# Guanosine Regulates Germinal Vesicle Breakdown (GVBD) in Mouse Oocytes

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## ABSTRACT

**Maturation of oocytes is maintained by complex procedures along with follicular genesis and is a critical step for embryonic development. Purine known as an oocyte maturation regulator is present in follicular fluid. In this study, the roles of guanosine as a strong inhibitor of GVBD and a modulator of cyclic GMP concentration in ooyctes were revealed. Denuded immature oocytes were treated with guanosine, and the maturation rates and cGMP concentration of oocytes were measured. GVBD was blocked in a concentration dependent manner by guanosine, but this effect was reversible. However, GVBD was lagged yet not significant by adenosine. Both guanosine and adenosine modified cGMP concentration in oocytes. The characteristic of the guanosine-treated oocyte was significantly higher cGMP compared with the adenosine-treated oocyes at initial time of the maturation. Based these results, guanosine may be a strong and reversible GVBD inhibitor. Although the precise mechanism of guanosine presently is unclear, the results suggest that guanosine may lead the accumulation of cGMP in oocyte cytoplasm, which in turn suppresses GVBD.**

**(Key words: Oocyte maturation, cGMP, Guanosine)**

## INTRODUCTION

Oocyte maturation is an important process involving regulation of nuclear maturation, as well as associated cytoplasmic changes required to achieve developmental potency. Fully matured oocytes are arrested at the G2 phase prior to ovulation, but the mechanism responsible for meiotic arrest is complex and poorly understood. At the completion of follicular development, the oocytes are stimulated to resume meiotic maturation by gonadotropin, especially LH. If LH is not surge at the proper time, the grown follicles and the oocytes within those follicles are degenerated. However, spontaneous resumption of oocyte maturation occurs after removal of the oocyte from the follicle (Edwards, 1965).

Cumulus cells surrounding the oocyte have a critical role in oocyte maturation. It has been suggested that meiotic resumption requires high cAMP levels in granulosa cells and low or decreasing levels in the oocyte, and that such opposing levels of cAMP may result from the selective expression and regulation of these phosphodiesterases in the two compartments of the cumulus-oocyte complex (Downs and Mastropolo, 1997; Tsafriri et al., 1996). Concentration of a membrane-permeable analog of cAMP, dbcAMP, that was below the dose required for complete meiotic inhibition had a greater inhibitory effect on the cumulus-oocyte complex (COC) than on the denuded oocytes (Eppig et al., 1983). cAMP concentration or MPF activity showed cell cycle dependent modification (Howlett, 1986).

The purines, which are compounds of follicular fluid, are considered to be important inhibitory substances of oocyte maturation (Downs, 1997; Downs and Mastropolo, 1997; Eppig et al., 1985). Adenosine and guanosine are precursors of cAMP, ATP, cGMP, and GTP (Kai, 1987; Tornell et al., 1990b). These metabolites is well known regulator of the cell activity, nuclear division, cytoplasmic division, and gene expression (Nureddin et al., 1990; Yamashita and Maller, 1990; Poueymirou and Schultz, 1987). Perturbants of purine metabolism can induce the resumption of maturation in meiotically arrested oocytes in hormone-independent fashion *In vivo* (Downs and Eppig, 1987) and *in vitro* (Downs, 1993).

In the mechanism controlling meiotic induction, cumulus cells are vital participants in the ligand-stimulated process. However, denuding the oocyte of investing cumulus cells inhibits it from duplicating the meiotic

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induction brought about by ligand treatment. Mentioned by Downs and coworkers (1988) it is suggested that the processes that regulate oocyte maturation are different in the denuded immature oocyte from those in the cumulus-enclosed oocyte. Although the studies about the role of purine during maturation have been did in oocyte-cumulus complex, it is little known that the role and mechanisms in the ooycte. Therefore, we studied the effects of purine, adenosine and guanosine on the GVBD, and on signal mediators like cGMP during *in vitro* maturation in the ooyctes using denuded-immature oocytes.

