

## Adenosine Modulates the Oocyte Developmental Competence by Exposing Stages and Synthetic Blocking during *In Vitro* Maturation

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**ABSTRACT :** Purine metabolism is known factor for nuclear maturation of oocytes through both follicle cells and oocyte itself. However, it is largely unknown the roles of purine metabolism in the oocyte competence for fertilization and early development. In this study, the effects of adenosine in oocyte competence for development were examined using adenosine and its synthetic inhibitors. Adenosine treatment from GV intact stage for 18 hr (fGV) caused of decrease the fertilization rate but of increase the cleavage rate compared from the other stage treatment groups. Hadacidin did not effect on fertilization rate but increased cleavage rate without stage specificity. Adenosine did not block the effects of hadacidin with the exception of fGV group. By the inhibition of purine synthetic pathways the fertilization rate was decreased in the fGV and fGVB groups but increased in the fMII group. Exogenous adenosine caused of decrease fertilization rate in the fGVB group but increase in the fMII group. Cleavage rate was dramatically increased in the adenosine treatment with synthetic inhibitors. It means that the metabolism of purine has stage specific effects on fertilization and cleavage. Exogenous adenosine had only can improve oocyte developmental competence when it treated at GV intact stage. On the other hand, endogenous synthesis in all maturation stage caused of increase the cleavage rate without effects on fertilization. These data suggest that adenosine at GV stage as a paracrine fashion and inhibitions of endogenous adenosine in all stage improve oocyte developmental competence..

**Key words :** Adenosine, *De novo* synthesis, Oocyte, Developmental competence

### INTRODUCTION

It is evaluated that the developmental competence of oocyte has been improved by modulation of cyclic adenosine monophosphate (cAMP) levels during *in vitro* maturation (Gilechrist, 2011; Ezoe et al., 2015). Addition of a cAMP modulator forskolin or 3-isobutyl-1-methyl-xanthine (IBMX) to the maturation media improves the developmental competence of vitrified-thawed GV oocytes as in natural GV oocytes (Ezoe et al., 2015). It based on

the LH or FSH works through cAMP mediated signaling pathways (Dekel & Kraicer, 1978; Sela-Abramovich et al., 2006; Conti et al., 2012) and maturation promoting factor (Ezoe et al., 2015).

In meiotic arrest, spontaneous maturation is observed after releasing from follicle (Edwards, 1962; Pincus & Enzmann, 1935). So, follicular environment is important for oocyte competence. From 1970s, it is suggested that maintenance of appropriate cAMP levels is critical factor for meiotic resumption regulation (Luciano et al., 2011;

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Richard 2007; Cho et al., 1974). cAMP levels are under the multiple factors such as G protein-coupled receptor 3 (GPR3), GRP8, cyclases, phosphodiesterases, and growth factors such as EGF-like peptides (Richard, 2007; Conti et al., 2005; Kawamura et al., 2004). Adenosine increases FSH-stimulated accumulation of cAMP in cumulus-oocyte-complex (COC) and markedly inhibits oocyte maturation in a dose-dependent manner (Miller & Behrman, 1986).

On the other hand, *de novo* synthesis of purines in somatic compartment of follicle and the timing and duration of such synthesis determines whether meiotic resumption will be suppressed or promoted in hormone mediated actions (Downs & Verhoeven, 2003; Downs, 2000). The purine synthesis by *de novo* pathway is important in maintenance of meiotic arrest under the hormone. Purine *de novo* synthesis is nearly doubled in OCC by FSH treatment, and this response was completely prevented by adenosine (Downs, 2000). FSH had no effect on hypoxanthine salvage, although adenosine reduced this activity by 98% (Downs, 2000). hCG-induced maturation is blocked by adenosine as effectively as by the purine *de novo* synthesis inhibitor, azaserine (Downs, 2000). Cheon et al. (1997) revealed that the azaserine, *de novo* synthetic inhibitor, at GV intact stage increases the nuclear maturation and polar body extrusion rates. On the other hand, it inhibited the polar body extrusion rate if it is treated from GVB stage. So it is suggested that purine metabolism may concern with the maturation process with other signaling mediators. Salvage synthesis of purine is so far confused in oocyte maturation, although it is used to recover bases and nucleosides and can be converted back into nucleotides. Downs suggested that salvage pathway may be not required for meiotic induction (1997a, b) but he could not conformed (Downs, 2000).

