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석사학위청구논문

Expression of lactate dehydrogenase and aquaporin genes in mouse embryo during preimplantation development

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성신여자대학교 대학원

생 물 학 과

신 수 정

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논 문 개 요

착상 전 배아에서의 포배강의 형성은 태반동물의 발생에 있어서 매우 필수적인 과정으로 내세포괴 (ICM)와 영양배엽을 갖는 포배로 분화된다. 포배강 형성은 Na^+와 Cl^-의 유입, K^+의 유출, H^+의 이동 등으로 생기는 이온의 농도 차이에 의해 형성되는 것으로 알려져 왔다. 그러나 이들 이온 농도차 형성의 원인과 수송 단백질 발현조절에 대한 이해는 미비한 실정이다. 젖산과 관련된 물질대사의 변화가 상실배와 포배시기에 알려져 왔고 연구실 사전 연구 결과에서 젖산이 포배강 형성과 관련되어 있는 것을 가정 할 수 있었다. 따라서 본 실험에서는 젖산 농도에 따른 초기배아 발생 특성분석, 젖산 생성관련 유전자 발현 정도 변화 등을 통하여 젖산이 포배의 발달에 관여함을 밝히고자 하였다. 젖산이 없거나 또는 저농도 젖산을 함유한 배양액에서 2-세포기 이후의 배아가 주어진 시간 내에 (hCG 주사 후 96 시간) 포배로 발생하는 율이 유의하게 감소하였다. 젖산 합성 대사 과정에서 중추적 효소인 lactate dehydrogenase (LDH)인 LDHA 가 상실배와 포배시기에 발현되고, LDHB 와 같은 경우는 난자단계부터 착상 전 모든 단계의 배아에서 발현되었다. LDHC 의 경우에는 2 세포기와 4 세포기에 발현되었다. LDHA, LDHB 의 발현은 배양액내 젖산의 농도에 의해 발현양이 조절되었다. 다른 한편 물의 수송과 관련되어 있는 유전자인 AQP 유전자 발현양상도 젖산 농도에 의해 발현 변화가 있음이 밝혀졌다. 본 실험에서 보게 된 AQP 의 발현양상은 AQP3 는 4 세포기와 상실배에 관찰되었으며, AQP8 은 부화시기에 발현하였다. 그리고 AQP9 은 4 세포기, 8 세포기, 포배시기에 발현하였다. AQP3 유전자는 젖산이 없는 환경에서 hCG 주사 후 120 시간의 배아에서 발현되었으며, AQP7 은 10mM 젖산 농도에서

hCG 주사 후 120 시간의 배아에서 발현하였다. 그리고 AQP8 의 발현은 20mM 의 젖산 농도에서 위와 같은 시기에 발현함을 알 수 있었다. AQP9 유전자는 비교군 배양액과 10mM 젖산 농도에서 배양한 상실배에서 발현하였다. 위의 실험 결과를 통해서 젖산이 포배 발달에 일정한 역할을 수행하고 있음을 추정 할 수 있다. 또한 배아는 LDH 의 발현 조절과 AQP 의 발현 조절을 통하여 포배강 형성에 있어서 중요한 조절 요소가 될 수 있음을 알 수 있다.

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REFERENCE

Atlante A, Bari L, Bobba A, Marra E, Passarella S. 2007. Transport and metabolism of L-lactate occur in mitochondria in from celebellar granule cells and are modified in cells undergoing low potassium dependent apoptosis. Biochi Biophy Acta 1767:1285-1299.

Barbehenn EK, Wales RG, Lowry OH, 1974. The explantation for the blockade of glycolysis in early mouse embryos. Proc Natl Acad Sci USA 71:1056-1060.

Barcroft LC, Offenberg H, Thomsen P, Watson AJ. 2003. Aquaporin proteins in murine trophectoderm mediate transepithelial water movements during cavitation. Dev Biol 256:342-354.

Biggers JD, Whittingham DG, Donahue RP. 1967. The pattern of enegy metabolism in the mouse oocyte and zygote. Proc Natl Acad Sci USA 58:560-567.

Borgnia M, Nielsen S, Engel A, Agre P. 1999. Cellular and molecular biology of the aquaporin water channels. Annu Rev Biochem 68:425-458.

Carbrey JM, Gorelick-Feldman DA, Kozono D, Praetorius J, Nielsen S, Agre P. 2002. Aquaglyceroporin AQP9: solute permeation and metabolic control of expression in liver. Proc Natl Acad Sci USA 100:2945-2950.

Cheon YP, 2008, Carbohydrate metabolism in preimplantation stage embryos and the role of metabolites. Dev Reprd 12:19-30.

Coonrod S, Vitare A, Duan C, Gould SB, Herr J, Goldburg E. 2006. Testis-specific lactate dehydrogenase (LD H-C4; Ldh3) in murine oocytes and preimplantation embryos. J Androl 27: 502-509.

Dumollard R, Ward Z, Carroll J, Duchen MR. 2007. Regulation of redox metabolism in the mouse oocyte and embryo. Development 134:455-465.

Edashige K, Sakamoto M, Kasai M. 2000. Expression of mRNAs of the aquaporin family in mouse oocytes and embryos. Cryobiology 40:171-175.

Hayashi Y, Edwards NA, Proescholdt MA, Oldfield EH, Merrill MJ. 2007. Regulation and function of aquaporin-1 in glioma cells. Neoplasia 9:777-787.

Ishibashi K, Sasaki S, Fushimi K, Uchida S, Kuwahara M, Saito H, Furukawa T, Nakajima K, Yamaguchi Y, Gojobori T, Marumo F. 1994. Molecular cloning and expression of a member of the aquaporin family with permeability to glycerol and urea in addition to water expressed at the basolateral membrane of kidney collection duct cells. Proc Natl Acad Sci USA 91:6269-6273.

