than those in higher oxygen tension in CCM-iBIS (77%; 733/951 and 88%; 835/951) and higher oxygen tension in CPO_2-2301 (84%; 84/100 and 86%; 86/100). However, there were no differences in developmental rates to the 2-cell stage 24 h after insemination between the three experimental groups. These results indicated that CCM-iBIS with lower oxygen tension better supported embryonic development after the 4-cell stage in mice. Table 2 shows the developmental stage of embryos every 24 h after insemination in CCM-iBIS and CPO_2-2301. Then, 48 h after insemination, 38% of embryos

developed beyond the 4-cell stage to the 5-cell stage in CCM-iBIS with lower oxygen tension. Only 10% and 4% of embryos showed the 5-cell stage in CCM-iBIS with higher oxygen tension and CPO_2-2301, respectively. Then, 72 h after insemination, 74% and 67% of embryos with compaction reached completion as morula in CCM-iBIS with lower and higher oxygen tension, respectively. Similarly, 96 h after insemination, 96% of embryos developed beyond the blastocyst stage to expanded or hatching blastocysts in CCM-iBIS with lower oxygen tension. However, only 64% and 33% of embryos showed expanded blastocysts or hatching blastocysts in CCM-iBIS with higher oxygen tension and CPO_2-2301, respectively. For CCM-iBIS and CPO_2-2301, both with higher oxygen tension, embryos cultured in CCM-iBIS showed a more advanced embryonic stage. For example, 58% of embryos showed hatching blastocysts in CCM-iBIS, while hatching blastocysts were only 5% in CPO_2-2301 96 h after insemination.

As shown in Table 3, when the average attainment time after insemination to each developmental stage was analysed by time-lapse cinematography, culture of in vitro fertilised eggs in lower oxygen tension improved embryonic development from the 2-cell stage throughout preimplantation development. Namely, the IVF embryos cultured in lower oxygen tension developed significantly earlier throughout preimplantation development, except for development to the morula stage, than in higher oxygen tension ($P < 0.05$). These results indicate that oxygen tension in culture is an important factor for the developmental potential of mouse embryos

fertilised in vitro. Fig. 1 shows a histogram of the number of IVF embryos at each developmental stage with the time lapse after insemination. Despite oxygen tension, the time of onset of cleavage was similar at each developmental stage. On the other hand, it took a considerable amount of time to cleave after the second cleavage (t3) in some embryos cultured under higher oxygen tension. The cleavage and development of IVF embryos were much faster and more synchronised in lower oxygen tension than in higher oxygen tension in CCM-iBIS.

To assess the influence of non-humidified culture conditions on embryonic development in vitro, embryos were cultured under higher oxygen tension in a conventional incubator (CPO_2-2301) without opening or closing the incubator door from the start of culture to the time of observation. Developmental rates of IVF embryos to the 4-cell stage 48 h after insemination and to the blastocyst stage 96 h after insemination were 84% (42/50) and 82% (41/50), respectively (Table 4). These values are not significantly different to those of the developmental rates of fertilised eggs to the 4-cell stage at 48 h (89%) and to the blastocyst stage at 96 h (77%) after insemination under higher oxygen tension in the non-humidifying incubator with time-lapse cinematography (CCM-iBIS). These results indicated that non-humidified culture conditions did not affect the development of mouse IVF embryos.

Fig. 2 illustrates the progression of cleavage of the IVF eggs cultured in lower oxygen tension in CCM-iBIS. The first cleavage occurred 17 h 27 min after insemination. The second

cleavage was initiated at 38 h 39 min after insemination. The time between the first and second cleavages was 21 h 12 min. The 3-cell stage was performed for 0 h 58 min. The second cleavage was completed 39 h 37 min after insemination. At 9 h 25 min after the second cleavage, the third cleavage was initiated; specifically, 49 h 2 min after insemination. The compaction of embryos was initiated 57 h 39 min after insemination and reached morula 70 h 0 min after insemination. Interestingly, compacting embryos during development to morula showed a phenomenon in which compaction was loosened due to an increase in the number of blastomeres accompanying cell division. Morula developed into blastocysts at 12 h 20 min; specifically, 82 h 20 min after insemination.