## MATERIALS AND METHODS

### Animals and Oocyte Collection

ICR mice were maintained on a 14-h light and 10-h dark cycle under standard vivarium conditions, and were supplied with food and water ad libitum. Virgin female mice 3 weeks old were injected with five units of gonadotropin from pregnant Mares serum (eCG, PMSG, Sigma) to enhance multiple follicular developments. At 46 h post eCG injection the females were sacrificed by cervical dislocation. Their ovaries were removed and transferred to Biggers, Whitten and Whittingham media (BWW; 94.6 mM NaCl, 4.78 mM KCl, 1.19 mM $KH_2PO_4$, 1.19 mM $MgSO_4 \cdot 7H_2O$, 1.71 mM calcium lactate, 21.58 mM sodium lactate, 0.3 mM sodium pyruvate, 25.07 mM $NaHCO_3$, 100 units/ml penicillin, 100 $\mu$g/ml streptomycin, pH 7.4, and 0.4% BSA). Oocytes were collected by ovarian follicular puncture with a needle under a dissecting microscope and cumulus masses were then removed by pipetting with a fine-bore pipette in the BWW medium. The entire procedure took place within 20 min and used for experiments. Only healthy immature oocytes were chosen for examination. These experiments were repeated at least 10 times and the minimum number of oocytes was 9 per treatment group.

### Culture of Oocytes and Chemical Treatments

Oocytes (8 - 10 oocytes / drop) were cultured in 10 $\mu$l drops of BWW medium in mineral oil (Sigma) for 18 h at 37℃ in a humidified atmosphere containing 5% $CO_2$. To study the concentration effects of adenosine and guanosine on oocyte maturation based on the report of Epigg et al (1985), immature oocytes were cultured in BWW media containing 50 and 750 $\mu$M of adenosine or guanosine respectively. To study a preparation time effects on GVBD, 750 $\mu$M guanosine treated within 20 min or after 40 min post follicle puncture. In order to measure the cGMP concentration, oocytes were treated with 750 $\mu$M adenosine or guanosine, and collected on the following time schedule: 0, 1.5, 3, and 6 h. The maturation stages were scored using a differential interference contrast microscope (Carl Zeiss, Germany).

### Measurement of cGMP Concentration

Oocytes were collected at 0, 1.5, 3, and 6 h to study the change of cGMP concentration during maturation. The collected oocytes were washed with PBS, dropped into liquid nitrogen, and stored frozen. Before the assay, 1 volume of tissue sample, still frozen, was mixed with 10 volumes of perchloric acid 1.07 N. Oocyte extracts were generated by multiple freeze-thaw cycles, and used in a cGMP radio immunoassay (Immunotech, Cat No; 1118), which is based on the competition between the succinylated cGMP of the sample and an $^{125}$I-labeled tracer for binding to polyclonal antibody coated onto tubes (anti-cGMP antibody). Assays were performed in triplicate following the supplier's instruction.

### Statistical Analysis

In the maturation assay, each experiment was performed a minimum of 10 times. Data from 10 replicates were pooled and analyzed by One-Way or Two-Way ANOVA. The results were considered to be statistically significant at $P<0.05$. In the study of cGMP concentration, each experiment was performed a minimum of 3 times. Data were analyzed by t-test or ANOVA and considered to be statistically significant at $P<0.05$.

## RESULTS

### Effects of Guanosine on the Denuded Oocyte Maturation

GVBD rate is very various by the culture conditions. In this studies, 77.6% of the oocytes showed GVBD after 1.5 h in control group. Most of oocytes (91.7%) were GVBD stage after 3 h and 98% after 6 h. In the 50 $\mu$M guanosine-treated group, GV intact oocytes significantly outnumbered the control at 1.5 h (59% vs. 22.7%) and 3 h (50% vs. 8.3%) after *in vitro* culture (Fig. 1A). In the 750 $\mu$M guanosine-treated group, maturation was inhibited completely. GVBD rates were 0% at 1.5h, 99% at 3h, and 98% at 6 h. These results showed that GVBD inhibition is dependent on the concentration of guanosine. Compared with the 50 $\mu$M guanosine, GVBD suppression is more pronounced in 750 $\mu$M at all times analyzed (Fig. 1A).

