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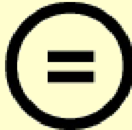

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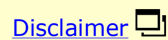

전 용 필 교수지도

석사학위 청구논문

Purine mediated meiotic arrest in mouse oocytes

2014

성신여자대학교 대학원

생 물 학 과

조 은 비

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이 논문을 석사학위논문으로 제출함

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성신여자대학교 대학원
생 물 학 과
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Purine mediated meiotic arrest in mouse oocytes

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Submitting a master's thesis of biology

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논 문 개 요

난자의 성숙은 난구와 난자간의 상호조절과 시스템 수준의 호르몬 등을 통한 복잡한 과정들에 의해서 조절된다. 성장하는 동안, 제1차 감수분열 전기에 머물러 있던 난자가 배란 직전에 감수분열을 다시 시작하는 것을 난자의 성숙으로 정의한다. 성장하는 동안 제1감수분열 전기에 멈춰져있던 난자는 황체형성호르몬의 급증을 통해 감수분열의 재개가 유도된다. 다 자란 난자가 난포에서 유리되면 호르몬의 자극 없이도 감수분열의 재개가 일어나는데 이를 바탕으로 난포가 감수분열 저지를 유지시키는 억제 물질들을 제공할 것이라고 제안되었다. 1970년대 이후 cAMP는 감수분열 저지에 있어서 중요한 역할을 한다는 것은 잘 알려져 있다. 높은 수준의 cAMP는 감수분열의 재개를 억제한다. 최근에는 cAMP뿐 아니라 퓨린을 포함한 다른 분자들이 감수분열의 조절자로서 제안되고 있다. 이전 연구에서 adenosine은 자발적 감수분열 재개를 늦추었으며 guanosine은 처리 농도에서 감수분열을 억제함을 밝혔다. 퓨린에 의한 감수분열 저지의 메커니즘은 난포내로 이동하여 cAMP 전구물로 되는 것 등이 제안되고 있으나 아직 많이 밝혀져 있지 않다. 신경세포 등에서 퓨린 수용체가 밝혀졌고 이들에 의해 신경세포 활동이 조절됨이 밝혀지고 있다. 이상의 여러 현상을 바탕으로 adenosine과 guanosine이 난포 내 그들의 수용체를 매개로 감수분열 재개를 억제할 가능성이 있음을 추론할 수 있었으므로 이를 바탕으로 이 실험에서는 퓨린의 감수분열 억제 기능이 난자 자체의 퓨린 수용체를 통하여 조절되는가를 알아보았다. Adenosine 수용체들 각각 그리고 있는 것으로 추정되는 guanosine 수용체에 특이적으로 작용하는 길항제와 촉진제를 GV 상태의 난자에 처리하여 핵 성숙에 미치는 영향을 알

아보았다. 미성숙 난자와 난구에서 adenosine 수용체의 발현을 real-time PCR과 whole-mount immunofluorescenc를 통하여 확인하였다. 난구-난자-복합형태의 난자의 핵 성숙은 A2a를 처리한 군에서 adenosine의 효과를 감소시켰으며, 난구가 제거된 난자에서는 모든 수용체의 길항제들이 adenosine의 효과를 감소시켰다. 전사수준에서 A1, A2a, A2b, A3는 난구에 비하여 100 배 이상 높았다. 모든 수용체가 검출되었으며 이중 A3의 전사수준이 제일 높았다. 전사수준에서의 발현뿐만이 아니라 단백질 수준에서도 발현이 되었는데 A1, A2a, A2b, A3 단백질 모두가 난자의 막과 핵에 위치하였다. A2b는 핵에 강하게 그러나 난자의 막에는 약하게 분포하는 특징을 보였다. 한편 guanosine은 농도 의존적으로 감수분열 재개를 억제하였으며 난포액내 guanosine의 양은 난포의 성장과 함께 증가하였다. Adenosine과 guanosine은 체외 성숙 동안에 난자 세포질내의 cAMP와 cGMP의 양적 변화를 유도하였다. 이러한 결과를 종합하여 볼 때 난자 내에 adenosine 수용체들이 존재하고 있으며 adenosine이 그 수용체들을 통하여 감수분열을 매개할 것이라고 생각하였다. 본 연구에서 guanosine이 수용체를 통하여 조절하는 것을 보이지는 못했으나 농도 의존적 반응을 통하여 그 가능성을 추정할 수 있었다. 향후 추가적 연구를 통하여 이를 밝히고자 하며, 이러한 연구는 포유동물의 감수분열 조절에 필요한 기반 지식을 구축하는데 도움이 될 것으로 사료된다.

CONTENTS

Abstract (Korean)

List of Tables

List of Figures

Introduction ········· 1

Materials and Methods ········· 4

Experimental Animals ········· 4

Oocyte collection ········· 4

Chemical treatment ········· 4

Total RNA isolation and first strand cDNA synthesis ········· 5

Real-Time RT-PCR ········· 6

Immunofluorescence ········· 6

Follicular fluid collection and analysis the amount of guanosine ········· 6

Statistics ········· 7

Results ········· 10

Effects of adenosine receptor antagonists in meiotic arrest in cumulus-enclosed oocytes and denuded oocytes ········· 10

Adenosine receptors expressed in both oocyte and cumulus cell ········· 13

Localization of adenosine receptors in oocyte ········· 15

Changes of cAMP and cGMP levels in mouse oocytes during in vitro maturation by adenosine and guanosine ········· 17

Effects of guanosine on meiotic resumption 19

Quantitative change of guanosine in mouse follicular fluid 21

Discussion 23

References 27

Abstract(English) 33

List of Tables

Table 1. Primers list for real-time PCR ananlysis··············· 7

Table 2. Thermal cycler schedule·· 9

List of Figures

Figure 1. Effects of various adenosine receptor antagonists on the meiotic maturation of mouse immature oocytes in vitro·················· 11

Figure 2. mRNA expression profiles of adenosine receptor genes in mouse oocyte and cumulus cell·· 14

Figure 3. Localization of adenosine receptor proteins in mouse oocytes·· 16

Figure 4. Change of cAMP and cGMP levels in mouse oocytes during in vitro maturation·················· 18

Figure 5. Guanosine suppressed in vitro maturation······· 20

Figure 6. Amount of guanosine in follicular fluid············ 22

INTRODUCTION

The growing mammalian oocytes are enclosed within follicle cells and arrested in prophase Ⅰ. The prophase 1 arrested oocyte acquires the competence to resume meiosis as it approaches its full size. By the grown of follicle, the granulosa cells can express LH receptor and can response the LH. The LH surge is reason of ovulation and trigger of meiosis resumption (Park et al., 2004). On the other hand, removal of the oocytes from their follicular environment causes resumption of meiosis (Pincus and Enzmann, 1935).