Jakubovicz DE, Klip A. 1989. Lactic acid-induced swelling in C6 glial cells via Na+/H+ exchange. Brain Res 485:215-224.

Jansen S, Esmaeilpour T, Pantaleon M, Kaye PL. 2006. Glucose affects monocarboxylate cotransporter (MCT)1 expression during mouse preimplantation development. Reprod 131:469-479

Kawagishi R, Tahara M, Sawada K, Morishige K, Sakata M, Tasaka K, Murata Y. 2004. Na+/H+ exchanger-3 is involved in mouse blastocyst

formation. J Experimental Zoology 301:767-775.

Kempski O, Staub F, Jansen M, Schodel F, Baethmann A. 1988. Glial swelling during extracellular acidosis in vitro. Stroke 19:385-392.

Khurana NK, Wales RG. 1989. Effects of oxygen concentration on the metabolism of [U-14C]glycose by mouse morulae and early blastocysts in vitrol. Reprod Fertil Dev 1:99-106.

Kitano T, Nisimaru N, Shibata E, Iwasaka H, Noguchi T, Yamada K. 2002. Lactate utilization as an energy substrate in ischemic preconditioned rat brain slices. Life Sci 72:557-564.

Lane M, Gardner DK. 2000. Lactate regulates pyruvate uptake and metabolism in the preimplantation mouse embryo. Biol Reprod 62:16-22.

Lane M, Gardner DK. 2005. Mitochondrial malate-aspartate shuttle regulates mouse embryo nutrient consumption. J Biol Chem 280:18361-18367.

Leese HJ. 1995. Metabolic control during preimplantation mammalian development. Hum Reprod Update 1:63-72.

Lemire J, Mailloux RJ, Appanna VD. 2008. Mitochondrial lactate dehydrogenase is involved in oxidative-energy metabolism in human astrocytoma cell (CCF-STTG1). PLoS ONE e1550.

Leppens-Luisier S, Denny S. 1997. Development, glycolytic activity, and viability of preimplantation mouse embryos subjected to different periods of glucose starvation. Biol Reprod. 56:589-596.

Lomneth R, Medrano S, Gruenstein EI. 1990. The role of

transmembrane pH gradients in the lactic acid induced swelling of astrocytes. Brain Res 523:69-77.

Manejwla FM, Cragoe EJ, Schultz RM. 1989. Blastocoel expansion in the preimplantation mouse embryo: Role of extracellular sodium and chloride and possible apical reutes of their entry. Dev Biol 133:210-220.

Moghaddam MA, Lindland H, Zelenin S, Roberg BA, Gundersen BB, Petersen P, Rinvik E, Torgner IA, Ottersen OP. 2005. Brain mitochondria contain aquaporin water channels: evidence for the expression of a short AQP9 isoform in the inner mitochondrial membrane. FASEB J 19:1459-1467.

Morishima T, Aoyama M, Iida Y, Yamamoto N, Hirate H, Arima H, Fujita Y, Sasano H, Tsuda T, Katsuya H, Asai K, Sobue K. 2008. Lactic acid increases aquaporin 4 expression on the cell membrane of cultured rat astrocytes. Neurosci Research 61:18-26.

Offenberg H, Barcroft LC, Caveney A, Viuff D, Thomsen PD, Watson AJ. 2000. mRNAs encoding aquaporins are present during murine preimplantation development. Mol reprod Dev 57:323-330.

Offenberg H, Thomsen PD. 2005. Functional challenge affects aquaporin mRNA abundance in mouse blastocysts. Mol reprod Dev 71:422-430.

Tsukaguchi H, Shayakul C, Berger UV, Mackenzie B, Devidas S, Guggino WB, et al. 1998. Molecular characterization of a broad selectivity neutral solute channel. J Biol Chem 237:24737-24743.

Watson AJ, Barcroft LC. 2001. Regulation of blastocyst formation. Front Biosci 6:708-730.

Watson AJ, Natale DR, Barcroft LC. 2004. Morecular regulation of blastocyst formation. Animal Repred Sci 82-83:583-592.

ABSTRACT

Expression of lactate dehydrogenase and aquaporin genes in mouse embryo during preimplantation development

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Differentiation from the morual to blastocyst is under the control of intrinsic and extrinsic regulaiton factors and is a key event in preimplantation embryonic development. Though a few mechanisms have been suggested as a reason for blastocoel formation, it is suspected that there are other mechanisms to control the blastocoel formation. From previous our laboratory results, we can suspect that carbohydrate metabolites may involve in development of morula to blastocyst. In lactate free condition, the 2-cell stage embryos did not properly develop to the blastocyst at 96 hr post hCG injection but there were no defect in development to morula stage at 84 hr post hCG injection. The successful development were concentration dependent and was

highest in medium containing 20mM lactate. In natural condition, the embryonic stage at 96 hr post hCG injection is blastocyst. Therefore we know that lactate is need to support the blastocoel formation. Also known that the embryo adaptation to the kinds of carbohydrate substrate. At 120 hr post hCG injection the most of the embryos reach to the blastocyst stage like in control group. In control group, the expression of LDH subunits various according to embryonic stages. LDHA was expressed morula and blastocyst stage, LDHB was expressed oocyte and preimplantation embry, and LDHC was expressed 2cell and 4cell stage embryo. But by the concentration of lactate the expression profiles were changed. LDHA was expressed morula stage in 10mM lactate and blastocyst stage in lactate free condition. LDHB was expressed morula stage in 20mM lactate and morula, blastocyst stage in 10mM lactate and all stage embryos from 120hr post hCG administration at control condition. On the other hand, AQP3 was expressed 4cell and morula stage. AQP8 was expressed hatching stage. AQP9 was expressed 4cell and 8cell and blastocyst stage. But these genes were different expression pattern in related of lactate. AQP3 was expressed all stage embryo in 120hr post hCG administration which was cultured control condition and lactate free condition. AQP7 was expressed all stage embryo in 120hr post hCG administration which was cultured 10mM lactate condition. Also AQP8 was expressed same stage embryo at 20mM lactate condition. AQP9 was expressed morula stage at control and 10mM lactate condition also all stage embryos from 120hr post hCG administration at control condition. Based on them it is suggested that concentration of lactate in environment and lactate synthesis in embryo are critical factor for blastocoels formation. In addition it is suggested that LDH

may involve the AQPs expression in embryos. Not only lactate amount in the media and but also lactate production capacity of embryo were associated with blastocoels formation gene, AQPs.