The possible roles of purine metabolism including adenosine on germinal vesicle breakdown is mediated the cAMP. cAMP levels also showed fluctuation during maturation (Webb et al., 2008; Hegele-Hartung et al., 1999). On the other

hand, it has been suggested a role as signaling mediator in oocyte maturation. However, it is largely un-known whether exogenous adenosine works as messenger during oocyte maturation or after maturation and its roles in oocyte developmental competence. In here, the possible roles of adenosine as messenger and its metabolism were evaluated with combination of purine synthesis metabolic inhibitors and adenosine.

## MATERIALS AND METHODS

### 1. Animals

All animals used in this study were CD-1 mice (6-8 weeks old) and were handled according to the National Institutes of Health Standards for the use and care of animals. The animal protocols were approved by Sunshin University Institutional Animal Care and Use Committee. Animals were maintained on a 14L:10D light dark cycle under standard conditions. Food and water were provided *ad libitum*. For IVF sperm was obtained from same strain male mice between 8 and 12 weeks old.

### 2. Oocyte collection and *in vitro* maturation (IVM)

Immature CD-1 mice (21-day-old) were injected with 5 IU of pregnant mare serum gonadotropin (Sigma) to stimulate the follicular development at 1:00 pm. Animals were sacrifice 46 hr later and oocytes were got through puncture the antral follicles with fine needle. The cumuli were removed physically using capillary pipet. Denuded germinal vesicle intact oocytes were pooled and washed three times in fresh medium and used. These processes were per-formed within 10 minutes. Oocytes were randomly allotted to treatment groups of about 100 per group. Groups were distinguished like followings: from GV intact group (fGV), from GVB group (fGVB), and from MII group (fMII). In fGV, chemicals were treated from GV intact stage and cultured for 18 hr. In GVB, chemicals were treated from GVB stage for 9 hr. In fMII, chemicals were treated

in MII oocytes for 2 hr (from 15 hr after maturation induction). After culture, the MII stage oocytes were washed in fresh medium at least 5 times. Groups of 10 washed denuded oocytes were matured in 10  $\mu$ L drops of equilibrated BWB medium containing 0.4% BAS covered with mineral oil at 37°C, 5% CO<sub>2</sub> in air. To evaluate the possible roles of adenosine and its synthetic inhibitors, 750  $\mu$ M adenosine (A), 20  $\mu$ M azacrine (Aza), 20  $\mu$ M hadacidine (Had), and 10  $\mu$ M mycophenolic acid (MA) were used.

### 3. IVF and embryo culture

The effect of adenosine and its synthetic inhibitors on oocyte developmental competence was assessed by examining the capacity of the oocyte to support preimplantation embryo development following *in vitro* maturation. All the media used were BWB medium containing 0.4% BSA. Data presented percentage of 10 replicate experiments. About 100 oocytes were used per treatment group. IVF was performed as described in the Manipulating the Mouse Embryo (Hogan et al., 1994). Fertilization was carried out for 4 hr and then the eggs were washed with medium to remove sperms. 10 zygotes were placed in 10  $\mu$ L drops of equilibrated BWB medium containing 0.4% BAS covered with mineral oil at 37°C, 5% CO<sub>2</sub> in air. Fertilization was assessed by the presence of second polar body and the development of 2-cell stage embryos the next day.

