INTRODUCTION

In mice, pre-implantation development commences with fertilization resulting in the formation of a single cell zygote and progress to the blastocyst stage after undergoing cleavage divisions. During the developmental period, critical cellular events, particularly zygotic genome activation (ZGA), which is a component of maternal to zygotic transition, occur at the two-cell stage. By the end of the two-cell stage, maternal factors of most RNAs and some proteins that are generated and accumulated within unfertilized oocytes are

Effects of pyruvate and dimethyl-α-ketoglutarate, either alone or in combination, on pre- and post-implantation development of mouse zygotes cultured in vitro

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Abstract

Purpose: Dimethyl α-ketoglutarate (dm-α-KG) promotes in vitro development to blastocysts of C57BL/6J × C3He F1 mouse zygotes cultured in medium lacking pyruvate. Here, we examined the effects of pyruvate and dm-α-KG on in vitro development to blastocysts of ICR mouse zygotes and their post-implantation developmental ability.

Methods: Zygotes were cultured in medium with pyruvate at 0-0.2 mmol/L in the presence or absence of 1 mmol/L dm-α-KG for 96 hours and evaluated for blastocyst formation rates. The resultant blastocysts were non-surgically transferred to surrogates and evaluated for birth rates.

Results: In medium lacking pyruvate, zygotes could not develop beyond the two-cell stage, in the presence or absence of dm-α-KG. However, the blastocyst formation rate in medium with 0.01 mmol/L pyruvate (12%) was markedly increased with addition of dm-α-KG (49%). Around 80% of embryos developed to blastocysts in medium with 0.2 mmol/L pyruvate, in the presence or absence of dm-α-KG. Importantly, birth rate was markedly improved by treatment with 0.2 mmol/L pyruvate and dm-αKG (31.0%), compared with those with pyruvate treatment alone (16.3%).

Conclusions: Pyruvate and dm-α-KG synergistically work during in vitro culture to markedly improve the blastocyst formation rate and post-implantation developmental ability of the resultant blastocysts in ICR mice.

KEYWORDS

dimethyl α-ketoglutarate, in vitro culture, mice, pre-and post-implantation development, pyruvate
MATERIALS AND METHODS

2.1 | Animals

ICR mice were obtained from Kiwa Laboratory Animals Co., Ltd. This study conformed to the Guide for the Care and Use of Laboratory Animals. All animal experiments were approved by the Committee for Ethics on Animal Experiments of the Graduate School of Agriculture, Kyoto University, Kyoto, Japan.

2.2 | Chemicals

All chemicals used were purchased from Sigma-Aldrich or Wako Pure Chemical Industries, unless otherwise specified.

2.3 | Collection of zygotes and in vitro culture

Female ICR mice, aged 6-8 weeks, were superovulated by injection of 7.5 IU equine chorionic gonadotrophin (ASKA Pharmaceutical) followed 48 hours later by 7.5 IU human chorionic gonadotropin (hCG; Yell Pharmaceutical). The females were subsequently mated with male mice of the same strain, and vaginal plug formation was confirmed on the next morning (day 1). Zygotes were collected 15 hours after administration of hCG from the ampullae of oviducts of superovulated females by tearing the ampullae with a hypodermic needle. After removal of cumulus cells by digestion with 0.1% (w/v) hyaluronidase for approximately 5 minutes, the embryos were placed in 100 µL of culture medium per well in U bottom 96-well plates (PrimeSurface; Sumitomo Bakelite Co., Ltd.) and cultured for 5 days at 37°C under 5% CO₂ in air. The culture media used were potassium simplex optimized medium (KSOM medium) without EDTA and pyruvate supplemented with 0.3% deionized BSA (dBSA), designated here as -P medium, and -P medium with or without various concentrations (0.01, 0.02, and 0.2 mmol/L) of pyruvate and 1 mmol/L dimethyl-α-KG (a membrane permeable α-KG, dm-α-KG; Tokyo Chemical Industry Co., Ltd.). Stock solution of dBSA was prepared as previously described.15-17 Briefly, BSA was dissolved in distilled water at a concentration of 12%. Approximately 360 mg of mixed ion-exchange resin beads (AG501-X8(D); Bio-Rad Laboratories, Inc) was then added to 10 mL of 12% BSA solution, and the mixture was incubated at room temperature with occasional stirring. When the beads changed color from blue-green to gold, fresh beads were replaced in the BSA solution for a total of three replacements. The supernatant was sterilized with filtration (0.45 µm; Merck Millipore) and stored at −20°C as 12% stock solution. The embryos were observed every 24 hours under a stereomicroscope. The culture efficiency was evaluated by determining the proportion of embryos reaching the two-cell (day 2), four-cell (day 3), and blastocyst (day 5) stages.