감사의 글

지난 2 년 동안의 연구실 생활을 이 한 권의 논문에 담는다는 것이 매우 아쉽지만 무사히 대학원 생활을 마치고 석사학위를 받을 수 있음에 감사드립니다.

너무나도 부족한 저를 이끌어 주신 지도교수님인 전용필 교수님께 감사를 표합니다. 교수님 덕분에 이렇게 부족했던 제가 석사 학위를 받을 수 있게 된 것 같습니다. 감사합니다. 그리고 부족했던 제 논문을 더 나은 논문으로 만들어 주시기 위해 심사를 해주신 김해권 교수님과 이동률 박사님께 깊은 감사를 표합니다. 두 분 덕분에 제 논문의 방향성이 제 자리를 찾을 수 있었습니다. 감사합니다.

4 년 동안의 학부생활에서 제가 대학원 생활을 잘 할 수 있도록 기본 지식을 많이 알려주시고 대학생으로써 캠퍼스 생활에 많은 도움을 주셨던 박경숙 교수님, 강혜순 교수님, 윤진호 교수님, 김인순 교수님 감사합니다.

2 년 동안의 연구실 생활에서 얻게 수 있었던 뜻 깊은 인연에도 감사합니다. 제가 논문 쓰는 데에 있어서 학문적, 인간적으로 도움을 주셨던 혜영언니, 비록 멀리 있지만 가끔씩 힘을 주셨던 성은언니, 동기로써 대학원 선배로써 그리고 소중한 친구로써 많은 도움을 주었던 희경언니, 같이 동거동락하며 친구로써 선배로써 소중한 기억을 많이 만들어주었던 자명이, 같이 논문 쓰며 힘든 마음을 많이 달래주었던 윤진언니 너무도 고맙습니다. 그리고 연구실 후배로써 실험을 많이 도와주고 친구로써도 많은 시간을 함께 했던 자영이, 미희, 소라, 자현이, 예아야 너무 고마워, 너희가 있어서 너무 든든했어. 앞으로도 계속 우리의 끈끈한 선후배 관계 지켜나가자.

저의 첫 직장에서의 좋은 추억과 즐거운 만남을 만들어 주셨던 권혁찬 부원장님, 박선애 선생님, 김보현 선생님, 윤효진 선생님, 박성백 선생님 너무 고맙습니다. 선생님들께서 계셔서 제가 대학원에서 든든할 수 있었습니다. 앞으로도 좋은 인연 이어가요.

제가 대학원 생활을 하는 데에 많은 응원을 해주신 부모님, 오빠 너무 고맙습니다. 그리고 대학원 생활과 논문 쓰느라 부족한 며느리였던 저를 딸처럼 아껴주시고 믿어주신 시부모님 너무 감사합니다. 그리고 실험하느라 몸과 마음이 힘들어 하던 저를 언제나 아껴주고 도와주었던 우리 남편 너무 고맙고 사랑해요.

대학원 다닌다는 핑계로 너무나도 소홀했던 친구들, 그동안 기다려주고 많이 챙겨주어서 미안하고 고맙습니다. 정신적으로 많은 도움을 줬던 수아언니, 너무나도 잘 맞았던 유진언니, 멀리 있지만 많은 응원을 해준 연우언니, 나를 친 동생처럼 많이 챙겨주었던 민기언니 너무 고마워요. 그리고 나의 친구들, 너무도 편한 효정이, 재미있는 추억이 너무 많은 수진이, 선영이, 민지, 자주는 아니었지만 가끔 만나면서 스트레스를 확 날려주었던 연희, 제연이, 예지 너무도 고맙다 얘들아.

이렇게 감사하는 마음을 몇 자의 글로 적는다는 것이 너무 부족하지만 모든 분들 감사합니다.

INTRODUCTION

Blastoceal formation is dramatic event in development of preimplantation stage embryo and is cue for implantation. It is accomplished by expressing a set of genes which facilitate the transport and retention of the blastocoel fluid as it accumulates in the nascent blastocyst cavity (Watson *et al.,* 2001). During cavitation, the trophoblast cells secrete fluid into blastocoel and finally escaped zona pellucida.

Na^+ and Cl^- in the medium imported through the trophectoderm into the blastocoel and this generates an osmotic gradient (Manejwala *et al.,* 1989). Na^+/H^+ exchanger (NHE) facilitate the transtrophectodermal Na^+ flux. The specific inhibitor of NHE-3 block the blastocyst formation in a dose dependent manner (Kawagishi *et al.,* 2004). In addition polarized mural trophectodermal Na/K-ATPase establishes and maintains an ionic gradient across the trophectoderm. The concentration gradient formed by those trasporter promotes the osmotic accumulation of water across the epithelium and result in formation of blastocoels (Watson *et al.,* 2004).

On the other hand, carbohydrate, energy substrate is important to support early embryonic development. The pattern of energy metabolism of the zygote is dependent on developmental stages and pyruvate is the main energy substrate which can be used directly by the oocyte and zygote (Biggers *et al.,* 1967). Lactate can support development from the two-cell stage and glucose can support after compaction (Leese, 1995).