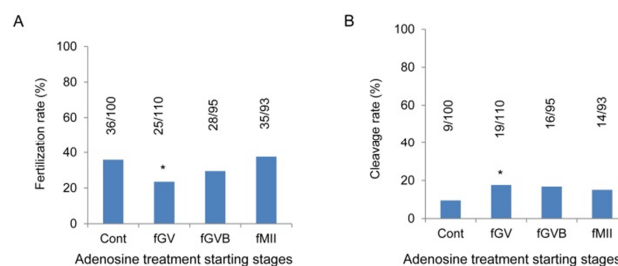
### 4. Statistical analysis

Data were represented as percentage. Data on fertilization and cleavage rate were analyzed using Chi-square test.  $P < 0.05$  was assumed to indicate statistical significance.

## RESULTS

### 1. Exogenous adenosine has no effect after GVB on

Most of the immature oocytes which were cultured with 750  $\mu$ M adenosine were failed to become MII stage oocytes

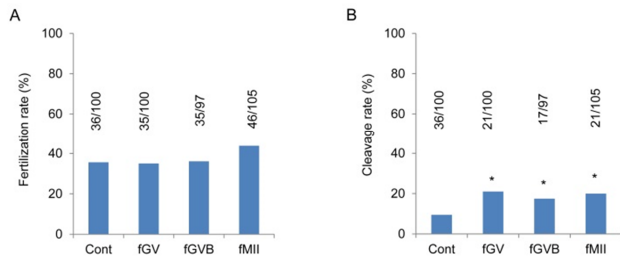


**Fig. 1. Effects of adenosine treated during maturation for 18, 9, or 2 hr on fertilization (A) and cleavage (B).** fGV is means that GV intact oocytes were cultured in the medium containing 750  $\mu$ M adenosine for 18 hr. fGVB is means that GVB oocytes were cultured in the medium containing 750  $\mu$ M adenosine for 9 hr. fMII is means that MII oocytes were treated 2 hr with 750  $\mu$ M adenosine. Cont, control; fGV, from germinal vesicle stage; fGVB, from germinal vesicle breakdown stage; fMII, from metaphase II stage. \*:  $P < 0.05$  control vs experimental groups ( $\chi^2$ -test)

but after GVB stage adenosine did not showed specific effects as mentioned in previous report (Cheon et al., 1997). In the fGV groups, adenosine was treated from GV intact stage for 18 hr to induce spontaneous maturation. In the MII stage oocytes getting at 18 hr after incubation (fGV) the fertilization was significantly low compared with other groups. However, the cleavage rate was significantly high compared with the other groups (Fig. 1A, B). Adenosine did not increase or decrease the fertilization rate or cleavage rate in the other groups (Fig. 1A, B). It showed that the effects of adenosine on oocyte competence are existing at GV stage.

### 2. Adenosine *de novo* inhibition enhancing the cleavage rate

To suppress the endogenous adenosine in oocyte, hadacidin was employed. Hadacidin can suppress synthesis of adenosine from inosine monophosphate (IMP). Hadacidin did not increase or decrease the fertilization rates by the treatment starting stages (Fig. 2A). However, the cleavage rate was