To determine the reversibility of the suppresion effects of guanosine, guanosine-treated immature oocytes were washed 6 times after 6 hr treatment with plain medium and transferred to the plain media. These washed oocytes were GVBD after transfer to the plain media as in control oocytes (Fig. 1B).

Usually maturation inhibitors are used to get immature oocytes *in vitro*. However in this study, to escape

the possible effects of inhibitors on maturation, those did not used. Therefore, to determine the preparation time effects, denuded oocytes were treated within 20 min or after 40 min. As depicted in Fig 1A and B, in the groups treated within 20 min, guanosine suppressed GVBD. However in the oocytes that were treated after 40 min the sppression of GVBD was not detectable (Fig. 2). From these results, it was conformed the reproducibility the procedure used in this study.

### Effects of Adenosine on the Denuded Oocyte Maturation

As mentioned above, most of the immature oocytes develop to GVBD stage, after removal of the oocyte from the follicle even if the cumulus masses were removed as previously reported. In our culture system, 77.6% of immature oocytes are GVBD after 1.5 h. Most of the oocytes (98%) were GVBD after 6 h of *in vitro* culture.

When oocytes were chronically treated with 50 $\mu$M adenosine, the GV stage oocytes outnumbered the control group at 1.5 h (29% vs 22.4%) and 3 h (21% vs. 8%), but it was not significant. However, after 6 h most of the immature oocytes (99%) had GVBD similar to the control (Fig. 3). In 750 $\mu$M adenosine-treated oocytes, the rate of arrested-oocyte at GV stage for each of the time points was not different from the control or 50 $\mu$M adenosine-treated group (Fig. 3). These results mean that adenosine only lagged the GVBD but did not inhibit GVBD.

One-way or two-way ANOVA was performed between time variation, adenosine, guanosine, and repetitions. There was no statistical significance by repetition and between control group and adenosine groups. However there was highly significance ($P<0.0001$) between adenosine groups and guanosine groups, as well as between control groups and guanosine groups (not

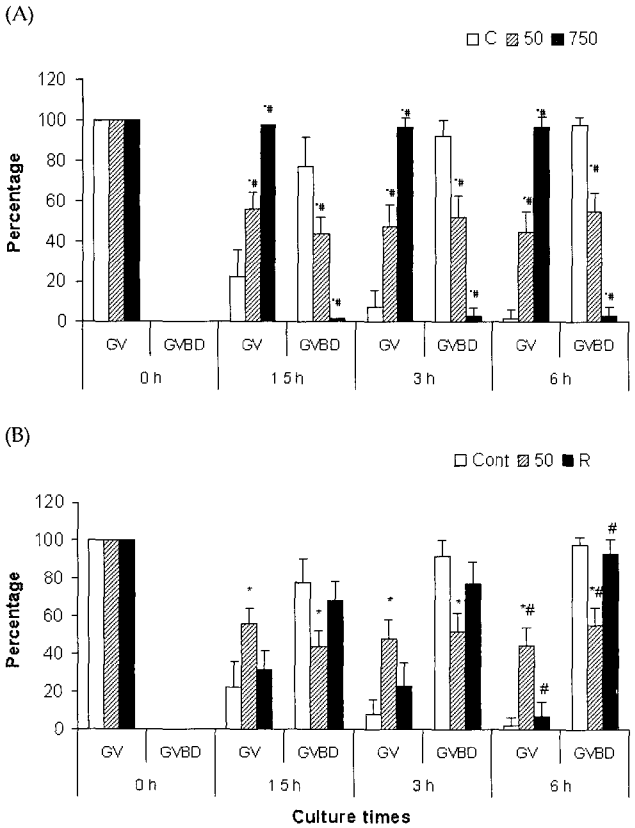


**Fig. 1. Effects of guanosine concentration on the maturation of the denuded oocyte.** (A). Effects of guanosine concentration on maturation. Maturational stages were determined following 1.5 h, 3 h, and 6 h culture in medium containing 50μ M or 70μ M guanosine. (B). Reversing the inhibitor effects of guanosine. Guanosine-treated oocytes were transferred to the untreated media and cultured. Data are given as mean± SEM of ten to eleven replications and represented with percentage. *, $P<0.05$ compared with control; #, $P<0.05$ compared with 50μ M and 750μ M. GV, germinal vesicle; GVBD, germinal vesicle breakdown.

