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Lactate metabolic regulation by NRs during early embryo stage development

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Lactate metabolic regulation by NRs during early embryo stage development

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ABSTRACT

Lactate metabolic regulation by NRs during early embryo stage development

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Environmental contribution in mammalian development is becoming more important topics these days. During embryogenesis, the microenvironment around the embryo supports one successful embryo development at a particular time through appropriate supporting of metabolites. A dramatical changes in metabolism is occurs during pre-implantation stage. The embryo of cleavage stages primarily uses pyruvate and lactate to maintain low levels of metabolism, and once compaction begins, pyruvate is converted to glucose-based metabolism. It also have a significant meaning on the preparing intermediate metabolites on the normal development of the embryo and become an important marker for developmental competency. It is known that lactate is not only used as an energy source but also one of the substrates whose proper production and utilization can affect embryonic development. Lactate dehydrogenase (LDH) catalyzes the interconversion between pyruvate and lactate. There are four

types of LDH gene: *Ldha*, *Ldhb*, *Ldhc* and *Ldhd*. First two genes code LDHA or LDHB subunit, respectively, which form five distinct tetrameric isoenzymes, LDH-1 to 5. LDH-1 (A0B4) catalyzes conversion L-lactate to pyruvate preferentially and LDH-5 (A4B0) catalyzes vice versa direction. LDH-2 (A3B1), LDH-3 (A2B2) and LDH-4 (A1B3) have intermediate enzyme activity. The *Ldhc* codes LDHC subunit forming LDH-C4 that catalyzes oxidation of L-lactate to pyruvate. Unlike others, *Ldhd* codes LDHD subunit that consists of dimer utilizing D-lactate. Nuclear receptors bind to ligands, function as transcriptional regulators in the nucleus, and can perform a variety of functions, including metabolic and developmental regulation, cell differentiation and homeostasis. Among them, the retinoid X receptor (RXR) play an important role in various biological processes because it can act as a dimer not only with RAR but also with other members of the nuclear receptor family such as Peroxisome Proliferator-Activated Receptor (PPAR). However, the studies of the role of signaling molecules and gene-regulated nuclear receptors in lactate metabolism during early embryonic development are currently lacking. In this study, the possible relation between RXR and lactate metabolism was examined. 2-cell stage embryos were used and analyzed developmental rate by culture condition. The mRNA levels of RXRs, PPARs, PGC1s and LDHs were evaluated at various treatment groups. Embryos cultured in lactate-free conditions showed developmental delays compared to controls. Conversely, embryos cultured under lactate-high conditions showed a faster development rate than control and

lactate-free conditions. The expression of *Ldha*, which converts pyruvate to lactate, showed a relatively higher mRNA expression level than that of *Ldhb*. *Rxr*s and *Pgc1*s also showed different expression depending on the concentration of lactate in the culture medium or the RXR agonist or antagonist. These results revealed that the rate of early embryo development can be affected by the concentration of lactate in the culture medium. In addition, based on these results, it was suggested that *Rxr*s may be involved in lactate metabolism and expression of lactate dehydrogenase through circuit forming for lactate metabolism.

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INTRODUCTION

During embryogenesis, the embryo is surrounded by a fluid that provides a suitable microenvironment such as temperature, pH, etc (Swain et al., 2011). Such microenvironment supports the development of a successful first embryo at a particular time through the proper metabolic chains. As mammalian pre-implantation embryos develop, a dramatic changes occur in metabolism. The modified early division stage maintains low levels of metabolism, primarily using pyruvate and lactate, and once compression is initiated, pyruvate is converted to glucose-based metabolism (Lain et al., 2000).

Unlike most cell types, late cleavage stage mouse embryo converts glucose consumed to lactic acid, even if there is enough oxygen to support complete oxidation (Cheon, 2008). During implantation, blastocysts have the property of releasing significant amounts of lactic acid into the surrounding microenvironment due to their unique metabolism, as demonstrated in some early studies (Wales and Whittingham, 1973; Clough and Whittingham, 1983; Gardner and Leese, 1990). Such aerobic glycolysis appears in the same pattern in human embryos. These metabolic changes can also have a profound effect on the normal development of the embryo and its subsequent viability (Krisher and Prather, 2012). Thus, lactate is one of the dispositions that not only is used as an energy source, but also prepare suitable environment for embryogenesis.

Lactate dehydrogenase (LDH) catalyzes the interconversion of lactic acid and pyruvic acid. It is known that there are four types of LDH genes: *Ldha, Ldhb, Ldhc* and *Ldhd*. First two genes code LDHA or LDHB subunit, respectively, which form five distinct tetrameric isoenzymes, LDH-1 to 5 (Porporato et al., 2011). LDH-1 (A0B4) catalyzes conversion L-lactate to pyruvate preferentially and LDH-5 (A4B0) catalyzes vice versa direction. LDH-2 (A3B1), LDH-3 (A2B2) and LDH-4 (A1B3) have intermediate enzyme activity. The *Ldhc* codes LDHC subunit forming LDH-C4 that catalyzes oxidation of L-lactate to pyruvate. Unlike others, *Ldhd* codes LDHD subunit that consists of dimer utilizing D-lactate (Drabkin et al., 2019). Nuclear receptors, on the other hand, are said to be able to bind ligands, act as transcriptional regulators in the nucleus, and perform a variety of functions, including regulation of metabolism and development, cell differentiation and homeostasis (Pawlak et al., 2021). Among them, the retinoid X receptors (RXRs) are an important member of the nuclear receptor steroid / thyroid hormone superfamily. RXR can play an important role in various biological processes because it can act as a dimer not only with RAR but also with other members of the ligand-gated nuclear receptor family such as peroxisome proliferator-activated receptor (PPAR) (Chandra et al., 2008). Heterodimers are functional units that bind ligands (retinoids), transcriptional co-regulators, and genetic networks that bind to DNA and control cell growth, differentiation, and death (Maire et al., 2019). Two main retinoid response pathways are known. Retinoic acid receptors (RARs) and RXRs heterodimerize and then activate transcription via retinoic acid (RA) response elements

consisting of direct repeats (DRs) spaced by 2 (DR-2) or 5 (DR-5) base pairs. (Zhang et al., 2015). Both the RAR and RXR partners of the heterodimer can be ligand activated in vivo, resulting in synergistic activation. RXRs also homodimerize, and this homodimerization contrasts with RAR-RXR heterodimerization in being ligand stimulated. RXR homodimers activate transcription from retinoid X response elements (RXREs) consisting of DRs spaced by 1 (DR-1) base pair (Pijnappel et al., 1998). In fact, RXR in skeletal muscle PGC-1α promotes RXR expression, which also reduces Myc by an unknown mechanism, resulting in a decrease in *Ldha* expression (Summermatter et al., 2013). In addition, it was found by previous studies that the expression of *Ldhb* can also be regulated by PGC-1a (Liang et al., 2016). Peroxisome proliferator-activated receptor-γ coactivator (PGC) -1α is a member of the transcriptional coactivator family, which plays a central role in the regulation of cellular energy metabolism. (Liang and Ward, 2006). PGC-1β is the closest homolog of PGC-1α and shares extensive sequence identity. PGC-1-related coactivator (PPRC1 or PRC), initially identified as a member of the PGC-1 family, is believed to regulate mitochondria biogenesis, respiration pathways, and cell proliferation. (He et al., 2012).