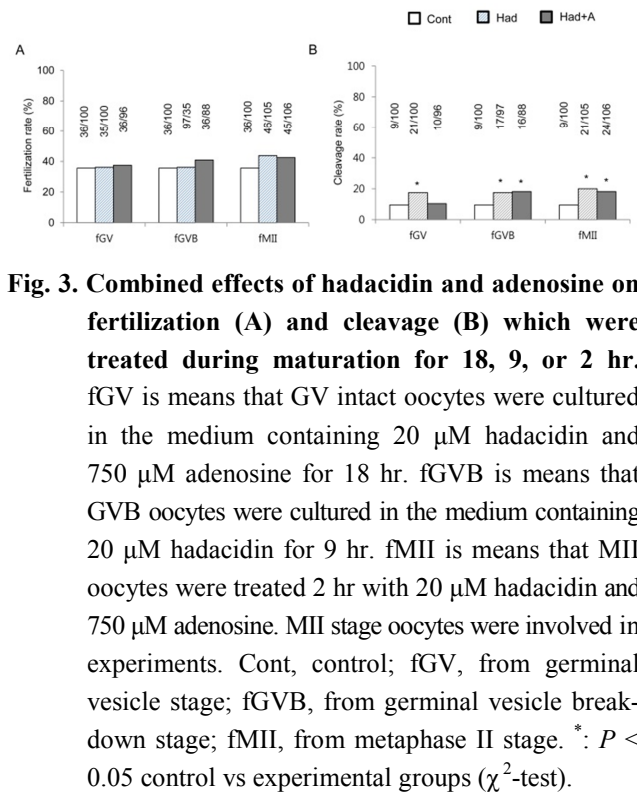
**Fig. 2. Effects of hadacidin on fertilization (A) and cleavage (B) which were treated during maturation for 18, 9, or 2 hr.** fGV is means that GV intact oocytes were cultured in the medium containing 20  $\mu$ M hadacidin for 18 hr. fGVB is means that GVB oocytes were cultured in the medium containing 20  $\mu$ M hadacidin for 9 hr. fMII is means that MII oocytes were treated 2 hr with 20  $\mu$ M hadacidin. MII stage oocytes were involved in experiments. Cont, control; fGV, from germinal vesicle stage; fGVB, from germinal vesicle breakdown stage; fMII, from metaphase II stage. \*:  $P < 0.05$  control vs experimental groups ( $\chi^2$ -test).

significantly higher in all three groups, fGV, fGVB and fMII compared with control (Fig. 2B). It clearly showed the inhibition of adenosine synthesis has positive effects on the improve oocyte quality.

### 3. Exogenous adenosine did not overcome with the concentration compensation manners of the hadacidin effects

To know whether exogenous adenosine can block the effects of hadacidin on the oocyte developmental competence, hadacidin and adenosine were co-treated as mentioned at Materials and Methods. Adenosine did not block the effects of hadacidin in fGVB and fMII groups. However, it blocked the effects of hadacidin at fGV. It means that adenosine does not work as quantity compensation manners at GV stage at least.

### 4. Purine synthesis had maturation stage specific effects on oocyte developmental competence



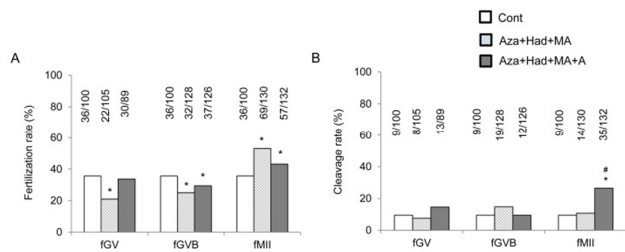
**Fig. 3. Combined effects of hadacidin and adenosine on fertilization (A) and cleavage (B) which were treated during maturation for 18, 9, or 2 hr.** fGV is means that GV intact oocytes were cultured in the medium containing 20  $\mu$ M hadacidin and 750  $\mu$ M adenosine for 18 hr. fGVB is means that GVB oocytes were cultured in the medium containing 20  $\mu$ M hadacidin for 9 hr. fMII is means that MII oocytes were treated 2 hr with 20  $\mu$ M hadacidin and 750  $\mu$ M adenosine. MII stage oocytes were involved in experiments. Cont, control; fGV, from germinal vesicle stage; fGVB, from germinal vesicle breakdown stage; fMII, from metaphase II stage. \*:  $P < 0.05$  control vs experimental groups ( $\chi^2$ -test).

To evaluate the possible role adenosine in the oocyte maturation, synthetic pathways were blocked with azacrine, hadacidin, and mycophenolic acid at one time with or without adenosine. Fertilization rates were decreased in fGV and fGVB groups by the blocking of synthesis but increased in fMII group. Adenosine had opposite effect for the synthesis blocking at fGVB and fMII groups (Fig. 4A). Fertilization rate were improved by the adenosine compared with control and synthesis blocking groups only in fMII group (Fig. 4B).