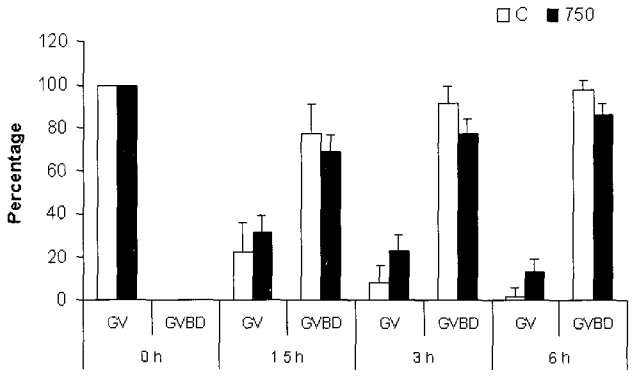


**Fig. 2. Effects of preparation times on maturation of the denuded oocytes.** Effects of preparation time on guanosine treated denuded oocytess. The bars indicate $\mu$M of guanosine. Data are given as mean± SEM of ten to eleven replications and represented with percentage. GV, germinal vesicle; GVBD, germinal vesicle breakdown.

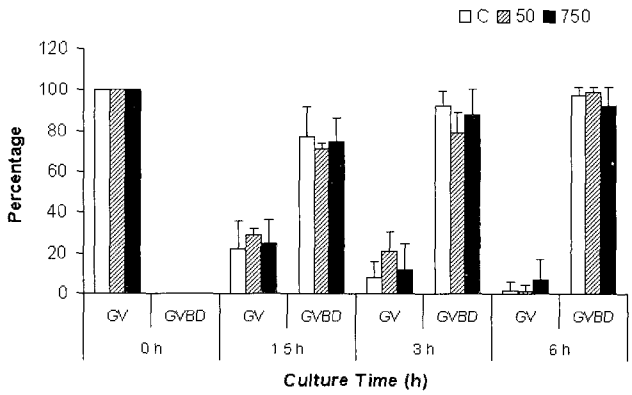


**Fig. 3. Effects of adenosine on the maturation of the denuded mouse oocytes.** Adenosine was added in 50μ M or 750μ M to culture medium and cultured 6 h. Maturation stages were observed at 1.5 h, 3 h, and 6 h during culture. Data are presented as the mean± SEM of ten to eleven repeats. Within each concentration, times with * or # are significantly different. *, $P<0.05$ compared with control; #, $P<0.05$ compared with 50μ M and 750μ M. GV, germinal vesicle; GVBD, germinal vesicle breakdown.