Meiotic inhibiting factors are synthesized both in growing oocytes or in the granulosa cells and accumulated in the oocyte. The known factors are including hypoxanthine, cyclic adenosine 3',5'-monophosphate (cAMP), cyclic guanosine 3',5'-monophosphate (cGMP) reactive oxygen species, protein kinase A, protein kinase C (Conti et al., 2002; Tripami et al., 2010).

Spontaneous resumption of meiotic division is suppressed by addition of dibutyryl cyclic adenosine 3',5'-monophosphate (dbcAMP) (Chen et al., 2006; Sato et al., 1985). In our laboratory, both of adenosine and guanosine have a inhibitory roles in meiotic resumption. Guanosine suppressed meiotic resumption in follicle free condition (Cheon et al., 1997; Hwang and Cheon, 2013).

Guanosine nucleotide-binding proteins (G proteins) induce various signals depend on ligands. Stimulatory heterodimeric GTP-binding protein consist with α, β and γ subunit. Its' activity is shutdowned

following hydolysis of GTP by intrinsic GTPase. When ligands are bound, α subunit dissociates from the βɣ complex and activated adenylate cyclase. Activation of adenylate cyclase increase the cAMP level, which activate cAMP-dependent protein kinase (PKA). Suppression of Gs activation by antibody in growing oocytes within a follicles result in meiotic resumption (Mehlmann et al.,2002). Gs activation of oocyte by G-protein coupled receptor 3 (GPR3) maintain cAMP accumulation above a threshold that precludes oocyte re-entry into the cell cycle. G-protein coupled receptor 3 knock-out ($Gpr3^{-/-}$) females have a phenotype of premature GVBD oocytes in the absence of LH stimulation (Ledent et al., 2005; Mehlmann et al., 2004). Similarly, downregulation of GPR3 or GPR12 causes spontaneous maturation in oocytes of mouse and rat, respectively (Hinckley et al., 2005; Mehlmann, 2005). In the same vein, growing ovarian oocyte in phosphodiesterase 3A knouckout ($Pde3a^{-/-}$) mice remain in meiotic arrest after releasing from preovulatory follicles. Besides, it stay at prophase Ⅰ after ovulation (Masciarelli et al., 2004). These are strongly confirm that the high cAMP levels by oocyte is a key mechanism the maintain meiotic arrest during prophase of meiosis Ⅰ.

It also known that cyclic guanosine monophosphate (cGMP) can activate or inactivate phosphodiesterases (PDEs). cGMP stimulates PDE2 activity but inhibits PDE3 activity (Juilfs et al., 1999; Wang et al., 2008) resulting in increase in cAMP levels (Nogueira et al., 2003), and block meiotic resumption. Production of cGMP in cumulus cell suppress meiotic resumption by transporting of cGMP into oocytes.

Natriuretic peptide receptor 2 (NPR2), the predominant guanylyl cyclase, in granulosa cell produce these cGMP (Wigglesworth et al., 2013). It is suggested that PDE3A is inhibited by the influx of cGMP from the cumulus (Noris et al., 2009).

Adenosine is a metabolite of cAMP in granulosa cells via PDE and 5'-nucleotides (Knecht et al., 1983). In addition, cAMP can be used as substrate for ATP. So, the nucleoside has a regulatory roles in a system highly depend upon cAMP. In gonadal tissues, adenosine has been shown to stimulate adenylate cyclase activity in Leydig tumor cells (Wolff and Hope Cook, 1977) and to increase cAMP and progesterone accumulation in human granulosa and rat luteal cells (Behrman et al., 1982; Brennan et al.,1983; Hall et al., 1981; Polan et al., 1983).

Approximately 80% of adenosine action in luteal cells is dependent upon the intracellular transport of adenosine, leading to increased cellular levels of ATP (Brennan et al., 1983). In PMSG primed maturing granulosa cells, adenosine amplified FSH and LH actions on cAMP accumulation, but the stimulation was inversely proportional to the stage of follicular development (Polan et al., 1983). In our laboratory studies results, purines disturbed the maturation of denuded oocytes (Hwang and Cheon, 2013). Put together, it is suggested that oocyte has its own system to produce cGMP and cAMP which are mediated by receptors. In this study, the expression and localization of purine receptors were evaluated and the roles of them were examined.

MATERIALS AND METHODS

Experimental animals

All experiment animals were studied according to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health and under the Experimental Animals Committee of Sungshin Women's University. Animals were maintained under the standard conditions at animals house in Sungshin Women's University with diurnal rhythm kept under the 14 L : 10 D 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 21 days after birth.

Oocyte collection

GV intact oocytes were collected from 21- to 28-day old female CD-1 mice 46 hr after intraperitoneal injection of 5 IU of pregnant mare's serum gonadotropin (PMSG). Oocytes were isolated from antral follicles using a 30-gauge needle in BWW medium containing 0.4% bovine serum albumin (BSA). Cumulus-enclosed oocyte were collected and denuded oocytes were collected after mechanically removed the cumulus .