2.4 | Embryo transfer

Pseudopregnant ICR females (8-12 weeks old) mated with proven sterile ICR males were used as embryo recipients and 17-18 embryos
that had developed to the blastocyst stage were non-surgically transferred into the uterus of the pseudopregnant females on day 3 using the NSET (Non-Surgical Embryo Transfer Device; ParaTechs) according to manufacturer’s instructions. Cesarean section and uterine analysis of implantation sites were performed in all recipients on day 20.

2.5 Immunofluorescence staining

Immunofluorescence staining was performed to determine cell numbers of blastocysts. Blastocysts were fixed in 3.7% paraformaldehyde in phosphate-buffered saline (PBS) for 1 hour at 4°C. After permeabilization with 0.5% Triton X-100 in PBS for 40 minutes at room temperature, the samples were blocked in blocking solution (0.02% Tween-20, 1.5% BSA and 0.2% sodium azide in PBS). To stain the inner cell masses (ICM), the samples were incubated at 4°C overnight in rabbit anti-OCT4 IgG (1:100 dilution; Santa Cruz Biotechnology), an ICM marker. After washing extensively in the blocking solution, they were incubated with Alexa-Fluor-488-labeled goat anti-rabbit IgG (1:100 dilution; Life Technologies) for 1 hour at room temperature. After washing with the blocking solution, the DNA was stained for 10 minutes with 10 mg/mL Hoechst 33258 and mounted on slides in 50% glycerol in PBS. The fluorescence signals of OCT4 and Hoechst were observed using a fluorescence microscope (FSX100; Olympus). The total number of cells was counted from the Hoechst image, and the number of ICM cells was counted from the OCT4 image. The number obtained by subtracting the number of ICM cells from the total number of cells was regarded as TE cell number.

2.6 Statistical analysis

Developmental rates to the two-cell, four-cell, and blastocyst stages of zygotes were analyzed by one-way ANOVA with subsequent Tukey’s multiple comparison tests. Data on blastocyst cell numbers were analyzed by Chi-square tests, and data on live offspring and implantation sites were analyzed by Fisher’s exact tests. Percentage data were subjected to arcsine transformation before statistical analyses. A value of $P < 0.05$ was considered to be significant. Each experiment was repeated at least three times.

3 RESULTS

We first examined the effects of various concentrations of pyruvate on the in vitro development to the blastocyst stage of zygotes. Zygotes were cultured for 96 hours in -P medium supplemented with pyruvate at concentrations of 0-0.2 mmol/L. As shown in Table 1, 76% of the embryos developed to the blastocyst stage in the medium with 0.2 mmol/L pyruvate. However, when cultured in the medium with 0.01 mmol/L pyruvate, the rate of development to the blastocyst stage (12%) was markedly reduced, and embryos cultured in medium with no pyruvate were completely arrested at the one- or two-cell stage. These results support earlier studies that have used slightly different media and different strains of mice.7,13,19