Generation of high-energy molecules (ATP and NADH) as cellular energy sources as part of aerobic respiration and anaerobic respiration. For aerobic

respiration or anaerobic respiration pyruvate is synthesized in cytoplasm. And, as a necessary, pyruvate is converted to lactate by lactate dehydrogenase (LDH) that concomitant conversion of NAD^+ to NADH in the cytosol (Lane *et al.,* 2005).

Recently, in cancer cells, it is known that LDH activity is increased in aerobic condition (Hayashi *et al.,* 2007). Interestingly it also known that in mice production of CO_2 and lactate increase in mourla and blastocysts through glucose metabolism (Khurana *et al.,* 1989). Taken together we can suspect that lactate may involve in cavitation of morula.

Transport of lactate across the plasma membrane of all cells is mediated by proton-linked monocarboxylate transporters (MCTs). The rate-limiting step for net lactic acid flux appears to be the return of the free carrier across the membrane which is required to complete the translocation cycle, and this is reflected in rates of monocarboxylate exchange being substantially faster than those of net transport. That is important in the critical regulation of pH and monocarboxylate transport, and implies a role for glucose in the control of MCT1 expression (Janse *et al.,* 2006).

Put together with our laboratory study, we could suspect that lactate may a factor for development of blastocoel. To evaluate the possible role of lactate in blastocyst formation, LDH expression and AQPs expression were examined in various lactate conditions.

MATERIALS AND METHODS

Animals

Six to eight week-old female CD-1 mice were used in this study. All experimental animals studies followed to the Guide for the Care and Use of Laboratory. Animals were maintained under standard conditions at Sungshin Women's University. Animals were fed a standard rodent diet and water ad libitum from weaning at 21 days of age. The condition is maintained by 14/10hrs light/dark cycle.

Superovulation induction and 2-cell stage embryo collection

Embryos were obtained from females that were superovulated with 5 IU of pregnant mares serum gonadotrophin (PMSG, Sigma) followed 48hr later by 5 IU of human chronic gonadotrophin (hCG, Sigma). Immediately after the hCG injection, females were placed with males of the same strain. The next morning of finding a vaginal plug was defined day 1 of pregnancy. Preimplantation mouse embryos were collected at 48hr post hCG. The embryos were flushed from oviducts by BWW medium containing 0.4% bovine serum albumin (BSA).

Embryos culture

The collected healthy two cell embryos were cultured in the 10 μl microdrops of BWW medium containing different concentrations of lactate. Embryos were cultured in groups according to the different lactate

concentrations: group 1, BWW (25mM lactate, control); group 2, 20mM lactate modified BWW; group 3, 15mM lactate modified BWW; group 4, 10mM lactate modified BWW; group 5, 5mM lactate modified BWW; group 6, 0mM modified BWW (Table 1). The embryos were cultured at 37℃ with 5% CO_2 in air until 144hrs post hCG injection. The embryo development was evaluated every 12 or 24hr under the inverted microscope (Olympus, IX70). The number and percentage of embryos reaching the 4-cell embryo, 8-cell embryo, morula, blastocyst, hatching or hatched blastocyst were recorded at 48, 72, 84, 96, 120 and 144hr post hCG injection.

Embryos sampling

The embryos were sampled to examine the expression of LDHs and AQPs. Sampling for morula stage embryos did at the 84hr post hCG administration embryos were quickly frozen using liquid nitrogen and stored at -80℃ until used. The blastocyst stage embryos were sampled by same manner at 120hr post hCG administration. And 120hr embryo post hCG injection were sampled as mentioned above. To analysis the express patterns of AQPs and LDH, the early stage embryos and oocytes were collected by time schedule. These were containing unfertile egg (UF, 16hrs post hCG injection in no mating female mouse), pronucleus (PN, 16hrs post hCG injection), two-cell, four-cell, eight-cell, morula, blastocyst and hatching embryo.

Table 1. Composition of media (mM)

Componant	BWW	Modified BWW; Lactate
NaCl	94.59	94.59
KCl	4.78	4.78
Ca-lactate	1.71	-
KH2PO4	1.19	1.19
MgSO4	1.19	1.19
NaHCO3	25.07	25.07
Na-pyruvate	0.25	0.25
Na-lactate	21.58	-
Glucose	5.56	5.56
CaCl2	-	1.71

	Na-lactate	NaCl
20mM	20	1.58
15mM	15	6.58
10mM	10	11.58
5mM	5	16.58
0mM	-	21.58

All medium containing antibiotics and phenol red and 0.4% bovine serum

albumin (BSA)

Total RNA extract and first cDNA synthesis

Total RNAs were extracted using SideStep™ Lysis & Stabilization Buffer (Strategene, cat #. 400901-21, CA, USA) according to the manufacturer's instruction. Total RNA measured OD value and stored at -80℃ until used. First strand cDNA was synthesized using First-Strand synthesis system (Stratagene, Cat #.200420, CA, USA). We used the following mixture for first-strand cDNA synthesis; reaction regent 2.0 μl standard buffer (10X), 1.5 μl oligo (dT) primer (0.5μg/μl), 0.5μl random primers (0.1μg/μl), 0.8 μl dNTP mix, 7 μl total RNA (5μg/μl), 1 μl Accutscript RT, 1 μl RNase Block Ribonuclease inhibitor and 6.2 μl RNase free water. Briefly the mixtures were incubated at 65℃ for 5 minutes and place tube at room temperature for 10 minutes for the primers to anneal to the RNA. And incubated at 42℃ for 60 minutes and incubated at 70℃ for 15 minutes to terminate cDNA synthesis.

Screening the mRNA expression for LDH, AQPs

Transcripts of target genes were amplified using PCR method (Table 2) with the LDH and AQPs specific primers (Bionics, Table 3). PCR products were analysed on 1% agarose gels and were stained with ethidium bromide.