Chemical treatment

Oocytes were cultured in BWW medium containing 0.4% bovine serum albumin (BSA) with or without containing following agents for 6 hr:

750 μM adenosine + 10 nM DCPCX (Tocris) (A1 receptor antagonist),
750 μM adenosine + 10 nM SCH44246 (Tocris) (A2a receptor antagonist),
750 μM adenosine + 10 nM MRS1706 (Tocris) (A2b receptor antagonist),
750μM adenosine+10nM MRS1334 (Tocris) (A3 receptor antagonist),
750 μM adenosine + 10 nM CGS15943 (Tocris) (nonselective adenosine receptor antagonist),
750 μM adenosine + mixed all antagonists,
50 μM guanosine ,
750 μM guanosine

Total RNA isolation and first strand cDNA synthesis

Total RNA of oocytes were extracted using RNeasy® Micro Kit (QIAGEN, CA USA) according to the manual of manufacturer. Total RNA 5 μg were used to perform reverse transcription. First strand cDNA was synthesized with First strand cDNA Synthesis Kit (Agilent, CA, USA) according to the manual of manufacturer. Briefly, reaction reagents are mixed with total RNA 5 μg, 5.0 μl Accuscript buffer (10X), 6.0 μl oligo dT primer (0.5 μg/μl), 1.0μl random primer (0.1 μg/μl), 2 μl dNTP mix (100 mM), and 1 μl RNase-free water. Reaction mixture was incubated at 65℃ for 5 min, placed the tube at RT to allow the primers to anneal to RNA for 10 min and then added 4.0 μl DTT (100 mM), 1 μl Accuscript multiple temperature RT, 2 μl RNase block ribonuclease inhibitor (40U/ml). The mixture was incubated at 42℃ for 1 hr and 72℃ for 15 min to terminate cDNA synthesis.

Real-Time RT-PCR

Transcripts of target gene were amplified using real time-PCR (TaKaRa, TP800) and the specific primers (Table 1). The primer parameters were 50% GC contents, avoiding repeat base pair and lengthening 20-24mer. For Quantitative RT-PCR (qPCR) was performed using SYBR Premix Ex Taq™ (TaKaRa) (Table 2). Each reaction was run in triplicate and consisted of 1.0 µl cDNA. Dissociation curves were run on all reactions to ensure amplification of a single product with the appropriate melting temperature. The fold change in gene expression was calculated using the ΔCt method with the housekeeping gene, β-actin.

Whole-mount immunohistochemistry

Mouse oocytes were fixed in 3.7% paraformaldehyde containing 0.15% picric acid(saturated) in PBS for 1 hr at room temperature (RT). Oocytes were permeabilized with 0.2% PBST for 1 hr at RT. Blocking in 1% BSA in PBS for 1 hr at RT. Oocytes incubated with the Rabbit polyclonal IgG A1R, A2aR, A2bR, and A3R (dilution 1:200) in 2% BSA in PBS for overnight in 4℃. After washing in 1% BSA in PBS, oocytes were incubated with biotinylated anti-rabbit IgG (dilution 1:200) in 2% BSA in PBS for 2 hr at RT. Oocytes were washed with 1% BSA in PBS and incubated with Hoechest 33258 (dilution 1:200) 15 min for counter staining and mount.

Follicular fluid collection and analysis the amount of guanosine

Follicular fluids were collected from the growing follicles and

preovulatory follicles (0hr, 24 hr, and 48 hr post PMSG; 6 hr and 12 hr post hCG (PMSG primed)), Follicles were punctured (40 follicles / oocyte) with syringe and centrifuges (> 10,000g, 10 min) and the supernatant was collected. The fluids were kept at −20℃ until analyzed. Amount of guanosine were measured by HPLC (Column: PACKED COLUM C_{18} UG 120 (5 μm) 4.6mm x 250 mm, Mobile Phase: 20 mM KH_2PO_4, Wavelength: 254 nm, Flow: 0.3 ml/min, 40 min)

Statistics

The *t*-test, chi-square test were used to evaluate the difference between control and experiment groups. Results were presented as mean±SEM. Values of $P < 0.05$ were considered significant.

Table 1. Primers list for real-time PCR analysis

Gene		Primer sequence	Amplified length (bp)
Adenosine A1 receptor	Forwad	TAT CAA CAT TGG GCC ACA GAC C	365
	Reverse	GCA CCC AGA CGA AGA AGT TGA A	
Adenosine A2a receptor	Forward	AAC CTG CAG AAC GTC ACC AAC T	294
	Reverse	GAA TGA CAG CAC CCA GCA AAT C	
Adenosine A2b receptor	Forward	ATC CCC TTT GCC ATC ACC AT	276
	Reverse	GCT GGT GGC ACT GTC TTT ACT GTT	
Adenosine A3 receptor (1)	Forward	ACC TTT GGC CAT TGC TGT CA	281
	Reverse	TTT GAG AGC TCG CTA AGG TTG C	
Adenosine A3 receptor (2)	Forward	TCC TTG GCT CTC TTT TCC GAT G	347
	Reverse	TTG GCC CGG TCT TCT CTA TTG T	
β-actin	Forward	ACC AGG CAC AGA CAG AAA TCC A	227
	Reverse	GCC ATC ATC CTC ATC TGC CAT CT	