As mentioned above, developmental arrest of C57BL/6J X C3He F1 mouse embryos at the one- or two-cell stage by pyruvate deprivation is completely alleviated by adding 1 mmol/L dm-α-KG into the medium lacking pyruvate. We thus examined whether this alleviating effect of

| Pyruvate (mmol/L) | No. of one-cell embryos examined | No. (%) of two-cell embryos | No. (%) of four-cell embryos | No. (%) of blastocysts |
|-------------------|---------------------------------|-----------------------------|-----------------------------|------------------------|
| 0                 | 79                              | 37 (46.8)                   | 1 (1.3)                     | 0 (0)b                 |
| 0.01              | 50                              | 46 (92.0)                   | 19 (38.0)                   | 6 (12.0)b              |
| 0.02              | 135                             | 131 (97.0)                  | 6 (12.0)                    | 5 (10.0)b              |
| 0.2               | 50                              | 43 (86.0)                   | 41 (82.0)                   | 38 (76.0)a             |

Note: Different superscripts (a, b) indicate that values within the same column are significantly different (Tukey’s tests, $P < 0.05$).

| Pyruvate (mmol/L) | dm-α-KG (mmol/L) | No. of one-cell embryos examined | No. (%) of two-cell embryos | No. (%) of four-cell embryos | No. (%) of blastocysts |
|-------------------|------------------|---------------------------------|-----------------------------|-----------------------------|------------------------|
| 0                 | 0                | 59                              | 27 (45.8)b                  | 2 (3.4)b                    | 0 (0)b                 |
| 1                 |                  | 60                              | 35 (58.3)b                  | 3 (5.0)b                    | 0 (0)b                 |
| 0.01              | 0                | 50                              | 46 (92.0)a                  | 6 (12.0)b                   | 5 (10.0)b              |
| 1                 |                  | 55                              | 53 (96.4)a                  | 46 (83.6)a                  | 27 (49.1)a             |
| 0.2               | 0                | 50                              | 43 (86.0)                   | 41 (82.0)                   | 38 (76.0)a             |
| 1                 |                  | 48                              | 47 (98.0)                   | 46 (95.8)                   | 43 (89.6)a             |

Note: Different superscripts (a, b) indicate that values within the same column are significantly different (Tukey’s tests, $P < 0.05$).
dm-α-KG is also exerted on embryos derived from the ICR strain. As shown in Table 2, when ICR strain mouse zygotes were cultured for 96 hours in -P medium supplemented with or without 1 mmol/L dm-α-KG, most of the embryos could not develop beyond the two-cell stage, in the presence or absence of dm-α-KG. However, when zygotes were cultured in 0.01 mmol/L pyruvate-containing medium without dm-α-KG, the embryos could develop to the blastocyst stage at a very low rate (10%), and this rate significantly increased (49%) upon adding dm-α-KG, suggesting the synergistic effect of pyruvate and dm-α-KG (Table 2). When cultured with 0.2 mmol/L pyruvate and 1 mmol/L dm-α-KG, the developmental rate (86%) was somewhat higher than that (76%) for pyruvate alone, although not significant (Table 2). Additionally, it was found that there were no significant differences in the total, ICM, and TE cell numbers between blastocysts generated in pyruvate-containing medium with or without dm-α-KG (Table 3).

We then evaluated the post-implantation developmental ability of blastocysts yielded by culturing in the medium with 0.2 mmol/L pyruvate alone or in combination with 1 mmol/L dm-α-KG. The combinatorial treatment significantly increased the implantation rate (46.2%), compared with that for pyruvate treatment alone (25.2%), when embryos at the blastocyst stage were transferred to surrogates. Importantly, birth rate was markedly improved by the combined treatment (31.0%), compared with that for pyruvate treatment alone (16.3%). Offspring at birth obtained by the combined treatment showed similar body weights to those for pyruvate treatment alone (Table 4). These results suggest that pyruvate and dm-α-KG synergistically work during in vitro culture to markedly improve the quality of IVP blastocysts in mice.