Statistics

The t-test was used to evaluate the difference between controls and experiment groups. Results were presented as MEAN ± SD. A p-value less than 0.05 were considered to be a significant difference.

Table 2. Thermal cycler schedule

Step	Temperature (℃)	Time	Cycle
Initial cycle	94	5 mins	1 cycle
	59	30 sec	
	72	1 min	
Denaturation	94	1 min	37 cycles
Annealing	59	30 sec	
Extension	72	1 min	
Final cylce	94	1 min	1 cycle
	59	30 sec	
	72	7 mins	
Hold	4	indefinitely	

Table 3. Sequences of primers

Gene		Primer sequence (5'-3')	Amplified length (bp)
AQP1	S	tgg ttt gag cat cgc tac tct g	347
	AS	tag tca atc gcc agc agg tgt	
AQP3	S	tga cct tcg caa tgt gct tc	379
	AS	tga aga ggc gag gtc caa agt	
AQP6	S	ttt acg ggg taa ctc cag gag gta	343
	AS	tag atc agc gaa gcc agg aca	
AQP7	S	gca gct atc tcg gtg tca act tg	340
	AS	acg aat gcc tca tcc agg aa	
AQP8	S	gat gcc gtg tgt tct ggt atg a	338
	AS	ctc tgg act cac cac ttt agc caa	
AQP9	S	tct gag ttc ctg ggc acc ttt	398
	AS	cct ggc acg gat aca aat ggt t	
LDH A	S	act gtg taa ctg cga act cca agc t	452
	AS	ctg ctt gtg aac ctc ctt cca	
LDH B	S	gct caa cct ggt gca gag aaa	344
	AS	ctg tcc cca ttt ctg gat tca g	
LDH C	S	cac ggc agt ctt ttc ctt agc act	399
	AS	gag tcc cca tgt tct cca aga a	

b-actin	S	cag ggt gtg atg gtg gga at	287
	AS	Tgt ggt acg acc aga ggc ata ca	

RESULTS

Lactate concentration in preimplantation stage embryo

The amount of lactate in culture media could not disturb the embryonic development until morula stage (Fig 1A). However, the development rate of morula to blastocysts was significantly reduced (respectively 26%, 25%) ($P < 0.05$) at 96hr post hCG injection in 10, 5 and 0 mM groups compared with control (Fig 1A).

As expected the embryos could adapt to the condition of nutrients. At 72 hr after incubation (120 hr post hCG injection) most of embryos developed to the blastocyst stage (Fig. 1B).

Profiles of LDH genes expression during early embryonic stage

LDHA, LDHB and LDHC were detected in embryos of various stages and concentration of lactate. LDHA was detected morula and blastocyst stage embryos in standard culture medium, BWW. The LDHA was detected morula stage embryo in 10mM lactate experiment group and blastocyst stage embryo in 0mM lactate experiment group. LDHB was detected all stage (UF, PN, 2-cell, 4-cell, 8-cell, morula, blastocyst and hatching-hatched) embryos. Also the LDHB was detected morula stage embryo at 20mM and 10mM lactate condition, and detected blastocyst stage in 10mM lactate condition. LDHC was detected only 2-cell and 4-cell embryos in control condition (Fig 2).

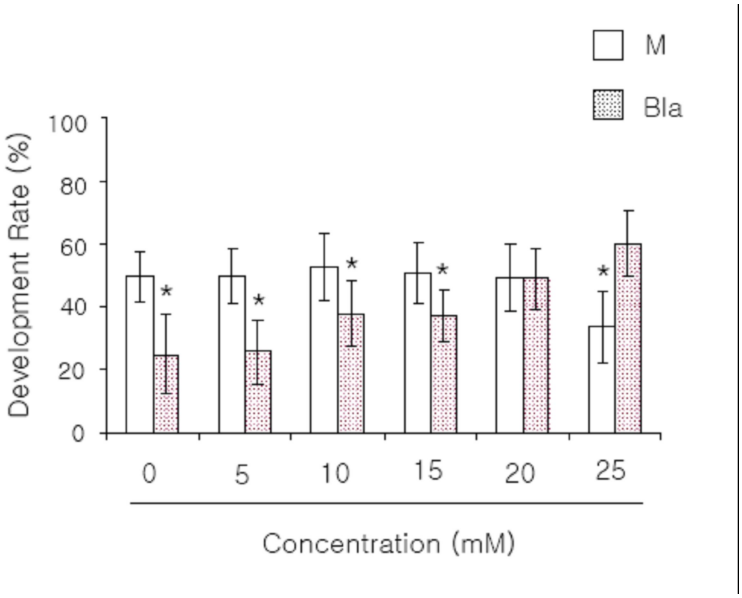
Expression profile of AQPs in preimplantation embryo

AQP1, AQP3, AQP6, AQP7, AQP8 and AQP9 were screened in various embryo stage and concentration of lactate. The embryos were cultured in BWW media. Then AQP3 was expressed at 4cell and morula stage embryo and AQP 8 was expressed hatching stage embryo but expression was very weak. And AQP9 was detected 4cell, 8cell and blastocyst stage embryo (Fig 3). AQP1, AQP6 and AQP7 were not detected in any stages.

AQPs expression in different lactate concentration

To examine, the lactate can effect on AQP expression in embryo, late 2-cell stage embryo were cultured in various conditional medium and collected as mentioned at Materials and Methods. AQP3 was detected in 120hr embryos in control group and 0mM lactate group. AQP7 was detected in 120hr embryos in 10mM lactate group. AQP8 was detected in 120hr embryos in 20mM lactate condition (Fig 4C). AQP9 was detected morula stage embryo in control group and 10mM lactate group (Fig 4A). Also AQP9 was detected from at 120hr embryo in control group. AQP1 and AQP 6 were not detected at this stage embryos.

