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RESEARCH NOTE

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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 X 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

1 | 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.[1,2] 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

depleted, and the subsequent development beyond the two-cell stage requires embryonic factors produced by ZGA gene burst.[3] These processes are dependent on highly coordinated epigenetic changes in the zygotic genome, and moreover, on the diverse energy requirements, such as α-ketoglutarate and acetyl-CoA, during the period of ZGA.[4-8]

Pre-implantation mouse embryos are able to be cultured in vitro from the zygote to blastocyst stages, and by embryo transfer of the in vitro-produced (IVP) blastocysts into the uterus of surrogates, they can implant into the uterine wall and develop to term. However, it is known that post-implantation development of IVP embryos is inferior to in vivo derived embryos.[9] The developmental competence of IVP embryos is influenced by in vitro culture conditions, indicating that subtle alterations in the culture medium composition can affect embryo metabolisms and viability.[10,11] Furthermore, the competence is different depending on the mouse strain.[12]

Metabolisms of pyruvate and lactate as energy sources in the early stage of pre-implantation embryos play important roles for their development. Zygotes fail to survive in the medium depleted of both pyruvate and lactate, and even if only pyruvate is removed from the medium, zygotes survive but fail to develop beyond the two-cell stage, the time of ZGA.[13] This requirement of pyruvate for the early stage of pre-implantation development is unexpected given the low energetic activity of the early-stage embryos.[8] However, recently, Nagaraj et al[7] reported that in the experiments using C57BL/6J X C3He F1 mice, a subset of mitochondrial enzymes, pyruvate dehydrogenase, and enzymes associated with the first half-cycle of the tricarboxylic acid cycle, including citrate synthase, aconitase 2, and isocitrate dehydrogenase 3A, are temporarily localized to and are active within the nucleus of the embryo during the ZGA, indicating the low metabolic activity of mitochondria particularly during ZGA. The nuclear localization of these enzymes is regulated by pyruvate and is essential for the production of important metabolites for epigenetic remodeling during ZGA, including acetyl coenzyme A (acetyl-CoA) and α-ketoglutarate (α-KG).

Interestingly, it was also reported that the developmental and nuclear transport defects caused by pyruvate deprivation can be rescued by exogenously provided α-KG and that energy (the amount of ATP and the ATP/ADP ratio) and redox (NAD^+/NADH ratio) levels critical for embryonic development are too low in the absence of pyruvate; whereas, such low energy and redox levels due to a lack of pyruvate remain unaltered even in the presence of α-KG.[7] These findings suggest that the mechanisms of action of α-KG may differ from those of pyruvate. Therefore, we may expect additional or synergistic effects of pyruvate and α-KG on in vitro development to the blastocyst stage of zygotes.

We thus examined the effects of pyruvate and α-KG, either alone or in combination, on in vitro development to the blastocyst stage of zygotes collected from ICR strain female mice, a popular outbred strain as a practical laboratory mouse used generally in the experiments on the development of in vitro culture system for pre-implantation embryos, and their post-implantation development by embryo transfer of the resultant blastocysts into the uterus of surrogates.

2 | 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% CO_2 in air. The culture media used were potassium simplex optimized medium (KSOM medium[14]) 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.[18] 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

TABLE 1 Dose effect of pyruvate on development of zygotes cultured in vitro

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)	107 (79.3)	70 (51.9)[a]
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, $P < 0.05$).

TABLE 2 Effects of pyruvate and dm-α-KG, either alone or in combination, on the development to the blastocyst stage of zygotes

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)[a]	41(82.0)[a]	38 (76.0)[a]
	1	48	47 (98.0)[a]	46 (95.8)[a]	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 F1mouse 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)[b]	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.[23] 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 oxidization of 5-methylcytosine (5mec) to 5-hydroxymethylcytosine (5hmec) by TET family enzymes,[24] and that this conversion is mostly observed at the zygote stage,[25] and thereafter an increase in the amount of 5hmec occurs again at the blastocyst stage, particularly in the ICM.[26] 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.[27] TET1 has an important function for the cellular specification of ICM at the blastocyst stage.[26] 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.[23,28-32] 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, Eibai 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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