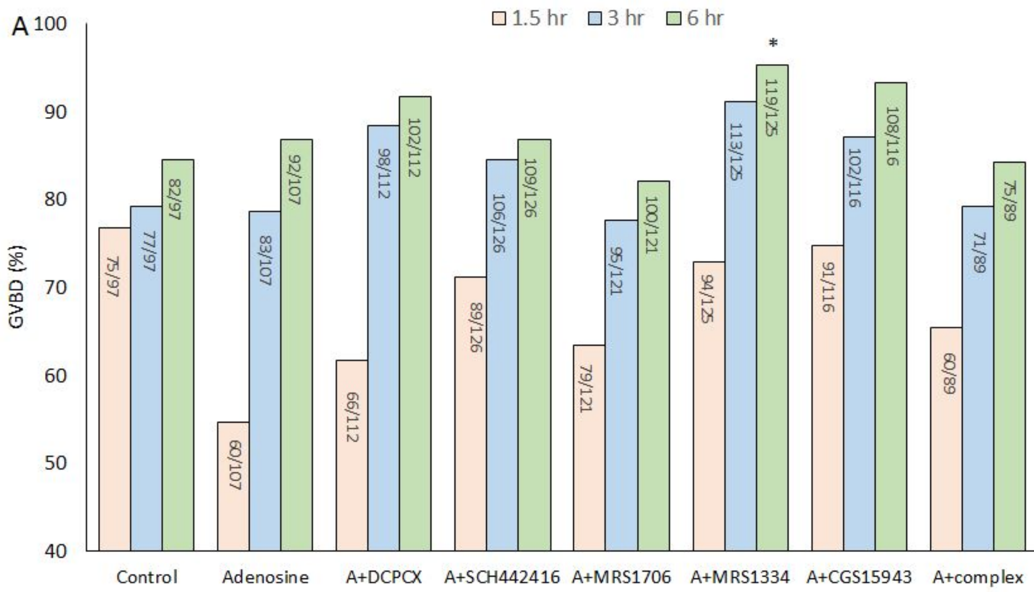
Table 2. Thermal cycler schedule

Step		Temperature (℃)	Time
Hold	Hold	94	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	5 min

RESULTS

Effects of adenosine receptor antagonists in meiotic arrest in cumulus-enclosed oocytes and denuded oocytes

To know the effects of adenosine receptor antagonist on meiotic arrest denuded oocytes or cumulus-enclosed oocytes were cultured in BWW medium containing 10nM adenosine receptor antagonists as mentioned in Materials and Methods. The state of meiotic arrest were assessed after incubation 0 hr, 1.5 hr, 3 hr, and 6 hr. 750 μM adenosine expanded duration for occuring GVBD both in DO and CEO. Meiotic resumption rates were similar between groups except SCH442416 group at 0.5 hr after incubation. Retardation of GVBD resumption by adenosine was blocked by SCH442416 at 0.5 hr after incubation (Fig. 1A).


A
1.5 hr
3 hr
6 hr
GVBD (%)
100
90
80
70
60
50
40
75/97
77/97
82/97
60/107
83/107
92/107
66/112
98/112
102/112
89/126
106/126
109/126
79/121
95/121
100/121
94/125
113/125
119/125
*
91/116
102/116
108/116
60/89
71/89
75/89
Control
Adenosine
A+DCPCX
A+SCH442416
A+MRS1706
A+MRS1334
A+CGS15943
A+complex

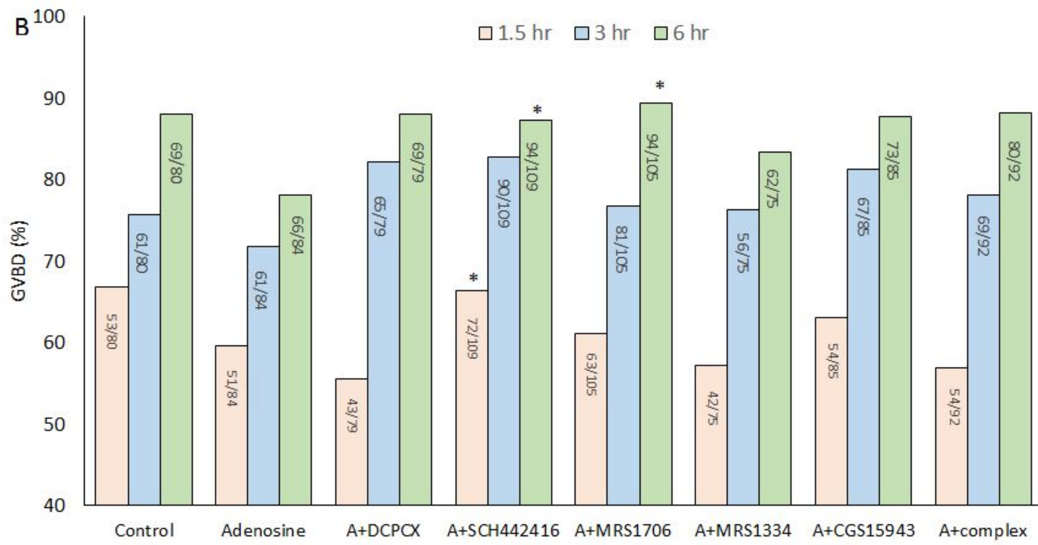

B
1.5 hr
3 hr
6 hr
GVBD (%)
100
90
80
70
60
50
40
53/80
61/80
69/80
51/84
61/84
66/84
43/79
65/79
69/79
72/109
*
90/109
94/109
*
63/105
81/105
94/105
*
42/75
56/75
62/75
54/85
67/85
73/85
54/92
69/92
80/92
Control
Adenosine
A+DCPCX
A+SCH442416
A+MRS1706
A+MRS1334
A+CGS15943
A+complex

Figure 1. Effects of various adenosine receptor antagonists on the meiotic maturation of mouse immature oocytes in vitro.

(A) Cumulus-enclosed oocytes and (B) denuded oocytes were cultured in BWW medium containing 10 nM adenosine receptor antagonists for 0 hr, 1.5 hr, 3 hr, and 6 hr. DCPCX; A1 receptor antagonist, SCH44246; A2a receptor antagonist, MRS1706; A2b receptor antagonist, MRS1334; A3 receptor antagonist, CGS15943; nonselective adenosine receptor antagonist. *$P<0.05$, control vs experimental group.

Adenosine receptors expressed in both oocyte and cumulus cell

The expression of adenosine receptors in oocytes and cumulus cells were examined by real-time RT-PCR. It is well known that neurons in brain express adenosine receptors. In this study, the brain was used as control for adenosine receptors. The expression profiles of adenosine receptors in cumulus cells were similar with them of brain but the levels were very low (Fig. 2A, B). In contrast to cumulus cell, the expression levels adenosine receptors mRNA were very high compared with cumulus or brain. Their mRNA expression levels were 2 times higher than cumulus cell and brain (Fig. 2C).