When the relationship between attainment time to 2-, 3-, 4-, and 5-cell as well as initiation of compaction and morula and attainment time to blastocyst stage was analysed, the correlation coefficients were identified as $r = 0.5016$, 0.6613, 0.6329, 0.7087, 0.5116, and 0.4754, respectively. Therefore, there were significant correlations between the attainment time to the 2-cell to the morula stage and the attainment time to the blastocyst stage ($P < 0.05$). The relationship between the attainment times of embryos cultured under higher oxygen tension was similar to those under lower oxygen tension (Fig. 3).

Discussion

As shown in Tables 1 to 3, the developmental speed of mouse embryos fertilised in vitro was much faster in embryos cultured under lower oxygen tension in CCM-iBIS than in those cultured under higher oxygen tension in CPO_2-2301 and CCM-iBIS. For infertility treatment in humans, the application of time-lapse monitoring together with an embryo-evaluating algorithm is associated with a significantly higher ongoing pregnancy rate, a significantly lower early pregnancy loss, and a significantly higher live birth rate [33, 34, 39, 40]. Therefore, the incubator with time-lapse cinematography appears to provide qualitative improvement of embryos in culture for both mice and humans. There are possible explanations for this improved embryonic development as follows: 1) non-humidifying incubation and 2) minimal handling of culturing embryos inside and outside the incubator.

Non-humidified culture conditions did not appear to influence the development of mouse embryos fertilised in vitro (Table 4). Developmental rates of mouse embryos from the 2-cell to blastocyst stage for 96 h in culture are not significantly different between humidifying and non-humidifying incubators [41]. Regarding the influence of handling embryos inside and outside the incubator, although there was no difference in the developmental rate to blastocyst stage between the CCM-iBIS and CPO_2-2301 incubators with higher oxygen tension, the embryonic development to the 4-cell stage at higher oxygen tension in CCM-iBIS was significantly higher

than that in CPO_2-2301 (Table 1). In addition, embryos cultured in CCM-iBIS showed a more advanced embryonic stage 96 h after insemination (Table 2). When embryos were cultured in CPO_2-2301 without opening or closing the incubator door from the start of cultivation to the time of microscopic observation, the developmental rates of embryos were not different from those cultured under higher oxygen tension in CCM-iBIS (Table 4). Therefore, the handling of embryos inside and outside the incubator as well as oxygen tension in the atmosphere in culture appears to influence the cleavage and development of embryos fertilised in vitro. The development of embryos was much faster and more synchronised under lower oxygen tension in culture (Tables 1-3 and Fig.1). In this study, we assessed the true effect of oxygen tension on embryonic development in vitro using a time-lapse incubator that does not require the culture dish to be frequently removed to observe the embryos.

Although Sutherland et al. [37], Karnaukhov et al. [42], Pribenszky et al. [35], and Kim et al. [36] applied a time-lapse monitoring system to evaluate mouse embryonic development in vitro, they used zygotes or 2-cell stage embryos derived from fertilisation in vivo. For the evaluation of preimplantation development in vitro with higher precision, analysis of in vitro fertilised eggs with synchronous ovulation and fertilisation is recommended [3, 38]. In this study, we precisely showed the timing of cleavage in IVF mouse embryos during preimplantation development through time-lapse cinematography (Tables 2 and 3, Figs. 1 and 2). As shown in Fig.

2, when IVF mouse zygotes were cultured in lower oxygen tension, they developed to 2-cell, 4-cell, morula, and blastocyst stages at approximately 17, 40, 70, and 82 h after insemination, respectively. Compaction, where all blastomeres flatten against each other eliminating the spaces between the cells, of embryos was initiated at an average of 57 h 39 min after insemination. Subsequently, the compaction partially unravelled with cleavage of embryos; in other words, with an increase in the number of blastomeres (Fig. 2) [43]. Cell division gives rise to two daughter cells that localise as one internal and one external cell or remain as two external cells with regards to the embryo. Therefore, when the daughter cells are localised externally to the embryo, compaction appears to be loose and incomplete. However, depending on the timing of observation, when the daughter cells are localized as one internal and one external cell, compaction appears to be complete. Although the embryos formed morula with full compaction at an average of 70 h after insemination (Table 3 and Fig. 2), approximately 30% of embryos did not fully compact at 72 h after insemination (Fig. 1). Therefore, it is likely that a conventional 24-h interval misjudges the developmental rate to morula. When the embryonic development of IVF mouse embryos was observed, the developmental rates to the 2-cell stage 24 h after insemination, 4-cell stage 48 h after insemination, morula 72 h after insemination, and blastocysts 96 and 120 h after insemination were used as an index. However, the determination of embryonic development 72 h after insemination might be more appropriate for the “compacting morula” rather than the “morula”

in mice. Although previous studies have observed compaction and subsequent embryonic development using time-lapse technology in human embryos, these focused on vacuolization in blastomeres [24], timing of morula formation [22, 23, 25, 26], and patterns of compaction with or without extruded blastomeres [22, 27]. There have been, to our knowledge, no previous studies involving the observation of a temporary loosening of compaction during the formation of morula beginning with the initiation of compaction in human embryos [44].