A


M
Bla
Development Rate (%)
100
80
60
40
20
0
*
*
*
*
*
0
5
10
15
20
25
Concentration (mM)

B

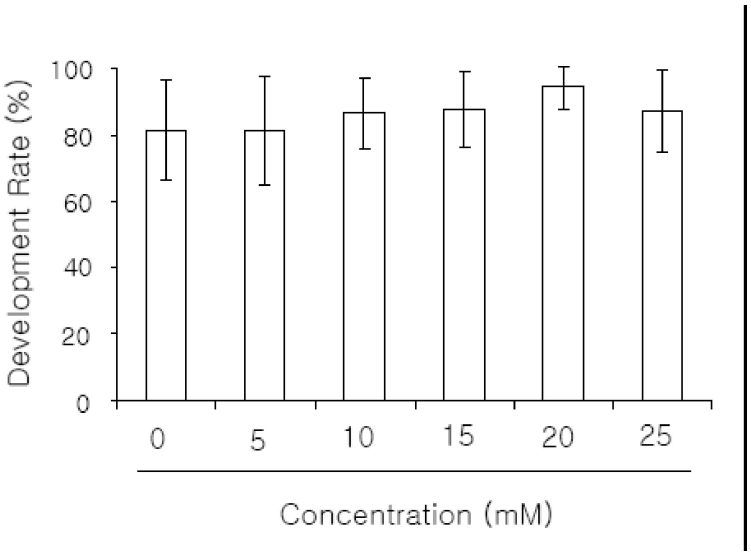
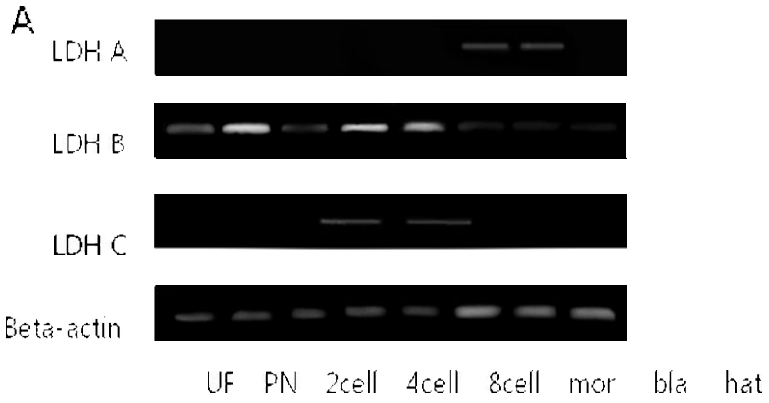
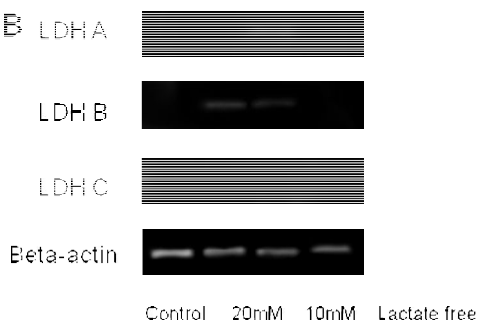


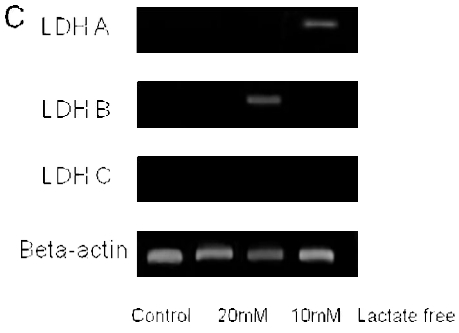
Figure 1. Effect of lactate on the in vitro development of mouse embryos at various stages.

A: Percentage of morula and blastocyst at 48 hr (96 hr post hCG injection) after incubation in conditioned media. B: Percentage of blastocyst at 72 hr (120 hr post hCG injection) after incubation in conditioned media. M; morula Bla; blastocyst.

* Significance: $p<0.05$ Control vs. experimental group


A
LDH A
LDH B
LDH C
Beta-actin
UF PN 2cell 4cell 8cell mor bla hat


B
LDH A
LDH B
LDH C
Beta-actin
Control 20mM 10mM Lactate free


C
LDH A
LDH B
LDH C
Beta-actin
Control 20mM 10mM Lactate free

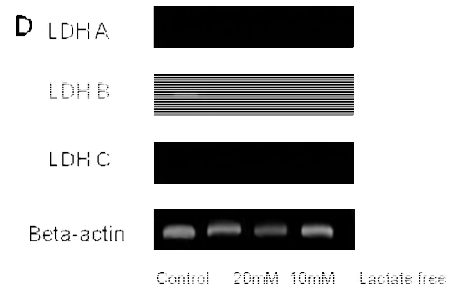


Figure 2. Expression of LDHs in periimplantation embryo

A: Expression profiles of LDHA, LDHB, and LDHC in unfertile eggs(UF), pronucleus stage embryos (PN), 2cell, 4cell, 8cell, morula, blastocyst, hatching stage embryo. These embryo cultured in BWW media. B: Expression profiles of LDHA, LDHB, and LDHC in morula stage in cultured different of lactate concentration. These embryos were cultured in 20mM lactate modified BWW, 10mM lactate modified BWW, lactate free modified BWW. C: Expression profiles of LDHA, LDHB, and LDHC in blastocyst and over stage in cultured different of lactate concentration. These embryos were cultured in 20mM lactate modified BWW, 10mM lactate modified BWW, lactate free modified BWW. D: Expression profiles of LDHA, LDHB, and LDHC in all stage embryo in timely 120hr post hCG administration. These embryos were cultured in 20mM lactate modified BWW, 10mM lactate modified BWW, lactate free modified BWW.