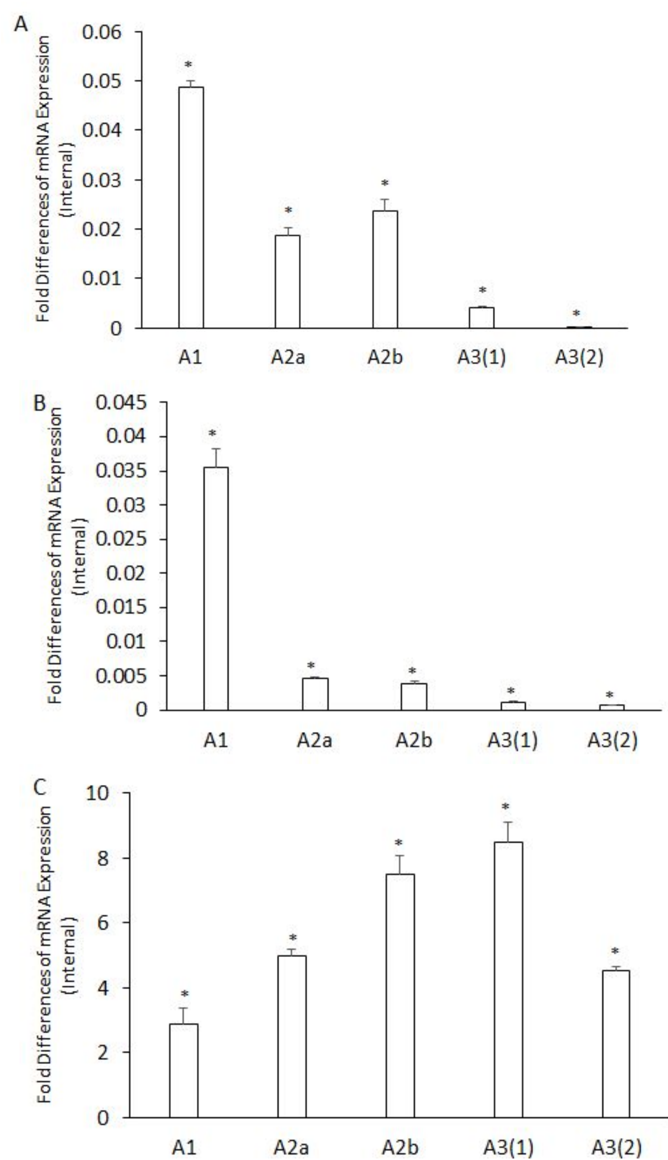


Figure 2. mRNA expression profiles of adenosine receptor genes in mouse oocyte and cumulus cell

The expression of adenosine receptors in denuded oocytes (A), cumulus cell (B) and brain (C) were examined by real-time RT-PCR. *$P<0.05$.

Localization of adenosine receptors in oocyte

Whole-mount immunofluorescence was performed to identify the localized area of adenosine receptors in immature oocytes. Adenosine receptor A1, A2a, A2b, and A3 was localized in nucleus and oocyte membrane (Fig3. A-L). Adenosine receptor A3 was mainly localized in cytoplasm (Fig3. J-L). In negative control (Fig. 3M-O), there was no any signal. The intensity of A2b protein was higher in nucleus than A1 and A2a. On the other hand, the intensity of A2b protein was lower in oocyte membrane than A1 an A2a. In A1 and A2a, the intensity was similar between nucleus and oocyte membrane.

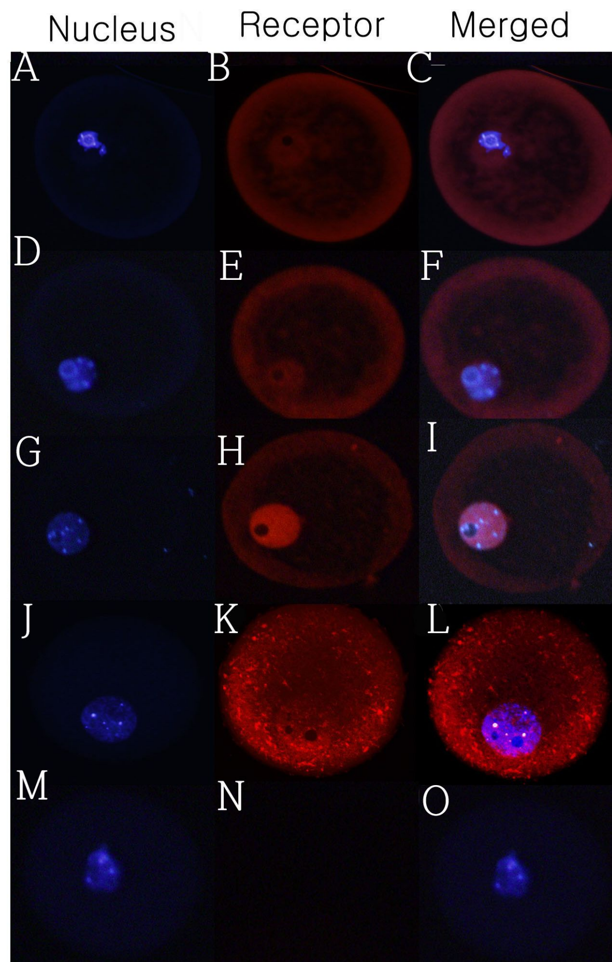


Figure 3. Localization of adenosine receptor proteins in mouse oocytes

The localization of adenosine receptors in immature oocyte were examined by whole-mount immunofluorescence. A-C: Negative control, D-F: adenosine A1 receptor, G-I: adenosine A2a receptor, J-L: Adenosine A2b receptor, M-O: adenosine A3 receptor. Rabbit IgG, Receptor is red(Cy3) and nucleus is blue (Hoechest33258).