In the present study, the attainment time of the first, second, and third cleavage and compaction was correlated with the probability of reaching the blastocyst stage, which is similar to the results of previous studies that used embryos fertilised in vivo [35, 36].

Procedures of oocyte collection and insemination, culture medium, and culture conditions are key to the success or failure of IVF and subsequent embryo transfer, but the accurate monitoring of embryonic development in vitro is also an important element. Over time, improvements have been made to the IVF method [1-3] and culture medium [3, 5, 8-13] making it possible to obtain stable and highly reproducible results. In addition, the advent of culture conditions, in particular the non-humidifying incubator with time-lapse cinematography [15], allows accurate monitoring of embryonic development in vitro. In particular, as a relatively long period of monitoring is required to accurately determine development to the morula stage, the incubator with time-lapse cinematography appears to function effectively. There is increasing

evidence that early cleavage blastomeres show bias to their development. Blastomeres at the 2-cell stage have an unequal distribution of mitochondrial ribosomal RNAs [45]. Some epigenetic regulators, such as Prdm14, Dnmt3b, and Dnmt3l [46], and cell fate regulator SOX21 [47] are highly heterogeneously expressed between blastomeres of the 4-cell embryo. As shown in Table 3, in vitro fertilised eggs of mice underwent a first cleavage at 17-18 h and a second cleavage at 39-40 h on average after insemination. However, in the first cleavage, all eggs were in the 2-cell stage 9-11 h after the first egg was in the 2-cell stage. In the second cleavage, it took 15 h under lower oxygen tension and 33 h under higher oxygen tension for all eggs to reach the 3-cell stage after the first egg was in the 3-cell stage (Fig. 1). Single-cell sequencing and live-cell tracking technology in combination with time-lapse cinematography provides the opportunity to understand the significance of cleavage speed for developmental competency of the embryo.

Materials and Methods

ICR (CLEA, Japan) female mice at 8-14 weeks of age had superovulation induced by intraperitoneal injections of 175 μL of CARD Hyper Ova (Kyudo Co. Ltd., Japan) and 5 i.u. of hCG (ASKA Pharmaceutical Co., Japan) 48 h apart. These female mice were euthanised by cervical dislocation 16 h after hCG injection, and the oviducts were removed and placed in a culture dish (35 mm, Falcon, Corning Incorporated, NY, USA) containing 400 μL of TYH (LSI Medience Corporation, Japan) covered with paraffin oil (Nacalai Tesque, Japan). The ampullar region of the oviduct was dissected with a needle, and the eggs surrounded by cumulus cells were introduced to the medium. Spermatozoa were collected from the cauda epididymis of ICR males at 18-26 weeks of age and suspended in 400 μL of TYH under paraffin oil. After preincubation for 2 h in an incubator (CPO_2-2301, HIRASAWA WORKS Inc., Japan) at 37°C in humidified 5% CO_2 in air, a small volume of sperm suspension was added to the medium containing eggs. The final concentration of spermatozoa was adjusted to 150 cells/μL. Then, 7 h after insemination, the eggs were transferred into 80 μL of KSOM medium supplemented with amino acids (KSOM, Ark Resource, Japan) covered with paraffin oil and washed twice. All media were equilibrated overnight at 37°C in humidified 5% CO_2 in air prior to use. After confirmation of the presence of the second polar body and both male and female pronuclei, the fertilised eggs were cultured for 113 h in a culture dish (LinKID micro DH-004PG, DNP, Japan) containing 60 μL of KSOM