So far, the studies on the role of signaling molecules and gene regulatory nuclear receptors in the metabolism of lactate during early embryonic development are currently lacking. RXR and lactate dehydrogenase are involved in the interconversion of lactate and pyruvic acid during early embryo development. In this study, by observing the expression levels of *Pgc1a, Pgc1b,*

Ldha, Ldhb, Rxra, Rxrb and *Rxrg* in culture with various lactic acid concentration conditions (lactate-free or high lactic acid) or in culture treated with an agonist and antagonist of RXR, the role of RXR in regulating lactate metabolism were studied.

MATERIALS AND METHODS

Laboratory animals

All experimental animals were conducted according to the Guide for the Care and Use of Laboratory Animals published by the National Institute of Health. Experiment protocols were approved by the IACUC (Institutional Animal and Care Committee) of Sungshin University (IACUC number: 2019-2-10). These animals were maintained under standard condition at Sungshin Women's University diurnal rhythm kept under the 14L : 10D schedule with light-on at 06:00 hr and clean room system. Animals were fed a standard rodent diet and water ad libitum from weaning at 28 days after birth catalyzes the conversion in the opposite direction.

Superovulation and embryo sampling

To induce superovulation in female mice, PMSG and hCG were diluted to 5 mg/0.1 ml with 0.85% saline. 6-8 weeks old female CD-1 mice were superovulated by PMSG (5IU, 0.1ml) intraperitoneal injection in followed 48hr later by hCG (5IU, 0.1ml). After that, after 5 pm, female mice are placed together with male mice of the same strain. The next morning, check for the presence of

a vaginal plug and consider pregnant. Each stage of preimplantation embryo including 4-cell, 8-cell, morula or early blastocyst were collected from oviduct or uterus by flushing with Biggers-Whitten-Whittingham (BWW) medium containing 0.4% bovine serum albumin (BOVOGEN, KE, Australia, Cat. No. BSA100) after 56, 64, 84 and 90 h of post-hCG injection, respectively. *In vitro* developed 4-cell, 8-cell, morula or early blastocyst were collected at 60, 72, 84 and 96 h of post-hCG injection, respectively.

Culture medium preparation

Control medium is Biggers-Whitten-Whittingham (BWW) medium. Lactate-free medium contained 21.58mM NaCl and 1.71mM $CaCl_2$ instead of 21.58mM Sodium DL-lactate and 1.71mM Calcium L-lactate. The composition is listed in Table 1.

Total RNA extraction and cDNA synthesis

Total RNA of 10 ea embryos was extracted using RNeasy® Micro kit (QIAGEN, CA, USA, Cat. No. 74004) according to the manual of manufacture. To synthesize irst strand cDNA, we used Accuscript first strand cDNA synthesis kit (Stratagene, CA, USA, Cat. No.) according to the manual of manufacture. Briefly,

reaction reagents were total RNA of 10ea embryos, 5.0 μl Accuscirpt buffer (10X), 1.0 oligo dT primer (0.5 μg/μl), 1.0 μl random primers (0.1 μg/μl), 2 μl dNTP mix (100mM), RNase-free water. Mixture was incubated at 65°C for 5 min, placed at RT to anneal RNA with primers for 10 min, after then 0.4 μl DTT (100mM), 2.0 μl RNase block ribonuclease inhibitor (40 U/ml), and 1.0 μl Accuscript multiple temperature RT were added. The reaction mixture was incubated at 42°C for 1 h and 70°C for 15 min. Finally, the products were kept at -20°C before used.

Real-time RT-PCR analysis

Real-time PCR was performed by AriaMx Real-time PCR System (Agilent, CA, USA) according to thermal cycler schedule in Table 2. Each reaction was run in triplicate and and consisted of 1 μl cDNA, 10 μl SYBR® Premix Ex Taq™ (TAKARA, Kyoto, Japan, Cat. No. RR420A) and the space target gene primers listed in Table 3. To ensure amplification of a single product at appropriate melting temperature, dissociation curve were run on all reactions. The fold change in gene expression was calculated using the ΔΔCT method with the housekeeping gene, a ribosomal protein, 36B4(RPLP0), as the internal control.

RXR agonist and antagonist treatment

To induce superovulation in female mice, PMSG and hCG were diluted to 5 mg/0.1 ml with 0.85% saline. 6-8 weeks old female CD-1 mice were superovulated by PMSG (5IU, 0.1ml) intraperitoneal injection in followed 48hr later by hCG (5IU, 0.1ml). After that, after 5 pm, female mice are placed together with male mice of the same strain. The next morning, check for the presence of a vaginal plug and consider pregnant. Each stage of preimplantation embryo including 4-cell, 8-cell, morula or early blastocyst were collected from oviduct or uterus by flushing with Biggers-Whitten-Whittingham (BWW) medium containing 0.4% bovine serum albumin (BOVOGEN, KE, Australia, Cat. No. BSA100) after 56, 64, 84 and 90 h of post-hCG injection, respectively. For *in vitro* culture, 2-cell stage embryos were collected from oviduct in BWW medium containing 0.4% bovine serum albumin (BSA). The 2-cell stage embryos were cultured in BWW medium containing 0.4% bovine serum albumin (BSA) and synthetic retinoid (HX630, UVI3003) for 96 h. *In vitro* developed 4-cell, 8-cell, morula or early blastocyst were collected at 60, 72, 84 and 96 h of post-hCG injection, respectively.

Statistics

The t-test was used to evaluate the difference between control and experimental group. Results were presented as mean ± SEM. Values of $P < 0.05$ were considered significant. The X^2 test was used to evaluate the development rate and experiment group development rate.