Changes of cAMP and cGMP levels in mouse oocytes during in vitro maturation by adenosine and guanosine

The levels of cAMP and cGMP were measured by enzyme immunoassay during spontaneous in vitro maturation. At 0, 1.5, 3, and 6 hr, cAMP levels were increased until 1.5 hour, decreased until 3 hour, and increased again thereafter in control group. In adenosine, and A+G treated groups, cAMP levels were not changed. In 50μM guanosine treated group, cAMP levels were increased from 1.5 hr to 3 hr and decreased after 3 hr of culture.

The pattern of cGMP level in control group was similar with cAMP level in control group. cGMP levels of guanosine treated oocytes were continuously increased by the incubation time. The levels of cGMP were higher In 750 μM guanosine than 50 μM guanosine (Fig 4B).

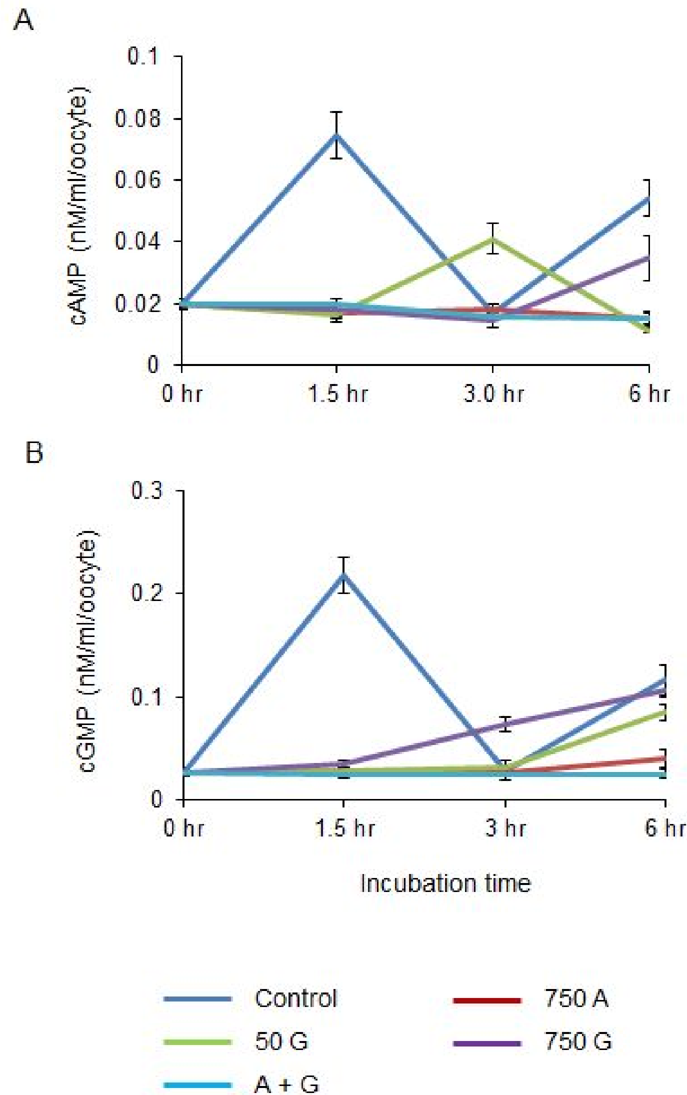


Figure 4. Change of cAMP and cGMP levels in mouse oocytes during in vitro maturation.

cAMP (A) and cGMP (B) levels during in vitro maturation were measured by enzyme immunoassay. The existence and level changes of signal mediators were analyzed using mass spectrometer. Abbreviation: A; adenosine, G; guanosine,

Effects of guanosine on meiotic resumption

To know whether is there guanosine control meiotic arrest, guanosine was treated during in vitro maturation. Most of the oocytes was undergo GVBD in control. The GV intact rates were decreased in guanosine treated groups in concentration dependent manner (Fig. 5).

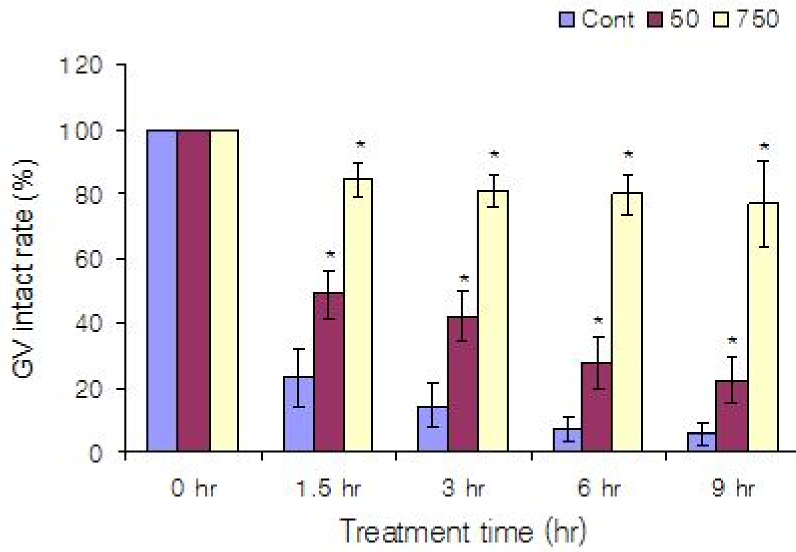


Figure 5. Guanosine suppressed in vitro maturation.

Guanosine was treated in denuded immature oocytes. GV intact oocytes were counted under the inverted microscope. *$P<0.05$, control vs experimental group.

Quantitative change of guanosine in mouse follicular fluid

Changes amount of guanosine were measured by high performance liquid chromatography (HPLC). Guanosine were measured in follicular fluid from different follicular stage. The amount of guanosine was peaked at 24 hr and maintained until 48 hr post PMSG injection. After then, its level decreased until ovulation.