covered with paraffin oil at 37°C in 5% CO_2 in air (higher oxygen tension) or 5% CO_2, 5% O_2, and 90% N_2 (lower oxygen tension) in a non-humidifying incubator with time-lapse cinematography (CCM-iBIS, ASTEC Co., Ltd., Japan). The CCM-iBIS had a non-humidifying system and was equipped with a 130 million-pixel CCD camera with a 10× objective lens, red LED lighting, silicone rubber heater with digital PID control, duty controlled gas pressure, and NAS image storage method. The CCD camera for the time-lapse microscope was set to acquire a single image every 15 min. Parameters regarding embryonic development were annotated as follows: t2, time of cleavage to 2-cell stage after insemination; t3, time of cleavage to 3-cell stage after insemination; t4, time of cleavage to 4-cell stage after insemination; t5, time of cleavage to 5-cell stage after insemination; tOC, time of the onset of compaction after insemination; tM, time of formation of fully compacted morula after insemination; tB, time of blastocyst formation with blastocoel at half volume of embryo after insemination; tExB, time of formation of expanded blastocyst developed to over 120 μm in diameter after insemination; tHB, time of the onset of hatching from zona pellucida after insemination. As a control, the fertilized eggs in vitro were cultured in a dish containing 50 μL of KSOM covered with paraffin oil at 37°C in higher oxygen tension under a humidified atmosphere in 5% CO_2 in air in a conventional CO_2 incubator (CPO_2-2301). The developmental stages of the embryos were observed under an inverted microscope 24 h apart in the control group.

The animals were kept in a barrier unit at 24 ± 1°C with a relative humidity of 50 ± 10% under a lighting regimen of 12L/12D (lights on 07:00 to 19:00). They were allowed free access to standard laboratory chow (CA-1, CLEA, Japan) and tap water. All experiments were carried out in accordance with the guidelines for the care and use of animals approved by the Obihiro University of Agriculture and Veterinary Medicine. The authors confirmed that this experiment complies with the ARRIVE guidelines. All animal experimental protocols were approved by the Institutional Animal Ethics Committee of Obihiro University of Agriculture and Veterinary Medicine.

Statistical analyses were performed using JMP software (SAS Institute, NC, USA). All data were analysed by logistic regression using the following model:

$$\ln(\alpha/1-\alpha) = \beta + \text{main factor (type of incubators)}$$

where α = frequency of positive outcomes and β = the intercept. The odds ratio with 95% confidence interval was calculated. Data for the timing of embryo cleavage were analysed by ANOVA. Differences were considered significant at $P < 0.05$. All graphs were drawn using DataGraph software (Visual Data Tools, Inc., NC, USA).

Acknowledgements

We would like to express our thanks to Fukunaga, N., Aoki, N., Kido, K., Ohashi, Y., Sanami, S., and Asada, Y. for their invaluable support in the analysis of preimplantation development of mouse embryos by means of a new in vitro culture system for time-lapse cinematography. We would also like to thank Editage for English language editing.

Author contributions

The research was designed by H.S. and H.W. Laboratory experiments were performed by H.I., A.S., and H.W. The manuscript was written by H.S. and H.W.

Competing interests

The authors declare no competing interests.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Figure legends

Fig. 1. Histogram of the number of embryos fertilised in vitro at each developmental stage with the lapse of time after insemination. Embryos cultured in higher oxygen tension and lower oxygen tension in CCM-iBIS are indicated in black and white, respectively. Values show the range of time to cleavage in each developmental stage. Cleavage and development of embryos fertilised in vitro were much more synchronised in lower oxygen tension than in higher oxygen tension in CCM-iBIS. Parameters regarding embryonic development are annotated as follows: t2, time of cleavage to the 2-cell stage after insemination; t3, time of cleavage to the 3-cell stage after insemination; t4, time of cleavage to the 4-cell stage after insemination; t5, time of cleavage to the 5-cell stage after insemination; tOC, time of the onset of compaction after insemination; tM, time of formation of fully compacted morula after insemination; tB, time of formation of blastocyst with blastocoel at half volume of the embryo after insemination.

Fig. 2. Timing of cleavage of mouse in vitro fertilised embryos during preimplantation development cultured in lower oxygen tension in a non-humidifying incubator with time-lapse cinematography (CCM-iBIS).

Fig. 3. Relationship between attainment time to 2-cell, 3-cell, 4-cell, 5-cell, initiation of

compaction and morula, and attainment time to blastocyst stage.