4 | DISCUSSION

This study investigated the effects of pyruvate and dm-α-KG treatments, either alone or in combination, during in vitro culture from the one-cell to blastocyst stages on blastocyst formation rates and post-implantation development of the resultant blastocysts after embryo transfer to surrogates in the ICR strain mice. As a result, we first found that developmental arrest at the one- or two-cell stage was caused by depleting the medium of pyruvate, and blastocyst formation rates were increased with increasing concentrations of pyruvate at 0-0.2 mmol/L, and the maximum rate reached at 0.2 mmol/L with around 80% of embryos developing to the blastocyst stage. Interestingly, Nagaraj et al.\(^7\) reported that although C57BL/6J X C3He F1 mouse zygotes that cultured in medium depleted of pyruvate (-P medium) could not develop beyond the two-cell stage, the developmental arrest was almost completely reversed by adding dm-α-KG into the -P medium, despite the energy and redox levels of the embryos cultured under these conditions being at similar levels to those cultured in -P medium. However, our results showed that the developmental arrest due to pyruvate deprivation could not be alleviated by dm-α-KG at all, whereas in the presence of small amounts of pyruvate (0.01 mmol/L), dm-α-KG promoted the development to the blastocyst stage by synergistic actions with pyruvate, suggesting that the energetic and redox stress caused by pyruvate depletion should be mitigated moderately, compared with C57BL/6J X C3He F1 mouse embryos. The reason for the difference in the effectiveness of dm-α-KG between our study and those of others remains unknown. However, it may be linked to the different mouse strains used, because strain-dependent differences in the ability of mouse embryos to metabolize particular substrates, including energy substrates, such as pyruvate, lactate, and glucose, which markedly influence their developmental competence, have previously been shown.\(^{13,20,21}\)

Furthermore, Nagaraj et al.\(^7\) reported that although α-KG can be generated with catabolism of non-essential amino acids, only proline and arginine among non-essential amino acids were capable of such a rescue, and similar to α-KG, proline promoted embryo progression beyond the two-cell stage even under energetic and redox stress. On the other hand, it is known that α-KG plays a critical role as a cosubstrate for several histone and DNA demethylases, including the jumonji domain-containing histone demethylase and the ten-eleven translocation (TET) family enzymes (TET1, TET2, and TET3),\(^{22}\) and that the process of ZGA that occurs at the

| TABLE 3 | Total, inner cell masses (ICM), and TE cell numbers in blastocysts cultured from zygotes in pyruvate (0.2 mmol/L) containing medium with or without dm-αKG |
|-----------------|-----------------|-----------------|-----------------|
| dm-α-KG (mmol/L) | No. of blastocysts examined | Total cell number (mean ± SEM) | ICM cell number (mean ± SEM) | TE cell number (mean ± SEM) |
| 0 | 12 | 100.1 ± 4.2 | 17.8 ± 0.8 | 82.3 ± 4.4 |
| 1 | 15 | 93.6 ± 5.2 | 19.9 ± 1.0 | 73.7 ± 4.8 |

| TABLE 4 | Post-implantation development of blastocysts cultured from zygotes for 96 h in pyruvate-containing medium with or without dm-α-KG after embryo transfer |
|-----------------|-----------------|-----------------|-----------------|
| dm-α-KG (mmol/L) | No. of embryos transferred (recipients) | No. (%) of live offspring | No. (%) of implantation sites | Average body weight (g) |
| 0 | 147 (8) | 24 (16.3)\(^a\) | 37 (25.2)\(^b\) | 2.00 |
| 1 | 158 (9) | 49 (31.0)\(^a\) | 73 (46.2)\(^a\) | 1.94 |

Note: Different superscripts (a, b) indicate that values within the same column are significantly different (Fisher’s exact tests, P < 0.05).
two-cell stage in mouse embryos involves epigenetic changes to paternal and maternal genomes for embryo development. These findings suggest that α-KG acts as an epigenetic regulator through the activation of α-KG-dependent histone and DNA demethylases, rather than as a metabolic substrate for energy production in mitochondria for its promoting effect on early embryo development. Recent studies have shown that after fertilization, DNA demethylation is induced by oxidation of 5-methylcytosine (5mec) to 5-hydroxymethylcytosine (5hmec) by TET family enzymes, and that this conversion is mostly observed at the zygote stage, and thereafter in the amount of 5mec occurs again at the blastocyst stage, particularly in the ICM. TET1 and TET3 play important roles in early embryo development. Failure of TET3 expression in early-stage embryos by conditional knockout caused an abnormal expression of Oct4 and Nanog due to improper demethylation on the promoter regions of the genes. TET1 has an important function for the cellular specification of ICM at the blastocyst stage. On the other hand, the jumonji domain-containing histone demethylases including H3K4 demethylase KDM5A and KDM5B, H3K9 demethylase KDM4C, and H3K27 demethylase KDM6B have been reported to play instrumental roles in regulating ZGA and early embryo development in mice. However, it still remains elusive how α-KG-dependent demethylases act on the development.