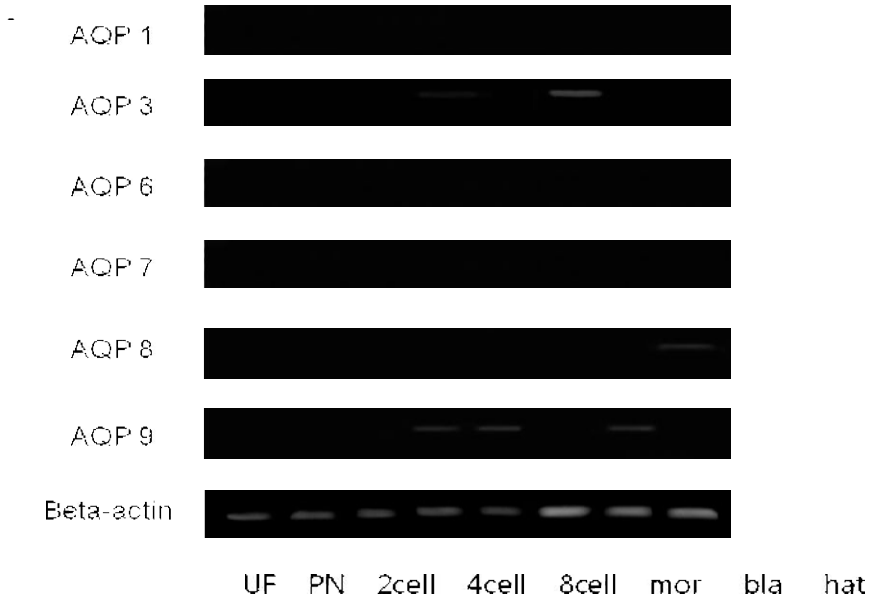


Figure 3. Modulation of AQPs expression patterns by lactate in preimplantation stages. AQP 1,3,6,7,8,and 9 expression in unfertile egg (UF), pronucleus (PN), 2cell, 4cell, 8cell, morula, blastocyst, hatching stage embryo. These embryos were cultured in BWW media.

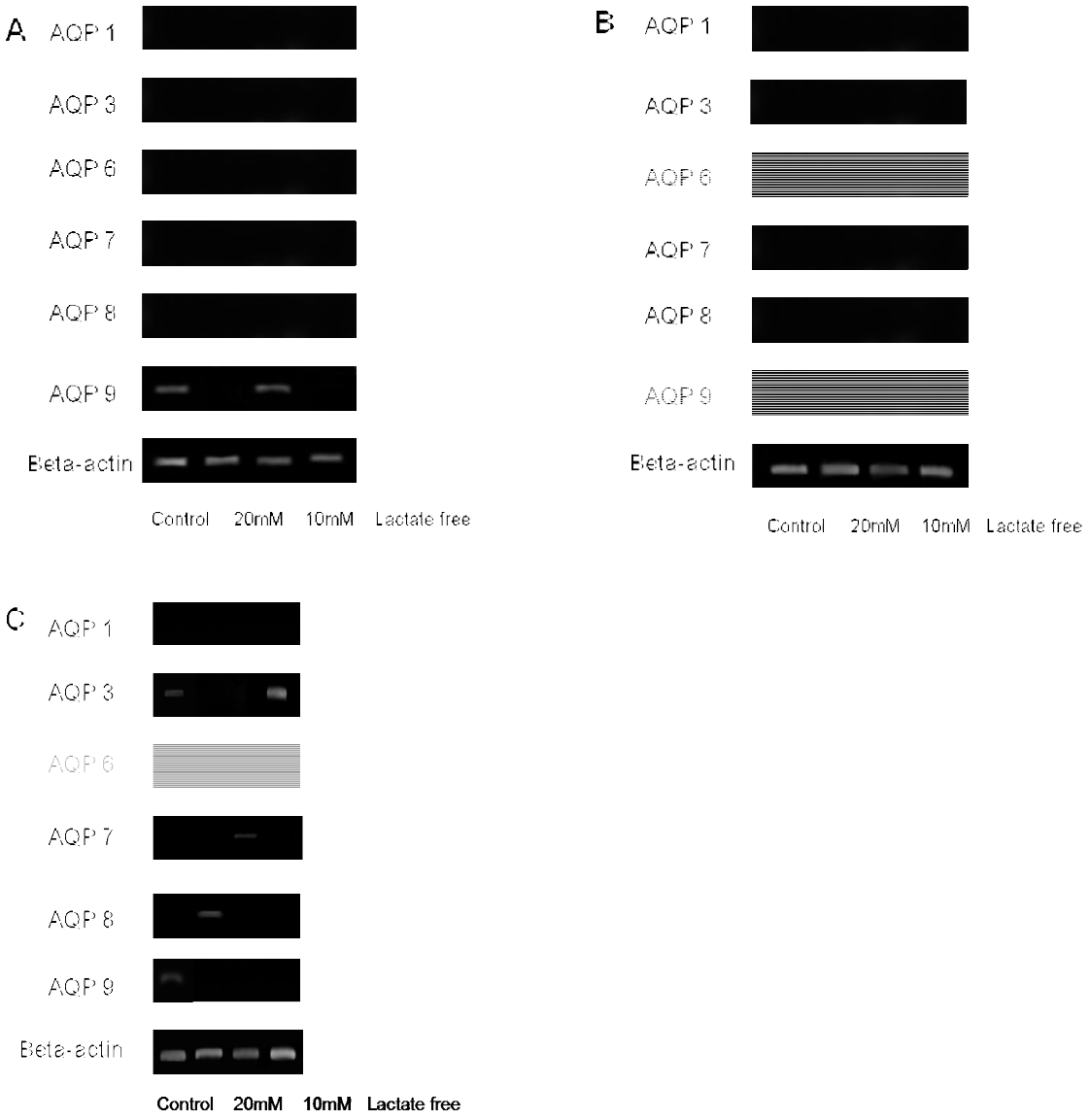


Figure 4. Lactate stimulated or inhibited the expression of AQPs in blastocyst A, AQP 1,3,6,7,8, and 9 expression pattern by lactate at morula stage embryo. These embryos were cultured in 20mM lactate modified BWW,

10mM lactate modified BWW, lactate free modified BWW.