Table 1. Results of in vitro cultivation of mouse embryos fertilized in vitro under high and low oxygen tension in a non-humidifying incubator with time-lapse cinematography (CCM-iBIS)

Incubator	No. of fertilized eggs cultured	No. (%) of development to:				
		2-cell (24 hr) [1]	4-cell (48 hr) [1]	Morula (72 hr) [1]	Blastocyst (96 hr) [1]	Blastocyst (120 hr) [1]
CPO_2-2301 [2] (5% CO_2 in air)	100	96 (96.0) [a]	79 (79.0) [a]	71 (71.0) [ab]	84 (84.0) [a]	86 (86.0) [a]
CCM-iBIS [3] (5% CO_2 in air)	951	940 (98.8) [a]	845 (88.9) [b]	588 (61.8) [a]	733 (77.1) [a]	835 (87.8) [a]
CCM-iBIS [3] (5% CO_2, 5% O_2, 90% N_2)	297	292 (98.3) [a]	288 (97.0) [c]	215 (72.4) [b]	280 (94.3) [b]	288 (97.0) [b]

[1] Hours after insemination.
[2] Humidifying conventional CO_2 incubator.
[3] Non-humidifying incubator with time-lapse cinematography.
a-c: Values with different superscripts are significantly different in the same column at $P<0.05$.

Table 2. Developmental stage of in vitro fertilized mouse embryos at every 24 hr after insemination.

Incubator	24 hr [1]			48 hr [1]		72 hr [1]		96 hr [1]			120 hr [1]		
	2-cell	3-cell	4-cell	4-cell	5-cell	Com	Morula	BL	ExBL	H-BL	BL	ExBL	H-BL
CPO_2-2301 [2] (high O_2 tension)	96 (100)	0 (0.0)	0 (0.0)	76 (96.2)	3 (3.8)	71 (100) [4]		56 (66.7)	24 (28.6)	4 (4.8)	6 (7.0)	15 (17.4)	65 (75.6)
CCM-iBIS [3] (high O_2 tension)	939 (99.9)	0 (0.0)	1 (0.1)	761 (90.1)	84 (9.9)	292 (33.2)	588 (66.8)	262 (35.7)	45 (6.1)	426 (58.1)	145 (17.4)	68 (8.1)	622 (74.5)
CCM-iBIS [3] (low O_2 tension)	291 (99.7)	1 (0.3)	0 (0.0)	178 (61.8)	110 (38.2)	75 (25.9)	215 (74.1)	12 (4.3)	147 (52.5)	121 (43.2)	4 (1.4)	143 (49.7)	141 (49.0)

[1] Hours after insemination. Com: Initiation of compaction, BL: Blastocyst, ExBL: Expanded Blastocyst, H-BL: Hatching Blastocyst.
[2] Humidifying conventional CO_2 incubator.
[3] Non-humidifying incubator with time-lapse cinematography.
[4] These embryos ("compacting" or "compacted" morula) were indistinguishable.

Table 3. Results of attainment time after insemination to each developmental stage of in vitro fertilized mouse embryos cultured under high and low oxygen tension in CCM-iBIS

Incubator	Hours after insemination [1]						
	2-cell (t2)	3-cell (t3)	4-cell (t4)	5-cell (t5)	Onset of compaction (tOC)	Morula (tM)	Blastocyst (tB)
CCM-iBIS [2] (high O_2 tension)	17.63 ± 0.04 [a]	39.83 ± 0.09 [a]	41.17 ± 0.12 [a]	51.97 ± 0.13 [a]	59.37 ± 0.14 [a]	69.54 ± 0.18 [a]	85.99 ± 0.29 [a]
CCM-iBIS [2] (low O_2 tension)	17.45 ± 0.07 [b]	38.66 ± 0.13 [b]	39.63 ± 0.15 [b]	49.04 ± 0.17 [b]	57.66 ± 0.19 [b]	70.01 ± 0.22 [a]	82.35 ± 0.27 [b]

[1] Data are shown as mean ± SEM in decimal. The embryos developed to the blastocyst stage within 120 hr after insemination were used for calculation (range: n=839-842 and n=280-289 in 20% and 5% O_2, respectively).
[2] Non-humidifying incubator with time-lapse cinematography.
a-b: Values with different superscripts are significantly different in the same column at $P<0.05$.

Table 4. Results of in vitro cultivation of mouse embryos fertilized in vitro in a non-humidifying incubator with time-lapse cinematography (CCM-iBIS) and a humidifying conventional CO_2 incubator (CPO_2-2301) without handling of culturing embryos inside and outside the incubator.