Table 1. Culture medium composition

A. BWW medium (Total volume : 500 ml)

Component	Concentration(mM)
NaCl (Sodium chloride)	94.59
KCl (Potassium chloride)	4.78
KH_2PO_4 (Potassium phosphate)	1.19
$MgSO_4$ (Magnesium sulfate)	1.19
Glucose (D-(+)-glucose)	5.56
Ca-lactate	1.71
Na-pyruvate	0.25
Na-lactate	21.58
Antibiotics (x1000)	-
Phenol red	-
$NaHCO_3$ (Sodium bicarbonate)	25.07
total	

B. Lactate-free medium (Total volume : 500 ml)

Component	Concentration(mM)
NaCl (Sodium chloride)	94.59
KCl (Potassium chloride)	4.78
KH_2PO_4 (Potassium phosphate)	1.19
$MgSO_4$ (Magnesium sulfate)	1.19
Glucose (D-(+)-glucose)	5.56
$CaCl_2 \cdot 2H_2O$	1.71
Na-pyruvate	0.25
NaCl	21.58
Antibiotics (x1000)	-
Phenol red	-
$NaHCO_3$ (Sodium bicarbonate)	25.07
total	

C. Lactate-high medium (Total volume : 500 ml)

Component	Concentration(mM)
NaCl (Sodium chloride)	82.305
KCl (Potassium chloride)	4.78
KH_2PO_4 (Potassium phosphate)	1.19
$MgSO_4$ (Magnesium sulfate)	1.19
Glucose (D-(+)-glucose)	5.56
Ca-lactate	1.71
Na-pyruvate	0.25
Na-lactate	38.29
Antibiotics (x1000)	-
Phenol red	-
$NaHCO_3$ (Sodium bicarbonate)	25.07
total	

Table 2. Thermal cycler schedule

step		Temperature	Time
Hold	Hold	95	30 sec
3 step PCR (45 cycle)	Denaturation	95	1 min
	Annealing	59	30 sec
	Extension	72	1 min
Dissociation	Denaturation	95	15 sec
	Annealing	60	30 sec
	Extension	95	15 sec
Hold		4	Indefinitely

Table 3. Primer for genes

Gene	Symbol	NCBI gene reference		Primer sequence (5’ -3’)	Amplified length (bp)
Retinoid X receptor alpha	Rxra	NM_011305.3	S	AAGGACCGGAATGAGAACGAG	246
			AS	TAGGGGCAGCTCAGAAAAGTGT	
Retinoid X receptor beta	Rxrb	NM_001205214.1	S	CCCTTCCCAGTCATCAGTTCTTC	230
			AS	CAGATTGCACAGAGCCGTTTG	
Retinoid X receptor gamma	Rxrg	NM_009107.3	S	GGAATGAACTGAGCAGCCAACA	231
			AS	ATACGGAGAGCCAAGAGCATTG	
Lactate dehydrogenase A	Ldha	NM_010699.2	S	ACTGTGTAACTGCGAACTCCAAGCT	452
			AS	CTGCTTGTGAACCTCCTTCCA	
Lactate dehydrogenase B	Ldhb	NM_008492.3	S	GCTCAACCTGGTGCAGAGAAA	344
			AS	CTGTCCCCATTTCTGGATTCAG	
PPARG Related Coactivator 1	Pprc1	NM_001081214.1	S	AGGGAACCCTGAAGCCTGAAGGAAT	237
			AS	AGCCCAATGACAACACAGGCTTGAT	
peroxisome proliferative activated receptor, gamma,	Ppargc1a (PGC1a)	NM_008904.2	S	CTCACACCAAACCCACAGAAAACAG	248
			AS	TTTATGAGGAGGAGTTGTGGGAGGA	

coactivator 1 alpha					
peroxisome proliferative activated receptor, gamma, coactivator 1 alpha	Ppargc1b (PGC1b)	NM_133249.3	S	GTGCCTGGGATTAACGTACTGCTTC	231
			AS	CAGCTCGAGTGGAACTCACTCAAAA	
Ribosomal protein, large, P0	Rplp0 (36B4)	NM_007475	S	CGACCTGGAAGTCCAACTACTTCCT	303
			AS	GCACCTTATTGGCCAACAGCAT	

RESULTS

Effect of lactate concentration in early embryo development stage in vitro

To observe the role of lactate during early embryo development, the development rates in lactate-free and lactate-high conditions were investigated with late 2-cell stage embryos. Development rates were checked at 60, 72, 84, 96, 120 and 144 h post-hCG (Fig. 1). At post-hCG 60 h, the development rate was delayed significantly in lactate-free medium cultured embryos (Fig. 1A). The developmental pattern of the lactate-free medium group lasted up to 120 h. There was no significant difference between the control and the lactate-high medium group. but after post- hCG 84 h, a higher lactate achieved more blastocyst rates (Fig. 1D). However, after 120 h of hCG, the highest percentage of blastocysts was consequently achieved in the control medium (Fig. 1E).

Also, the hatching rate after 144 h was significantly different from that of the control group in both groups, indicating a high level. (Fig. 1F).