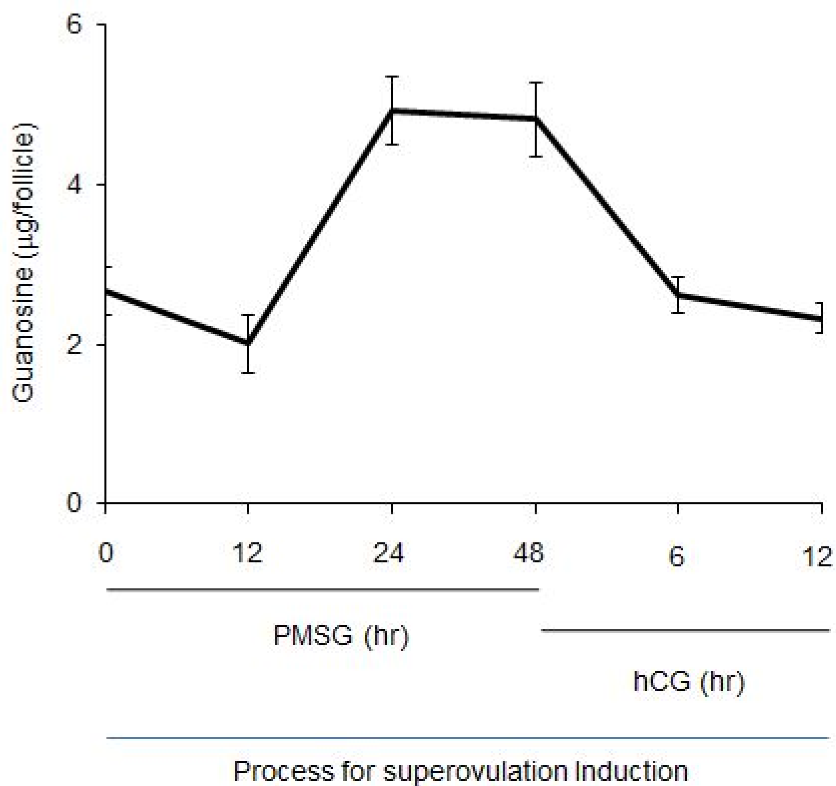


Figure 6. Amount of guanosine in follicular fluid

Amount of guanosine were measured by HPLC. Follicular fluids were collected from follicles during superovulation induction.

Column: PACKED COLUM C_{18} UG 120 (5 μm) 4.6mm x 250 mm, Mobile Phase: 20 mM KH_2PO_4, Wavelength: 254 nm, Flow: 0.3 ml/min, 40 min.

Discussion

Mammalian oocytes are arrested in meiotic prophase during growth. LH surge triggers the resumption of meiosis. Interestingly, when fully grown competent oocytes are removed from their follicles, they resume the meiosis without hormonal stimulation, suggesting that the follicle provides an inhibitory factors to maintain meiotic arrest. It is well established that cAMP is a key molecule in meiotic arrest. However, recently other molecules including purine have been suggested as a meiotic regulator. The mechanisms of meiotic arrest or delay by purine is not much uncovered. In this study it was evaluated the possible mechanisms of purines in meiotic arrest.

The mammalian ovarian follicle maintains the oocyte in meiotic arrest until the preovulatory surge of gonadotropins. Controversy has surrounded the proposed existence of a low molecular weight substance in ovarian follicular fluid that maintains the oocyte in meiotic arrest. Although such substances have been reported to be present in pig, hamster, sheep, cow, and human follicular fluid (Chang, 1955; Tsafriri and Channing, 1975; Gwatkin and Andersen, 1976; Jagiello et al., 1977; Tsafriri et al., 1977; Chari et al.,1983), the existence of these substances has been controversial, since some investigators have failed to obtain an inhibitory effect of follicular weight (Downs and Eppig, 1984). Eppig and his colleague have shown further that the principal inhibitory component of this low molecular weight PFF fraction is hypoxanthine (Downs et al., 1985a). In addition

to hypoxanthine (1.4 mM), the preparation of PFF analyzed contained high concentrations of other purines and pyrimidines including adenine (0.06 mM), uracil (0.44 mM), and 7-methylinosine (0.19 mM). However, these purines and pyrimidines did not appear to contribute significantly to the maturation-arresting activity of the PFF fraction (Downs et al., 1985a). Nevertheless, it is suggested that other purines, not detected in the analyzed preparation of PFF, may participate in the maintenance of meiotic arrest and other aspects of follicular function.

Adenosine is of particular interest because it has been shown to elevate granulosa cell cAMP levels (Polan et al., 1983), and the elevation of cumulus cell cAMP levels has been positively correlated with the maintenance of meiotic arrest. So it is also suggested as PFF (Eppig et al., 1983; Schultz et al., 1983a; Ekholm et al., 1984; Racowsky, 1984). The known mechanisms is mediated the granulosa cells, but purine receptors is suspected as a one mechanism in meiotic arrest from various low data in our laboratory

Treatment with adenosine receptor antagonists to the culture medium delayed GVBD in denuded oocyte and cumulus-enclosed oocyte. Adenosine receptor antagonists prevent the inhibitory effects of adenosine both in denuded oocyte and cumulus-enclosed oocyte. In denuded oocyte, A1, A2a, A2b, and A3 receptor antagonists inhibited the effects of adenosine in oocyte maturation in vitro. It means that adenosine regulate meiotic arrest through the receptors in cumulus and oocyte.

A1, A2a, A2b, and A3 mRNAs were expressed in oocytes and cumulus. Their expression levels in oocytes were very high compare with cumulus and the positive control, brain. In denuded oocyte, the meiotic arrest by adenosine was significantly inhibited in A3. In the other groups, the effects of adenosine on meiotic arrest was inhibited by other antagonist, although there was no statistical significance. This result shows that one of the methods in meiotic arrest is the purine signaling pathways in oocyte itself.

In neuron, the receptors of adenosine is G protein coupled receptor and localized in membrane (Sahin et al., 2007). However, interestingly in oocyte, A1, A2a, 2b, and A3 were localized in membrane and nucleus. In the case of A2b, it was localized strongly in nucleus compared with the membrane. Besides, the effects of adenosine was inhibited by A2b antagonist. In addition, Highly expressed A3 localized mainly in oocyte membrane and A3 antagonist suppressed the effects of adenosine in meiotic maturation. GPR3 is mainly expressed in oocytes, rather than in the follicle cells in the mouse (Mehlmann, 2005; Mehlmann et al., 2004) an localized both in the plasma membrane and in throughout the cytoplasm of the oocyte (Katie et al., 2011). Although further studies are need, based on them, it is suggested that the adenosine receptors which are localized both in membrane and nucleus are involved in meiotic arrest.