Incubator	No. of fertilized eggs cultured	No. (%) of development to:	
		4-cell (48 hr) [1]	Blastocyst (96 hr) [1]
CPO_2-2301 [2]	50	42 (84.0) [a]	—
	50	—	41 (82.0) [a]
CCM-iBIS [3,4]	951	845 (88.9) [a]	733 (77.1) [a]

[1] Hours after insemination.

[2] Humidifying conventional CO_2 incubator at 37°C, 5% CO_2 in air.

[3] Non-humidifying incubator with time-lapse cinematography at 37°C, 5% CO_2 in air.

[4] Data are from Table 1.

Values with the same superscripts are not significantly different in the same column at $P<0.05$.

Figures

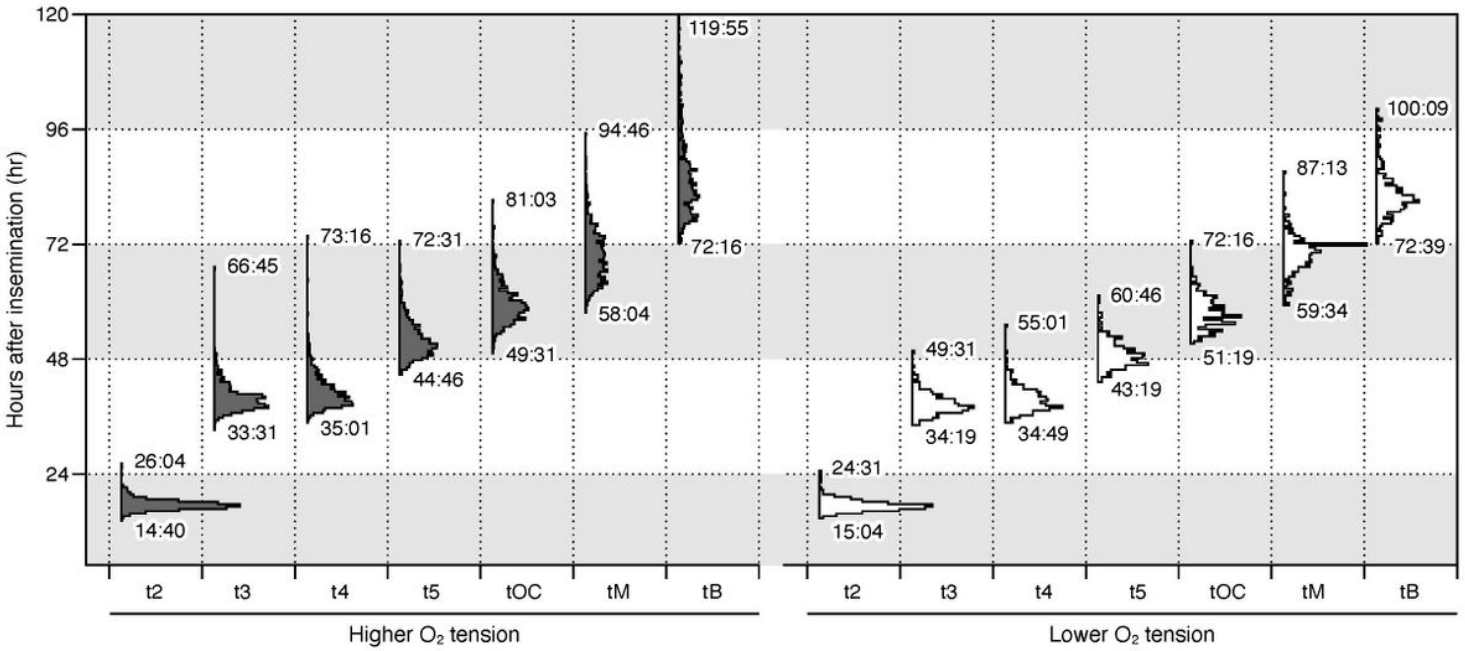

Figure 1

Histogram of the number of embryos fertilised in vitro at each developmental stage with the lapse of time after insemination. Embryos cultured in higher oxygen tension and lower oxygen tension in CCM-iBIS are indicated in black and white, respectively. Values show the range of time to cleavage in each developmental stage. Cleavage and development of embryos fertilised in vitro were much more synchronised in lower oxygen tension than in higher oxygen tension in CCM-iBIS. Parameters regarding embryonic development are annotated as follows: t2, time of cleavage to the 2-cell stage after insemination; t3, time of cleavage to the 3-cell stage after insemination; t4, time of cleavage to the 4-cell stage after insemination; t5, time of cleavage to the 5-cell stage after insemination; tOC, time of the onset of compaction after insemination; tM, time of formation of fully compacted morula after insemination; tB, time of formation of blastocyst with blastocoel at half volume of the embryo after insemination.