B, AQP 1,3,6,7,8, and 9 expression pattern by lactate at blastocyst over stage embryo. These embryos were cultured in 20mM lactate modified BWW, 10mM lactate modified BWW, lactate free modified BWW.

C. AQP 1,3,6,7,8, and 9 expression pattern by lactate at all stage embryo in timely 120hr post hCG administration. These embryos were cultured in 20mM lactate modified BWW, 10mM lactate modified BWW, lactate free modified BWW.

DISCUSSION

Carbohydrate metabolism in early embryos shows substrate, specificity and metabolic adaptation (Leese, 1995; Cheon, 2008). Low level of lactate in medium did not inhibit the development of 2-cell to morula stage. However, blastocoels formation rate was much low in the media containing less lactate at 96hr post hCG injction. Blastocoel formation rate was dependent on lactate concentration in media. Besides in lactate free condition, blastoceal formation rate was dramatically reduced. Based on them in is suggested that lactate is a factor to control the embryonic development to blastocyst.

Metabolic adaptation of early stage embryos have been well known, even though the quality of embryos is different by the nutrient condition. Interestingly, as expected, those embryos formed blastocoel and reached to the blastocyt stage at 120hr post hCG injection. Therefore it is suggested that the critical point that may be regulated by lactate may morula stage.

The metabolic pathway, lactate dehydrogenase is a key enzyme to produce lactic acid. In mammals, LDH is constructed by assembly in homo- and heterodimer of Ldha, Ldhb and Ldhc. Differential expression of these genes determines the lactate dehydrogenase (LDH) isozyme composition of tissues. A subunits predominate in skeletal muscle and B subunits are abundantly produced in brain and heart. In oocytes, the LDH2 isozyme is most abundant form. The C peptide is the primary LDH of spermatozoa (Coonrod *et al.,* 2006).

The embryonic stage specific isoform was examined in this study. In our

result, LDHs were detected in oocyte and preimplantation embryos. Establishment, LDHA was expressed in morula stage and blastocyst stage very weak. This result suggests that LDHA related blastoceal formation. And LDHB was expressed all stage in preimplantation embryo and oocyte. So we know that LDHB was important in preimplantation embryo and oocyte. LDHC was expressed only 2cell and 4cell in preimplantation embryo.

The embryo was cultured in conditioned media express the different pattern of LDHs compared with control. LDHA detected morula stage in 10mM lactate modified media and blastocyst stage in lactate free modified media more strong expression than control media cultured embryo. We know that LDHA was associated with blastocoel formation, establishment in lactate free condition. LDHB was expressed at morula stage by cultured in 20mM, 10mM lactate modified media. Also this gene expressed at blastocyst stage by cultured in 10mM lactate modified media and all stage embryos sampled from 120hr post hCG administration at control condition. LDHC did not expression at morula and blastocyst stage in cultured associated of lactate. These results were similar control cultured embryo.

Recently, many studies have indicated that the water channel protein aquaporin (AQP) is involved in the homeostasis of water level in the brain and the development and reduction of brain edema. On the other hand, there are few reports about the regulation mechanisms of AQPs in the brain. Here, these have demonstrated that lactate increases the AQP4 expression level on the membrane of cultured rat astrocytes, and it may be a new regulation mechanism of AQP4 in the brain (Morishima *et al.,* 2008). As for any

substrate transported by aquaporins, the driving force for lactate through AQP9 is provided by its concentration gradient. The concentration gradient would be particularly pronounced in ischemia and hypoxia, which are characterized by an increased lactate formation and a reduction in cytoplasmic pH (Moghaddam *et al.,* 2005). We experiment AQPs expression pattern in embryo associated with lactate concentration to know that AQPs can these rule in the embryo.

AQP 1,3,6,7,8, and 9 was examined oocyte and preimplantation embryos. AQP3 was expressed 4cell and morula stage embryo. And AQP8 was expressed only hatching stage embryo. And AQP9 was detected 4cell, 8cell and blastocyst stage embryo. But AQP 1,6,7 was not detected any stage. These results show that oocyte does not express AQPs. In our experiment of associate with lactate culture, AQP expression pattern have some different with control cultured embryo. AQP9 was expressed in morula stage embryo in control and 10mM lactate modified condition also detected from all stage embryos 120hr post hCG administration at control condition. AQP3 was detected at all stage embryos at 120hr post hCG administration at cultured control condition and lactate free modified condition. That result suggests that AQP 3 was regulated by lactate at after blastocyst stage. AQP7 was expressed at over blastocyst stage embryo cultured in 10mM lactate modified condition. AQP8 was detected at over blastocyst stage embryo cultured in 20mM lactate modified condition. Not only the lactate free condition cultured embryo analysis of LDH expression pattern, these result shows LDH3 expressed from all embryonic stage 120hr post hCG administration but also

AQP expression pattern was exchanged by lactate. So we suggest that lactate may regulate LDH and AQP genes.

In summary, the blastocyst formation was very important in preimplantation embryo. Lactate could effect to development of the preimplantation stage embryos to blastocyst stage. In addition the expression patterns of LDHs and AQPs were different by the lactate concentration. Based on them it is suggested that lactate could induce the LDH gene expression and AQPs expression in early stage embryos.