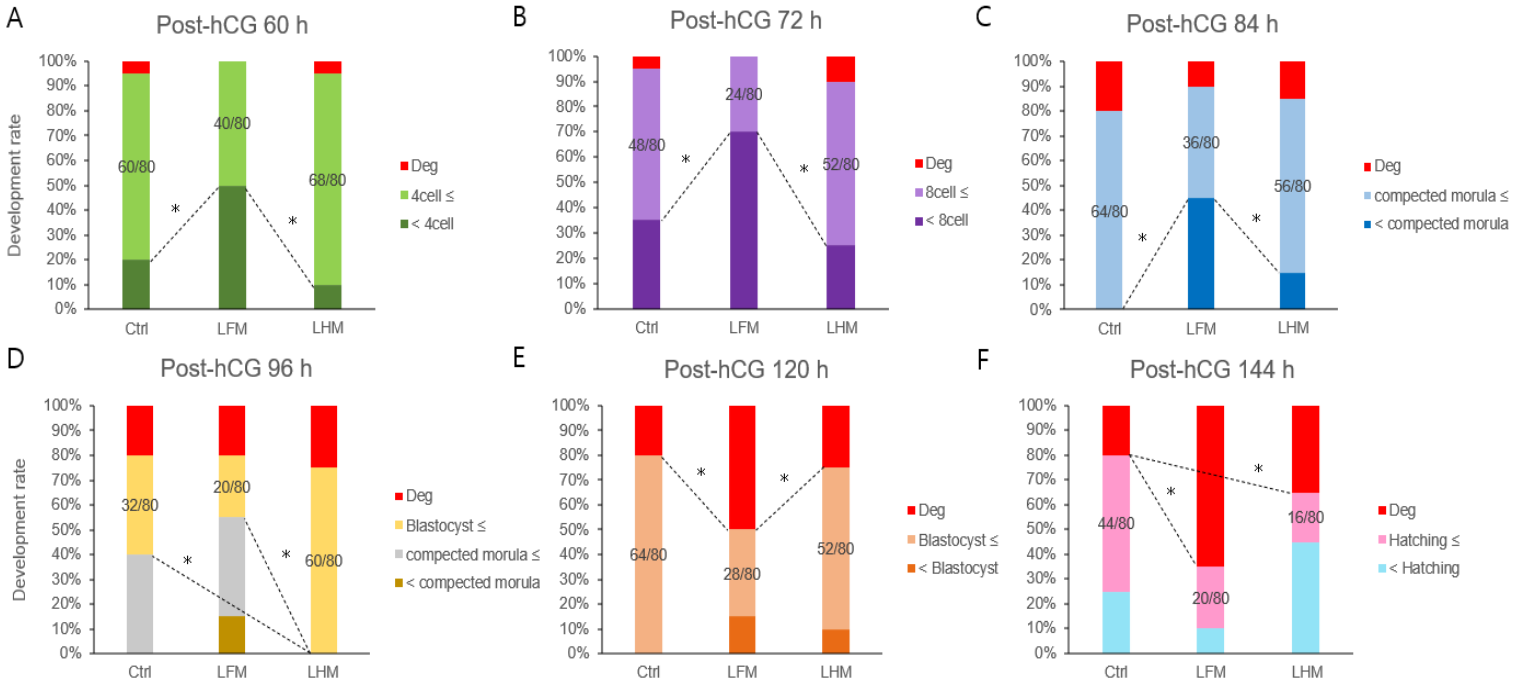

Figure 1. Development ratio of embryo in control BWW or lactate-free BWW medium or lactate-high BWW medium.

A-F: Developmental ratio of control (BWW), lactate-high (LHM) and lactate-free (LFM) cultured embryos at each observation time. Late 2-cell stage embryos were collected from oviduct at post-hCG 48 h and divided to three groups. Developmental rate was checked at 60, 72, 84, 96, 120 and 144 h post-hCG. *, $P < 0.05$ control vs. lactate-free or lactate-high of the same time pointy.

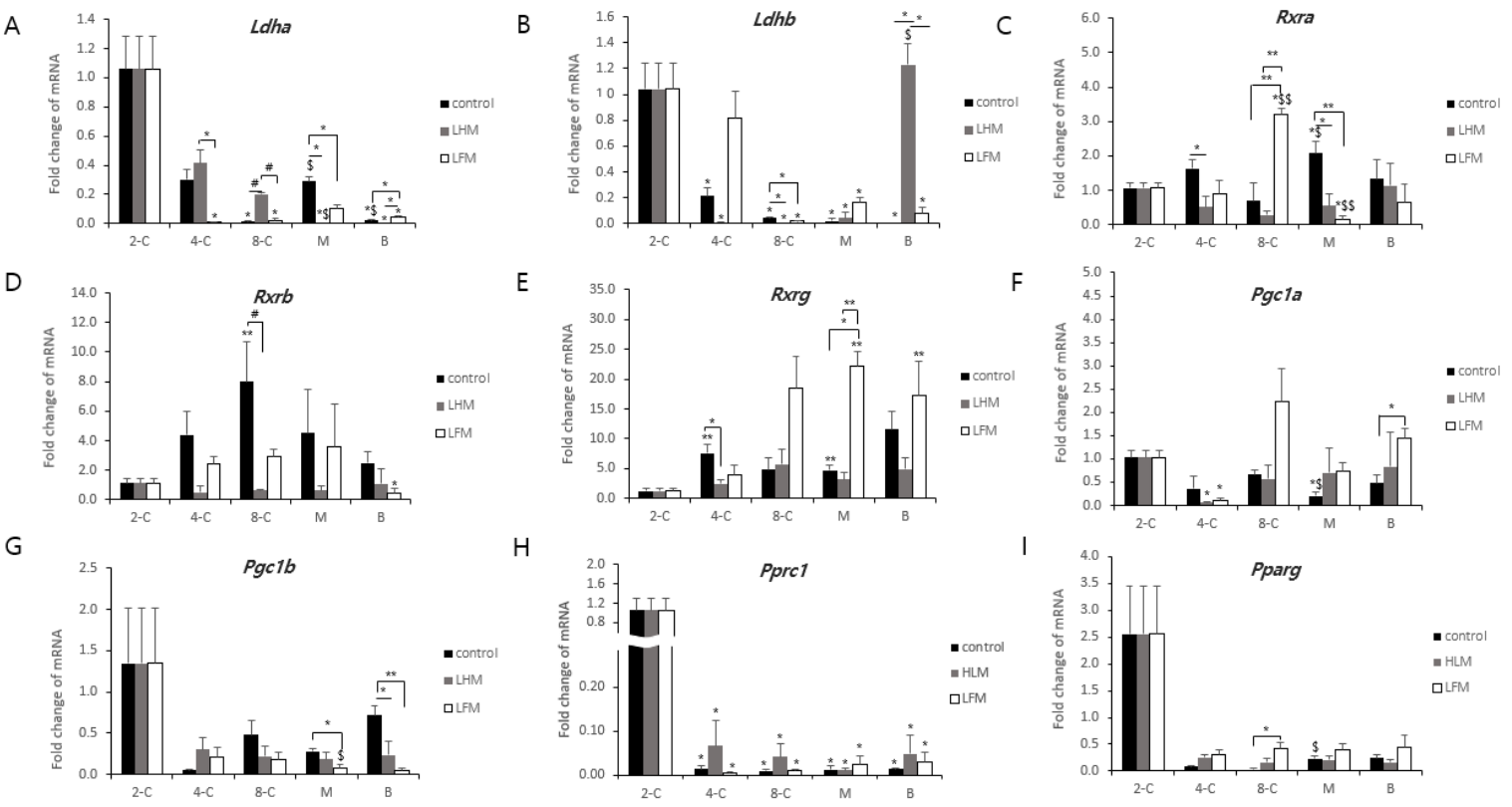
A
Ldha
B
Ldhb
C
Rxra
D
Rxrb
E
Rxrg
F
Pgc1a
G
Pgc1b
H
Pprc1
I
Pparg
Fold change of mRNA
control
LHM
HLM
LFM
2-C
4-C
8-C
M
B

Figure 2. Expression of mRNA levels in preimplantation embryos according to lactate concentration in culture media. Gene expression was measured by RT-qPCR. Each stage of the embryo was collected at 48, 60, 72, 86 and 96 h after culture. *, $P < 0.05$ control vs. lactate-free or lactate-high of the same time pointy.