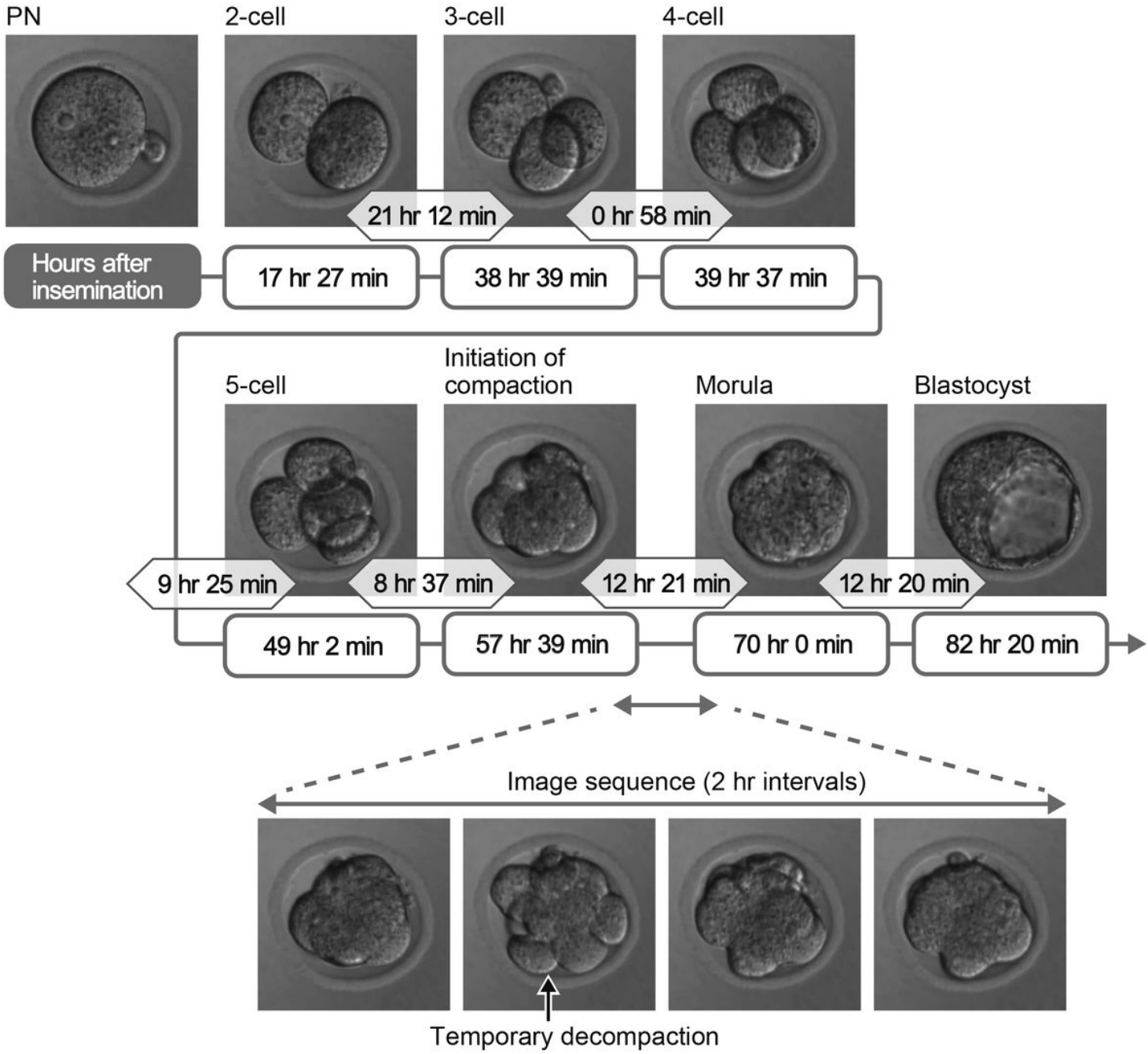

Figure 2

Timing of cleavage of mouse in vitro fertilised embryos during preimplantation development cultured in lower oxygen tension in a non-humidifying incubator with time-lapse cinematography (CCM-iBIS).