Importantly, here we found for the first time that the post-implantation developmental ability of blastocysts yielded by culturing zygotes for 96 hours in 0.2 mmol/L pyruvate combined with dm-α-KG was significantly enhanced, compared with those cultured in 0.2 mmol/L pyruvate alone, even though there were no significant differences in blastocyst formation rates and in the total, ICM, and TE cell numbers of the resultant blastocysts between treatments both with pyruvate and dm-α-KG and with pyruvate alone. Our findings may be utilized to improve the in vitro culture system for in vitro fertilized human and livestock embryos.

**DISCLOSURES**

**Conflict of interest:** Eun Sol Choi, Koga Kawano, Misaki Hiraya, Elbai Matsukawa and Masayasu Yamada declare that they have no conflict of interest. **Human rights statement and informed consent:** This article does not contain any study with human participants that was performed by any of the authors. **Animal studies:** All the experiments in this research were approved by the Committee for Ethics on Animal Experiments of the Graduate School of Agriculture, Kyoto University, Kyoto, Japan.

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**REFERENCES**

1. Bultman SJ, Gebuhr TC, Pan H, Svoboda P, Schultz RM, Magnuson T. Maternal BRG1 regulates zygotic genome activation in the mouse. Genes Dev. 2006;20(13):1744-1754.

2. Aoki F, Worrad DM, Schultz RM. Regulation of transcriptional activity during the first and second cell cycles in the preimplantation mouse embryo. Dev Biol. 1997;181(2):296-307.

3. Li L, Zheng P, Dean J. Maternal control of early mouse development. Development. 2010;137(6):859-870.

4. Weaver JR, Susiarjo M, Bartolomei MS. Imaging and epigenetic changes in the early embryo. Mam Reprod. 2009;20(9-10):532-543.

5. Hardivillé S, Hart GW. Nutritional regulation of signaling, transcription, and cell physiology by O-GlcNAcylation. Cell Metab. 2014;20(2):208-213.

6. Martínez-Pastor B, Cosentino C, Mostoslavsky R. A tale of metabolites: the cross-talk between chromatin and energy metabolism. Cancer Discov. 2013;3(5):497-501.

7. Nagaraj R, Sharpley MS, Chi F, et al. Nuclear localization of mitochondrial TCA cycle enzymes as a critical step in mammalian zygotic genome activation. Cell. 2017;168(1-2):210-223.

8. Leese HJ. Metabolism of the preimplantation embryo: 40 years on. Reproduction. 2012;143(4):417-427.

9. Truong TT, Soh YM, Gardner DK. Antioxidants improve preimplantation embryo development and viability. Hum Reprod. 2016;31(7):1445-1454.

10. Feuer S, Rinaudo P. Preimplantation stress and development. Birth Defects Res C Embryo Today. 2012;96(4):299-314.

11. Schwarzer C, Esteves TC, Araúzo-Bravo MJ, et al. ART culture conditions change the probability of mouse embryo gestation through defined cellular and molecular responses. Hum Reprod. 2012;27(9):2627-2640.

12. Zanon M, Garagna S, Redi CA, Zuccotti M. The 2-cell block occurring during development of outbred mouse embryos is rescued by cytoplasmic factors present in inbred maternal cell cytoplasts. Int J Dev Biol. 2009;53(1):129-134.

13. Brown JJ, Whittingham DG. The roles of pyruvate, lactate and glucose during preimplantation development of embryos from F1 hybrid mice in vitro. Development. 1991;112(1):99-105.