Profiles of target genes mRNA in mouse preimplantation embryo in vitro medium of different lactate concentration

In order to observe the mRNA expression of genes during early embryo development, a study using quantitative real-time PCR analysis was performed. Genes for analysis include *Ldha*, *Ldhb*, three retinoid X receptors (RXR-α, RXR-β and RXR-γ), the PGC1 family (PGC1-α, PGC1-β, PPRC1) and PPARg. Gene expression was confirmed in embryo samples collected at 60, 72, 84, and 96 h at post-hCG 48 h. The mRNA expression of the 2-cell stage was determined as 1, and the mRNA expression was confirmed. The expression of *Ldha* mRNA showed a significant decrease in 8-cells and blastocyst stages under control culture conditions. As a result of observing the expression of other stages based on the 2-cell stage, *Ldha* showed the highest expression in 4-cells under the lactate-high condition. And it showed a gradually decreasing pattern, and compared to other conditions, it showed significantly higher expression in 8-cells. In the absence of lactate, the expression was higher in 8-cells than in the control group, but lower in morula than in the control group. And compared to the lactate-high conditions and the control group, the expression was higher in the blastocyst (Figure 2A). *Ldhb* could not be detected initially under lactate-high conditions, but increased significantly in the blastocyst. In the lactate-free conditions, expression was higher in 4-cells and morula compared to other conditions, and similarly, it shows a pattern that gradually decreases as development progresses (Fig. 2B). *Rxra* showed high expression in the 8cell stage compared to the control group under the lactate-free condition and then

decreased at morula. In lactate-high conditions, 4-cells and morula showed significantly lower expression than in the control group, but in blastocyst, there was a significant difference from other conditions (Fig. 2C). The expression levels of *Rxrb* mRNA gradually increased in the control group and gradually decreased after showing the highest expression in 8-cells. Under the high or no lactate concentration conditions, all of them showed lower expression than the control group. In particular, lactate-high conditions showed lower expression than other conditions (Fig. 2D). The expression of *Rxrg* mRNA increased from 8 cells in the absence of lactate, showing the highest expression in morula, and then decreased. Control and lactate-high conditions showed similar expression values except for 4 cells (Fig. 2E.) *Pgc1a* was significantly decreased in 4-cells with high or no lactate conditions. The control group and the lactate-free condition showed a similar expression pattern, which increased in 8-cells, decreased in morula, and then increased in blastocysts. In the lactate-high condition, the expression pattern gradually increased after 4-cells, and when compared to the control group, the expression was significantly higher in the blastocyst in the lactate-free condition (Fig. 2F.). The expression of *Pgc1b* mRNA showed no significant difference in all stages of the condition with or without lactate. Compared with the control group, there was a significant difference in the absence of lactate, but both showed a significant difference in the blastocyst stage. (Fig. 2G). In *Pprc1*, the expression level was significantly low in all conditions. (Fig. 2H). There was no significant difference in *Pparg*, except that

the expression was significantly greater in the lactate-free condition than in the control group in 8 cells. (Fig. 2I).

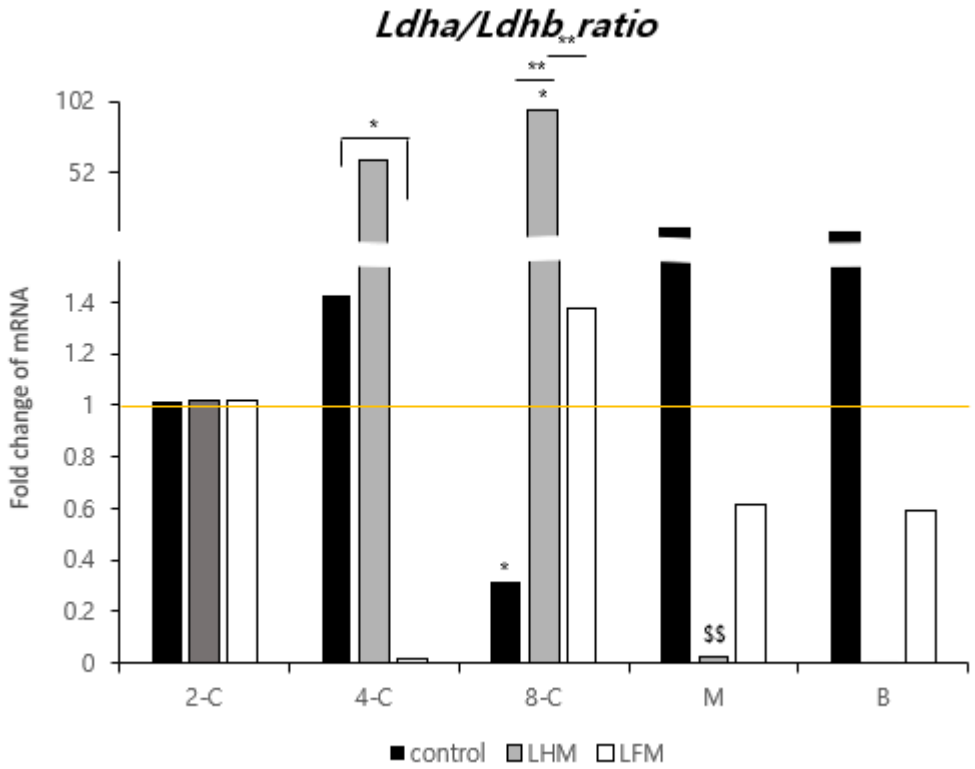

Figure 3. Ldha/Ldhb mRNA expression ratios in control BWW or lactate-free BWW medium or lactate-high BWW medium.

LDHA mainly reduces pyruvate to lactate, whereas LDHB catalyzes the reverse reaction, lactate oxidation to pyruvate. LDH tetramers can form 5 isoenzymes, each different depending on the ratio of LDHA and LDHB subunits. Therefore, To understand which sub-strategies are needed at each stage by identifying the ratios of the expression of *Ldha* mRNA and *Ldhb* mRNA for each stage of development. Therefore, by checking the ratio of the expression levels of *Ldha* mRNA and *Ldhb* mRNA for each development stage, tried to understand what substrate is more required at each stage. The ratio of the expression levels of *Ldha*/*Ldhb* is shown in Figure 3. Looking at the results, it can be seen

that in the control group, except for the 8-cell stage, *Ldha* was expressed higher than *Ldhb* mRNA. However, under the condition of high lactate, it was confirmed that *Ldha* mRNA expression was higher in the early stages of 2-cell and 4-cell development, and in the 8-cell stage when compaction occurs. Later stages showed higher expression in *Ldhb* mRNA. In the culture condition without lactic acid, the expression of *Ldhb* mRNA was higher in 4-cells, but higher in *Ldha* mRNA in the stage of 8-cells. However, morula and blastocyst again showed higher expression values in *Ldhb* mRNA compared to *Ldha* mRNA.