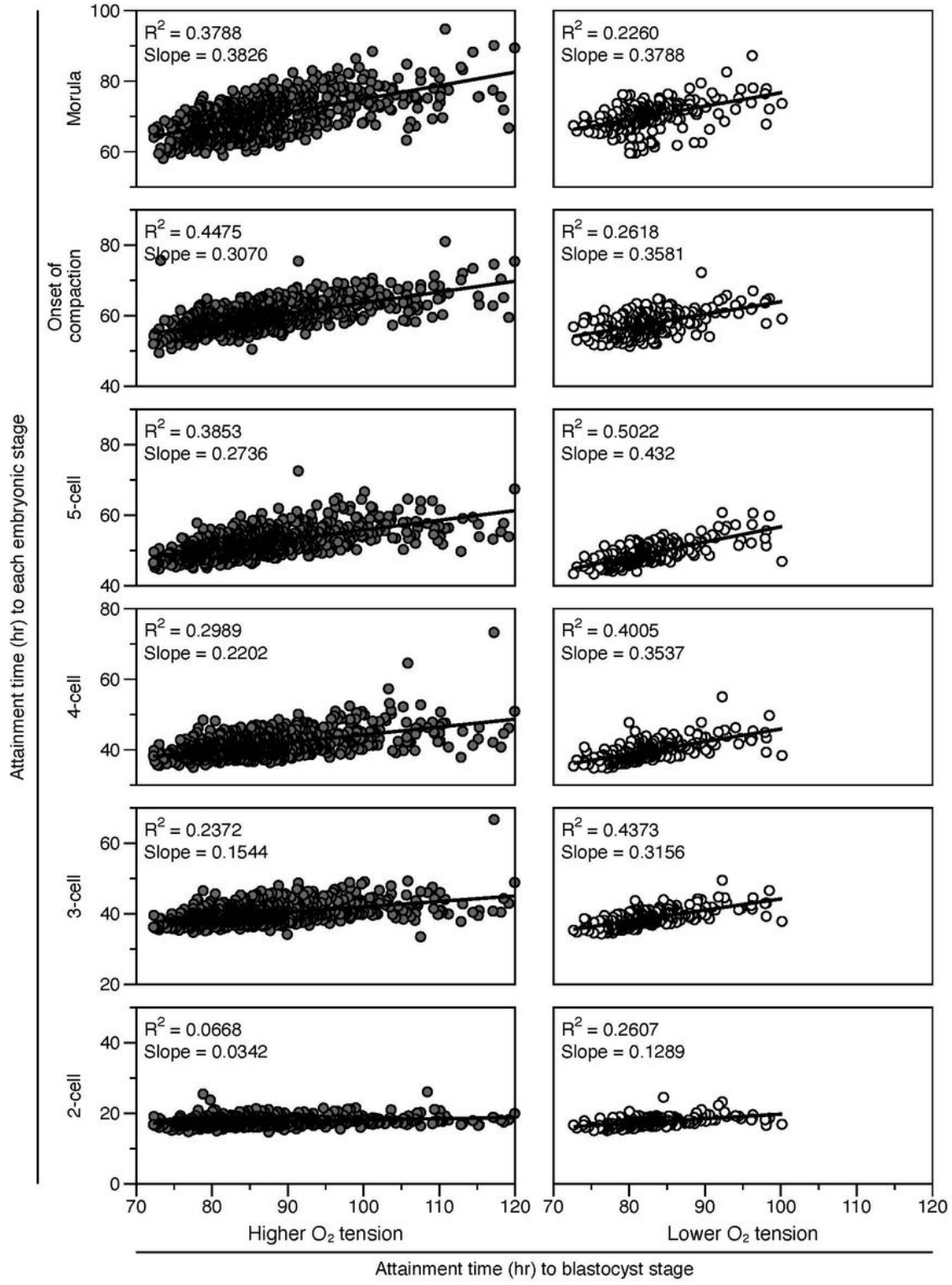

Figure 3

Relationship between attainment time to 2-cell, 3-cell, 4-cell, 5-cell, initiation of compaction and morula, and attainment time to blastocyst stage.