Hypoxanthine and adenosine are present in preparations of mouse ovarian follicular fluid, and these purines maintain mouse

oocytes in meiotic arrest in vitro (Eppig et al., 1985). In denuded oocytes, adenosine had little inhibitory effect on GVBD at this time point but sustained higher levels of cAMP. Hypoxanthine maintained 80% of cumulus cell-enclosed oocytes in meiotic arrest and also sustained higher cAMP levels in the oocytes. The addition of adenosine to hypoxanthine-containing medium increased the percentage of oocytes maintained in meiotic arrest, and increased the amount of cAMP in the oocytes above that maintained by either hypoxanthine or adenosine alone (Downs et al., 1989). Interestingly, during in vitro maturation, cAMP levels were increased during early time and then showed fluctuation. The levels of cAMP in oocyte were changed dramatically by exogenous adenosine and guanosine with different patterns. It conformed that the suggestion of this study, oocyte express its own purine receptors and regulate their meiotic arrest.

On summary, adenosine receptor antagonists prevent of inhibitory effects of adenosine on the meiotic maturation of mouse oocytes. Adenosine receptors are localized both in oocyte membrane and nucleus. The levels of cAMP and cGMP were modulated by exogenous adenosine and guanosine in denuded oocytes. Guanosine inhibited in vitro maturation of oocytes in concentration dependent manner. The concentration of guanosine is decreased after LH surge. Taken together, it is suggested that purines may regulate meiotic arrest through their receptors in oocyte itself.

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ABSTRACT

Purine mediated meiotic arrest in mouse oocytes

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Mammalian oocytes are arrested in meiotic prophase during growth. LH surge triggers the resumption of meiosis. Interestingly, when fully grown competent oocytes are removed from their follicles, they resume the meiosis without hormonal stimulation, suggesting that the follicle provides an inhibitory factors to maintain meiotic arrest. It is well established that cAMP is a key molecule in meiotic arrest. However, recently other molecules including purine have been suggested as a meiotic regulator. The mechanisms of meiotic arrest or delay by purine is not much uncovered. In this study we evaluated the possible mechanisms of purines in meiotic arrest. GV intact oocytes were collected after 46 hr of 5iu pregnant mare's serum gonadotropin (PMSG) administration. GV intact oocytes were cultured in BWW medium with or without adenosine receptor antagonists or guanosine pathway inhibitors; A1 receptor antagonist, DCPCX; A2a receptor antagonist, SCH44246; A2b receptor antagonist, MRS1706; A3 receptor antagonist, MRS1334;

nonselective adenosine receptor antagonist or CGS15943. cAMP and cGMP level of oocytes were analyzed with Direct EIA Kit. GVBD was temporally inhibited by adenosine with concentration dependent in both COCs and DOs. Adenosine receptors were detected both in transcription and translation levels. Their expression levels were very high compared with cumulus and brain. The antagonists of adenosine receptors, A1, A2a, A2b, and A3 blocked the effects of adenosine on meiotic arrest of immature oocytes. Levels of cAMP and cGMP were changed by purines. Guanosine suppressed the meiotic resumption in concentration dependent manner. The concentration of guanosine in follicular fluid was decreased after LH surge. Based on these results it is suggested that purines in follicular fluid may be one of the important regulation factors of meiotic arrest and they may work through their own receptors on oocyte itself.

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발생방, 사랑합니다♡ 너네가 없었다면 내 졸업도 없었어. 이건 한치의 거짓도 없이 모두 진심이야! 너희 같은 후배들을 만나서 내 대학원 생활이 즐거웠고, 행복했어. 빛과 소금같은 내 후배들, 나 떠난다고 파티하고 그러지마. 그리고 대망의 장다예♡ 허구헌날 바보같은 하소연 들어주고, 내 편 들어주느라 니가 더 고생했어. 답답할 때 너한테 얘기하면 다 해결^^♡ 학교를 떠나도 우리 사이는 변치 않을거야! 넌 내꺼니까. 내 가난한 대학원 시절을 먹여살려준 다예쁘니, 다 보답할게 기대해! 호호, 백수경언니♡ 졸업하고 나서도 매일 질문하는 나 때문에 졸업한 거 같지도 않았지? 귀찮게 해도 언제나 친절하게 대답해줘서 사랑해^_^ 언니마음=나의마음bb 만날 때마다 밥 사주고

커피사주고 언니는 정말 날 사랑하는 거 같아. 주말마다 나랑 데이트해준다 해놓고 자꾸 시집갈 생각하는데 그러지 않기로 해. 내가 보내줄 거 같아?

나의 사랑, 나의 보물, 나의 행복, 나의 활력소 엄마, 아빠, 빈이, 비비! 말하지 않아도 알지? 헤헤 여러분의 사랑과 뒷바라지(...)가 없었다면 오늘도 없었다! 언제나 내 편인 김향미♥ 내가 누구 욕을 하던 같이 욕해줘서 고마워 ㅋㅋ 내가 입버릇처럼 나 같은 딸 세상 어디에도 없다고 말하지만(이건 진실) 세상 어디에도 우리 엄마 아빠같은 부모님도 없을 거야. 내가 하고 싶은 일을 할 수 있게 언제나 뒤에서 지켜봐주고, 응원해줘서 고마워♡ 그리고 우리 할아버지랑 큰삼촌, 작은삼촌, 외숙모, 서영이, 서우도 고맙고 사랑합니다^_^ 내가 사랑하고, 날 사랑해주는 우리 가족이 없었다면 아마 아무것도 하지 못했을 거야. 정말정말 사랑합니다♥

그 외에 위에서 얘기하지 못한 도움을 주신 모든 분들, 정말 감사합니다.