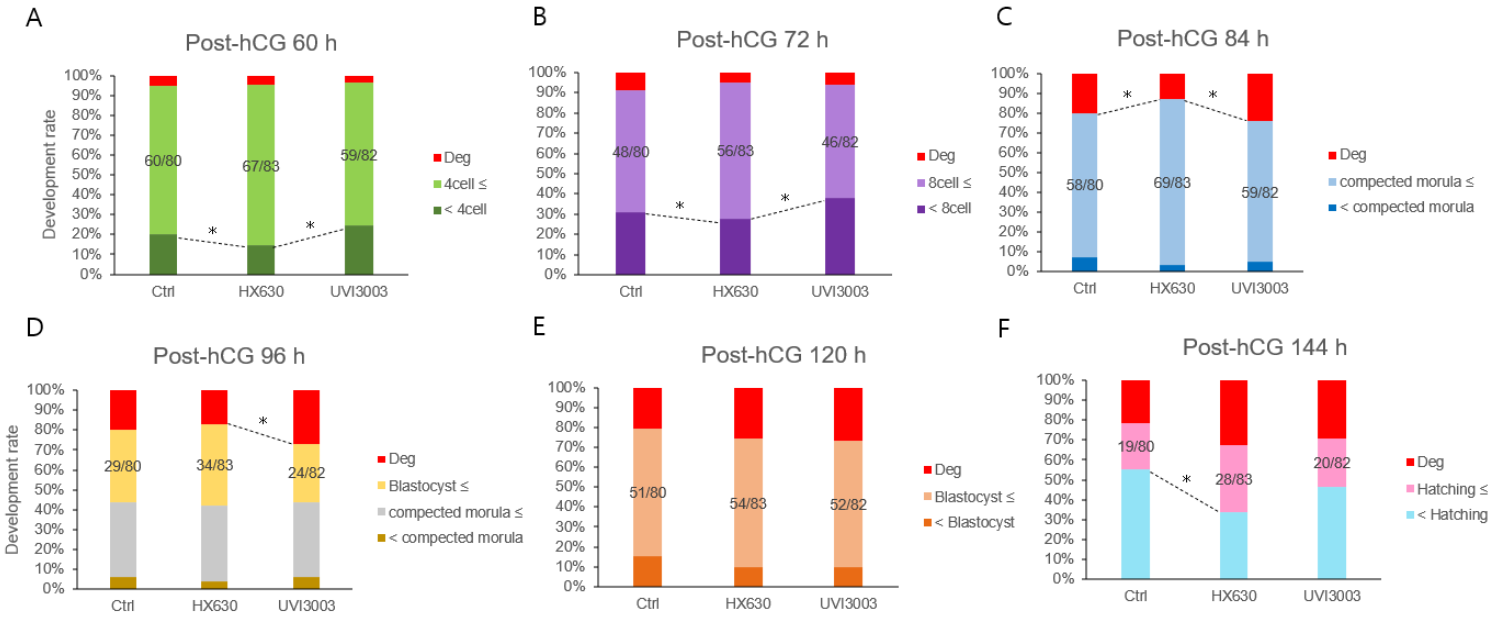

Figure 4. Effects of RXR agonist and antagonist on early-stage embryo development. A-F: Developmental ratio of control (BWW), RXR agonist (HX630) and antagonist (UVI3003) cultured embryos at each observation time. Late 2-cell stage embryos were collected from oviduct at post-hCG 48 h and divided to three groups. Developmental

rate was checked at 60, 72, 84, 96, 120 and 144 h post-hCG. *, P < 0.05 control vs. lactate-free or lactate-high of the same time pointy.

Effect of RXR agonist and antagonist treatment in early embryo development stage in vitro

To observe the role of RXR during early embryo development stage, we cultured late 2-cell stage embryos in a medium treated with the RXR agonist HX630 and the antagonist UVI3003 chemicals to determine the developmental rate. Development rates were checked at 60, 72, 84, 96, 120 and 144 h post-hCG (Fig. 4). After 60 h of hCG, the development rate of the group treated with the agonist HX630 was significantly faster than that of the other two groups. (Fig. 4A). This difference persisted up to 84 h. (Fig. 4B, C, D). At 96 h, the difference from the control group disappeared and only the antagonist UVI3003 showed a significant difference. At 120 h, all three groups showed similar development rates, and then at 144 h, only the control group and HX630 showed significance (Fig. 4E, F). This suggests that the agonist HX630 may have a greater impact on development.

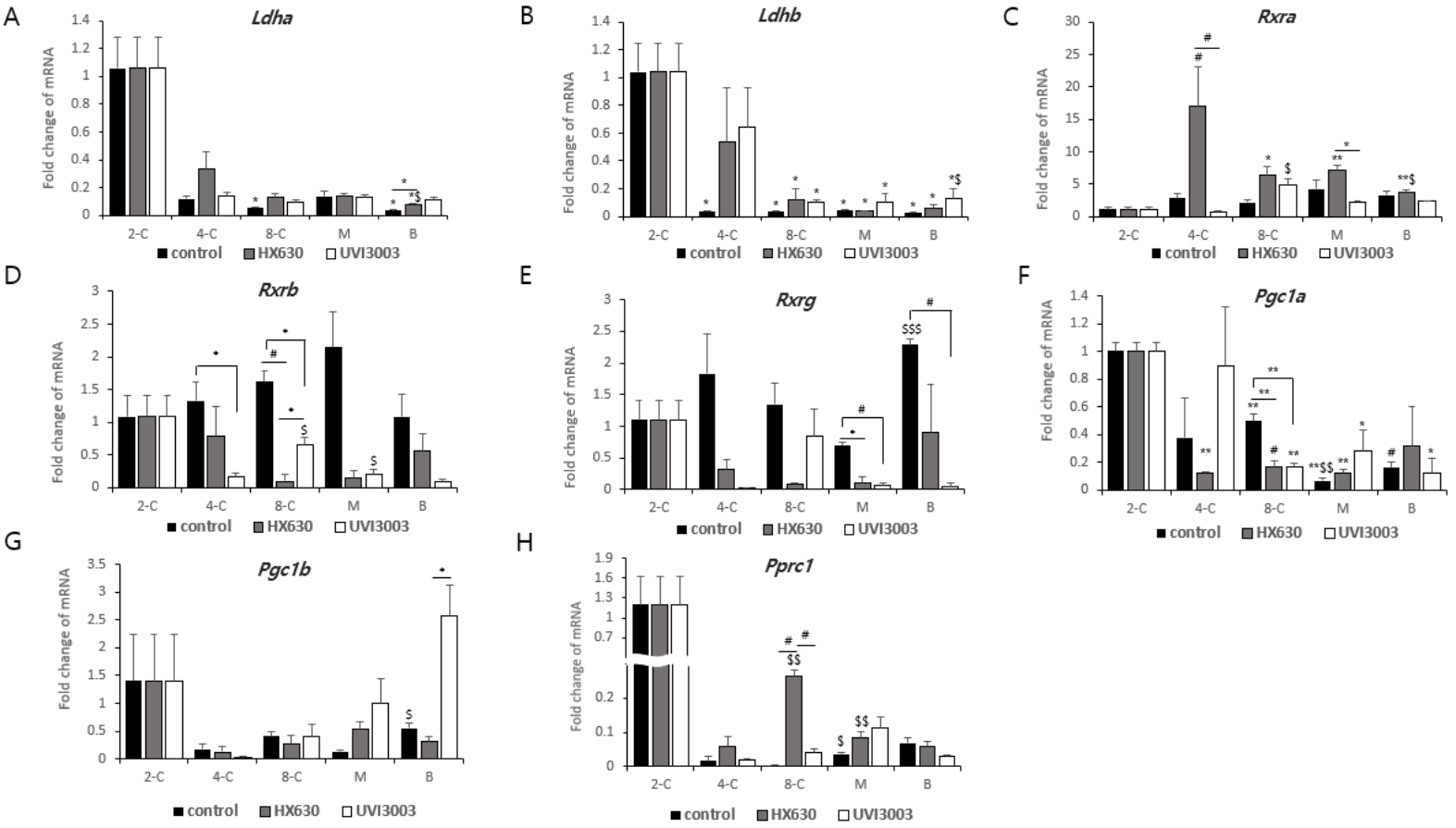
A
Ldha
B
Ldhb
C
Rxra
D
Rxrb
E
Rxrg
F
Pgc1a
G
Pgc1b
H
Pprc1
Fold change of mRNA
2-C
4-C
8-C
M
B
control
HX630
UVI3003