14. Erbach GT, Lawitts JA, Papaioannou VE, Biggers JD. Differential growth of the mouse preimplantation embryo in chemically defined media. Biol Reprod. 1994;50(5):1027-1033.

15. Ng ES, Davis R, Stanley EG, Elefanty AG. A protocol describing the use of a recombinant protein-based, animal product-free medium (APEL) for human embryonic stem cell differentiation as spin embryoid bodies. Nat Protoc. 2008;3(5):768-776.

16. Miyamoto K, Tajima Y, Yoshida K, et al. Reprogramming towards totipotency is greatly facilitated by synergistic effects of small molecules. Bioopen. 2017;6(4):415-424.

17. Azuma R, Miyamoto K, Oikawa M, Yamada M, Anzai M. Combinational treatment of trichostatin A and vitamin C improves the efficiency of cloning mice by somatic cell nuclear transfer. J Vis Exp. 2018;134:e57036.

18. Van Thuan N, Wakayama S, Kishigami S, et al. Injection of somatic cell cytoplasm into oocytes before intracytoplasmic sperm injection impairs full-term development and increases placental weight in mice. Biol Reprod. 2006;74(5):865-873.

19. Biggers JD, Whittingham DG, Donahue RP. The pattern of energy metabolism in the mouse oocyte and zygote. Proc Natl Acad Sci U S A. 1967;58(2):560-567.

20. Brown JJ, Whittingham DG. The dynamic provision of different energy substrates improves development of one-cell random-bred mouse embryos in vitro. J Reprod Fertil. 1992;95(2):503-511.

21. Feuer SK, Rinaudo PF. Physiological, metabolic and transcriptional postnatal phenotypes of in vitro fertilization (IVF) in the mouse. J Dev Orig Health Dis. 2017;8(4):403-410.

22. Xiao M, Yang H, Xu W, et al. Inhibition of α-KG-dependent histone and DNA demethylases by fumarate and succinate that are accumulated in mutations of FH and SDH tumor suppressors. Genes Dev. 2012;26(12):1326-1338.
23. Dahl JA, Jung I, Aanes H, et al. Broad histone H3K4me3 domains in mouse oocytes modulate maternal-to-zygotic transition. Nature. 2016;537(7621):548-552.
24. Tahiliani M, Koh KP, Shen Y, et al. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science. 2009;324(5929):930-935.
25. Inoue A, Zhang Y. Replication-dependent loss of 5-hydroxymethylcytosine in mouse preimplantation embryos. Science. 2011;334(6053):194.
26. Ito S, D’Alessio AC, Taranova OV, Hong K, Sowers LC, Zhang Y. Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specification. Nature. 2010;466(7310):1129-1133.
27. Gu TP, Guo F, Yang H, et al. The role of Tet3 DNA dioxygenase in epigenetic reprogramming by oocytes. Nature. 2011;477(7366):606-610.
28. Xhabija B, Kidder BL. KDM5B is a master regulator of the H3K4-demethylome in stem cells, development and cancer. Semin Cancer Biol. 2018; pii: S1044-579X(18)30073-30077
29. Wang J, Zhang M, Zhang Y, et al. The histone demethylase JMJD2C is stage-specifically expressed in preimplantation mouse embryos and is required for embryonic development. Biol Reprod. 2010;82(1):105-111.
30. Liu X, Wang Y, Gao Y, et al. H3K9 demethylase KDM4E is an epigenetic regulator for bovine embryonic development and a defective factor for nuclear reprogramming. Development. 2018;145(4):pii: dev158261.
31. Chung N, Bogliotti YS, Ding W, et al. Active H3K27me3 demethylation by KDM6B is required for normal development of bovine preimplantation embryos. Epigenetics. 2017;12(12):1048-1056.
32. Yang L, Song LS, Liu XF, et al. The maternal effect genes UTX and JMJD3 play contrasting roles in mus musculus preimplantation embryo development. Sci Rep. 2016;6:26711.

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