## DISCUSSION

Metabolism of purine nucleotides in oocyte or cumulus is suggested as one of the factors for meiotic arresting. Purine *de novo* or salvage syntheses are involved in regulation mechanism of oocyte maturation. Purine salvage synthesis supplies adenine or guanine from intermediates in the degradative pathway for nucleotides. Salvage pathways are

#### Stage Specific Effects of Adenosine on Oocyte Competence



**Fig. 4. Combined effects of azacerine, hadacidin, mycophenolic acid, and adenosine on fertilization (A) and cleavage (B) which were treated during maturation for 18, 9, or 2 hr.** fGV is means that GV intact oocytes were cultured for 18 hr. fGVB is means that GVB oocytes were cultured for 9 hr, fMII is means that MII oocytes were treated 2 hr with 20  $\mu$ M azacerine, 20  $\mu$ M hadacidin, mycophenolic acid, and 750  $\mu$ M adenosine. MII stage oocytes were involved in experiments. Cont, control; fGV, from germinal vesicle stage; fGVB, from germinal vesicle breakdown stage; fMII, from metaphase; Aza, azacerine; Had, hadacidin; MA, mycophenolic acid; A, adenosine. II stage. \*:  $P < 0.05$  control vs experimental groups ( $\chi^2$ -test).

used to recover bases and nucleosides. *De novo* synthesis begins with simple molecules such as amino acids and bicarbonate (Berg et al., 2002). Salvage synthesis is much less costly than the *de novo* synthesis. Adenylosuccinate synthetase catalyzes the first committed step in the biosynthesis of AMP from IMP. Adenine phosphoribosyl transferase catalyzes the formation of adenylylate, whereas hypoxanthine-guanine phosphoribosyl transferase catalyzes the formation of inosinate and guanylate. It has been evaluated that these purine metabolism is important in meiotic arrest.

Previous data showed that adenosine treatment from GV intact stage significantly disturbed the maturation processes after GVB (Cheon et al., 1997). Interestingly, however, in the MII oocytes which were matured in the media containing adenosine, the cleavage rate was significantly higher than the other groups. Exogenous adenosine did not inhibit or accelerate of fertilization rate and cleavage rate

of MII stage oocytes which were got from fGV and fMII eggs. It can be supported by the previously evaluated results; meiotic suppression by cAMP-upregulation agents improves subsequent oocyte developmental competence (Luciano et al., 2011; Nogueira et al., 2003a, b). It means that adenosine work mediator of meiotic arrest as well as oocyte competence.

It has been suggested that adenosine blocks meiotic induction via its pronounced inhibition of the purine and pentose phosphate metabolic pathways (Downs, 2000). On the other hand, it also has been suggested that salvage synthesis of purine is not required for meiotic induction (Downs, 1997a, b). Interestingly, present study showed that the blocking of endogenous adenosine synthesis causes of increase of the cleavage rate. Besides, co-treatment of hadacidin and adenosine did not support quantitative compensation. Those suggest that exogenous adenosine work as messenger not as metabolic substance. It also can be supported by Fig. 4.

Previously we suggested that adenosine receptors in oocyte may regulate the maturation (Hwang & Cheon, 2013). It is supported by the present study. In GV intact oocytes adenosine improved fertilization rates without inhibit the effects of hadacidin. It suggests that the adenosine mediate signaling is involved in cytoplasmic maturation and improve developmental competence.

On summary, adenosine improved the cleavage rate when it treated from GV intact stage but not form GVB or MII. By the blocking the endogenous adenosine synthesis, the fertilization rates were increased in all groups, fGV, fGVB, and fMII. Besides, adenosine did not compensation the hadacidine in fGVG and fMII group except in fGV group. Those suggest that adenosine work with paracrine fashion during GV intact stage for oocyte development competence. Blocking *de novo* synthesis had the treatment stage specific effects on fertilization rate and adenosine only improved the cleavage rate. Put together those results suggest that

adenosine works with paracrine fashion and adenosine metabolism has stage specific effects on oocyte developmental competence, although further studies in the molecular level are needed.

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