Figure 5. Effects of RXR agonist and antagonist on early stage embryo development. Gene expression was measured by RT-qPCR. Each stage of the embryo was collected at 48, 60, 72, 86 and 96h after culture. *, $P < 0.05$ control vs. lactate-free or lactate-high of the same time pointy.

Profiles of target genes mRNA in mouse preimplantation embryo in vitro RXR agonist and antagonist

During early embryo development, mRNA expression in embryos collected from the medium treated with the RXR agonist HX630 and the antagonist UVI3003 was confirmed. In order to observe the mRNA expression of genes during early embryo development, a study using quantitative real-time PCR analysis was performed. Genes for analysis include *Ldha*, *Ldhb*, three retinoid X receptors (RXR-α, RXR-β and RXR-γ) and the PGC1 family (PGC1-α, PGC1-β, PPRC1). Gene expression was confirmed in embryo samples collected at 60, 72, 84, and 96 h at post-hCG 48 h. The mRNA expression of the 2cell stage was determined as 1. *Ldha* mRNA expression level was significantly decreased in 8-cells and blastocyst stage in control culture condition. As a result of observing the expression of different stages based on the 2-cell stage, *Ldha* mRNA showed higher expression in the blastocyst stage of the HX630 treat condition than in the control group. Except for this, there was no significant difference in all three groups. (Fig. 5A). *Ldhb* mRNA was not detectable in the control group at an early stage of development. However, the chemical-treated group showed a decrease at the 4cell stage, and after that, all three groups showed a small amount of *Ldhb* mRNA expression overall. (Fig. 5B). *Rxra* mRNA showed high expression in UVI300 and morula stage in HX630 treat condition. (Fig. 5C). *Rxrb* mRNA expression was significantly decreased at the 4-cell stage in the HX630 treated condition compared to the control group and the UVI3003 treated group. UVI3003 treated group maintained low expression level except for 8-cells. (Fig.

5D). *Rxrg* mRNA showed a significantly lower expression level in morula in all chemical treatment groups compared with the control group. UVI3003 maintained this significant difference even up to the blastocyst stage. (Fig. 5E). The expression of *Pgc1a* mRNA was significantly lower in the HX630 treated group and slightly increased in the blastocyst. UVI3003 was significantly reduced significantly in 8-cell. (Fig. 5F.) The expression of *Pgc1b* mRNA was significantly increased in UVI3003 at the blastocyst stage compared to the control group maintaining low expression and HX630. (Fig. 5G). The expression of *Pprc1* mRNA was maintained low in all three groups, and the expression of HX630 treated group was significantly higher in 8-cells compared to the control group and UVI3003 treated group. (Fig. 5H).

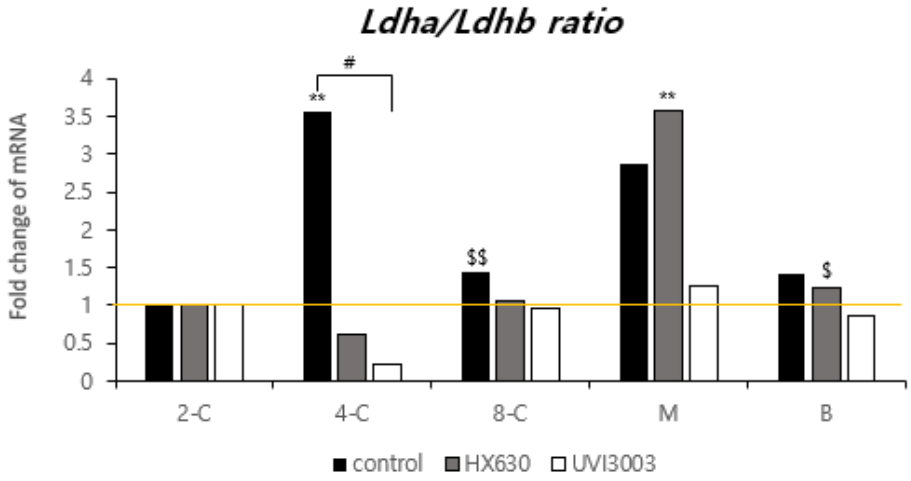

Figure 6. Ldha/Ldhb mRNA expression ratio in culture medium treated with RXR agonist and antagonist

LDHA primarily reduces pyruvate to lactate, whereas LDHB catalyzes the reverse reaction of oxidation of lactate to pyruvate. LDH tetramers can form five isoenzymes, each of which depends on the ratio of LDHA to LDHB subunits. Therefore, we identify the ratios of the expression levels of *Ldha* to *Ldhb* for each stage of development to understand which sub-strategies are needed at each stage. Therefore, we wanted to determine the ratio of *Ldha* to *Ldhb* for each development step to determine which more substrates are needed for each step. The ratio of *Ldha*/*Ldhb* is shown in Figure 6. It can be seen that *Ldha* was expressed higher than *Ldhb* in all control stages. In the medium treated with HX630, the expression of *Ldhb* was higher than that of *Ldha* only in 4 cells. In the medium treated with UVI3003, it was confirmed that *Ldhb* expression was higher in 4-cell, 8-cell and blastocyst stages.

Discussion

Recently, it is widely accepted that the metabolites work as signaling molecules in mammals. During early embryonic stage of the mammalian embryo, carbohydrate metabolism undergoes rapid changes as it moves from the 8-cell to the blastocyst and increase activity of some metabolic chains. The amount of glucose consumed gradually increases as the development stage progresses, and lactic acid also increases until blastocyst. Conversely, consumption of pyruvic acid was high as it plays an essential role in the initial stage of division, and then the consumption gradually decreases as develop to the blastocyst stage. As such, energy substrates show different aspects in early developmental stages, which act as important events in embryonic development. There was a significant delay in developmental rate in LFM cultured embryos at 60 h post-hCG, which was maintained up to 120 h. Also, after 84 h of post-hCG, LHM achieved a higher blastocyst ratio, but as a result, it was confirmed that the highest proportion of blastocysts was achieved in the control medium after 120 h. These results may lead to the conclusion that both endogenous and exogenous lactate are required for early embryo development. On the other hand, it is suggested that the embryo which develop in lactate free condition could develop to blastocyst stage with compensational regulation.

To investigate the relationship between lactate and RXR in the development process, the mRNA expression levels of *Ldh*s and *Rxr*s were observed. As a result, the expression of *Ldha* in the medium with high lactate concentration

decreased as development progressed toward the blastocyst, and, conversely, the expression of *Ldhb* rapidly increased during the blastocyst stage. The expression of *Rxra*, *Rxrb*, and *Rxrg* all showed different expression patterns, suggesting that the three *Rxr*s play different roles in early embryo development. The expression of *Rxr* showed a significantly higher expression level in LFM, not in LHM. This seems to be related to the previous findings that the expression of *Rxr*s consequently decreased the expression of *Ldha*. *Pgc1a* showed a significantly higher expression level in 8-cells of LFM culture, the same as *Rxr*s. These results showed that *Rxr*s may be involved in the conversion of pyruvate and lactate.

In addition, by culturing in a medium treated with RXR agonist and antagonist, the mRNA expression rate equal to the development rate was investigated. As a result, in *Ldh*, an elevated expression of *Rxra* in the group treated with HX630 and a significant decrease in *Rxrg* in the group treated with UVI3003 were confirmed. After confirming the ratio of *Ldha* and *Ldhb*, it was found that the amount of expressed *Ldha* or *Ldhb* may vary depending on the concentration of lactate. These findings could suggest that lactate dehydrogenase is associated with the RXR in early embryo development.

In summary, expression levels of *Ldh*s were controlled by the exogenously supplied lactate and the activity of RXRs. The expression levels of *Pgc1*s also modulated by the concentration o lactate and the chemically modifid activity of RXRs. These results suggest that depending on the conditions of lactate, PGC1a can affect RXRb and consequently regulate the expression of *Ldh*s. In

addition, it is suggested that a circuit for lactate metabolism could be formed during early stage embryo development.

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논 문 개 요

포유류 발달에 대한 환경적 기여는 오늘날 더욱 중요한 주제가 되고 있다. 배아 발생 동안, 배아 주변의 미세 환경은 대사 산물의 적절한 지원을 통해 특정 시간에 하나의 성공적인 배아 발달을 지원한다. 신진대사의 극적인 변화는 신진대사에 착상 전 단계가 진행되는 동안 발생한다. 첫 번째 난할 단계는 주로 낮은 수준의 대사를 유지하기 위해 피루브산과 젖산을 사용하고 일단 밀착이 시작되면 피루브산은 포도당 기반 대사로 전환된다. 대사 변화는 또한 중간 대사 산물 중 하나로서 배아의 정상적인 발달에 중요한 의미를 가질 수 있으며 발달 능력의 중요한 지표가 될 수 있다. 따라서 젖산은 에너지원으로 사용될 뿐만 아니라 적절한 생산과 활용이 배아 발달에 영향을 줄 수 있는 기질 중 하나라고 볼 수 있다. 젖산 탈수소효소(LDH)는 피루브산과 젖산 사이의 상호전환을 촉매한다. LDH 유전자에는 *Ldha, Ldhb, Ldhc* 및 *Ldhd* 의 4 가지 유형이 있다. 처음 2 개의 유전자는 각각 LDHA 또는 LDHB 서브유닛을 코딩하며, 이는 5 개의 별개의 사량체성 동효소인 LDH-1 에서 5 를 형성한다. 핵 수용체는 리간드에 결합하고 핵에서 전사 조절자로서 기능하며 대사 및 발달 조절, 세포 분화 및 항상성을 비롯한 다양한 기능을 수행할 수 있다. 그 중 레티노이드 X 수용체는 RAR 뿐만 아니라 PPAR 과 같은 핵 수용체 패밀리의 다른 구성원과도 이량체로 작용할 수

있기 때문에 다양한 생물학적 과정에서 중요한 역할을 할 수 있다. 이 연구에서는 RXR 과 젖산 대사 사이의 가능한 관계를 조사했다. 2-세포기 배아를 사용하여 배양 조건에 따른 발달 속도를 분석하고, 다양한 젖산 농도의 배지에서 *Rxrs, Pgc1s, Ldhs* 및 *Pparg* 의 mRNA 수준을 조사하였다. 젖산이 없는 조건에서 배양된 배아는 대조군에 비해 발달 지연을 보였다. 반대로, 높은 젖산 조건에서 배양된 배아는 대조군 및 젖산이 없는 조건보다 더 빠른 발달 속도를 보였다. pyruvate 를 lactate 로 전환시키는 *Ldha* 의 발현은 *Ldhb* 보다 상대적으로 높은 mRNA 발현 수준을 보였다. *Rxrs* 및 *Pgc*1s 는 또한 배양 배지 내 젖산 농도 또는 RXR 작용제 또는 길항제 처리에 따라 다른 발현을 보였다. 이러한 데이터로부터 초기 배 발달 속도는 배양 배지의 젖산 농도에 영향을 받을 수 있음을 알 수 있다. 또한, 이러한 결과를 바탕으로 *Rxrs* 는 젖산 대사 및 젖산 탈수소효소의 발현에 관여할 수 있음을 확인하였다. 이러한 결과는 초기 배아의 적절한 발달을 위해 젖산 대사 회로가 형성될 수 있음을 시사한다.

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