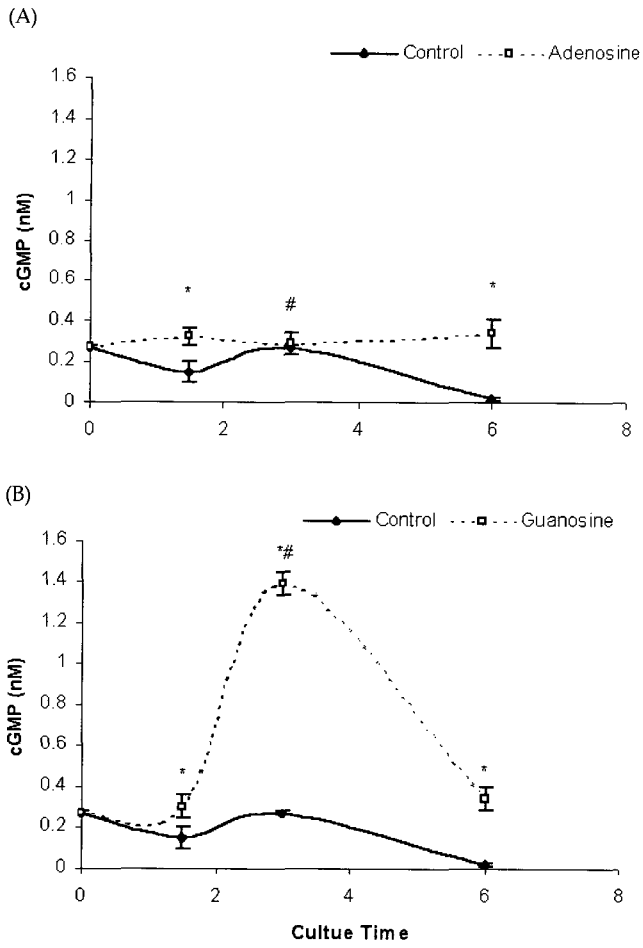


**Fig. 4. Change of the cGMP concentration during *in vitro* maturation.** (A). cGMP concentration in adenosine treated oocytes during maturation. (B). cGMP concentration in guanosine treated oocytes during maturation. Oocytes were collected during culture by the maturation stage and measured the cGMP concentration as mentioned at Materials and Methods. Data are presented as the mean SEM and RIA did three or four times repeats. Within each times with * or # are significantly different. *, $P<0.05$ compared with control; #, $P<0.05$ compared with adenosine and guanosine.

shown, compare between Fig. 1A and Fig. 3). Taken together, these results showed that adenosine has no inhibitory effects on GVBD and there were no concentration effects on the maturation in denuded oocytes. However guanosine has inhibitory effects on GVBD and there were concentration effects on the maturation.

### Change of [cGMP] during Oocyte Maturation

For the study of the cGMP modulation by the purine, we measured its concentration during *in vitro* maturation. As depicted in Fig. 4, cGMP concentration showed fluctuation during maturation. At 1.5 h time point, cGMP concentration decreased about 2 times, but it increased to the 0 h level at 3 h post incubation. Thereafter it decreased about 18 times at 6 h post incubation compared with that of 0 hr.

In adenosine-treated oocytes, cGMP concentration slightly increased at 1.5 h but it was significantly higher compared to that of the control. After 1.5 h, cGMP concentration was not much changed (Fig. 4A). Interestingly, in guanosine treated oocytes, cGMP concentration increased continuously until 3 h. It was 5 times higher than that of 0 hr. After then, the concentration level decreased and maintained similar levels to adenosine -treated oocytes (Fig. 4B).

For the further study, we did statistical analysis with one-way or two-way ANOVA. Significance between adenosine and guasnoine is $P<0.0001$ at 3 h (Fig 4A vs B). From the results we know that cGMP concentration can fluctuate during maturation dependently to the maturation stage. Furthermore, cGMP concentration can be modulated by both purines, particularly guanosine was a potent stimulator in denuded oocytes.

## DISCUSSION

The arrest of meiotic maturation is essential to achieve not only the potency required for oocyte growth, but also the genetic substances and organelles needed for proper maturation. It was proposed that the inhibition of oocyte maturation is mediated by a factor of granulosa/cumulus cells origin, which does however require cAMP for its activity and/or generation, as well as an intact intercellular coupling pathway between cumulus and the oocyte (mouse, Eppig et al., 1983; bovine, Richard et al., 1997). However, during meiotic maturation, opposing fluctuations of cAMP levels were observed between the somatic granulosa cells and germ cells. The oocyte expresses cGMP-inhibited type 3 phosphodiesterase (CGI -PDE), while cAMP-specific type 4 PDE is mainly expressed in granulosa cells (Tsafriri et al., 1996). These mean that maturation of ooyctes regulated by with microenvironment and its own regulation system.

It was suggested that purine nuclotide-generating pathways are vital participants in the mechanisms regulating hormone-induced meiotic maturation, and that either the de novo or salvage pathway can fulfill this nucleotide requirement (Downs 1997; Downs and Mastropolo, 1997). What happen when the denuded immature oocytes are exposed to exogenous purines? Previously, it is suggested that adenosine is metabolized within oocyte cytoplasm or binds to the adenosine receptors (Billig et al., 1989; Dascal et al., 1985; Downs, 1999; Kobayashi et al., 2002; Lotan et al., 1985; Salustri et al., 1988). Labbe et al. (1988) suggested that immature oocytes contain an inactive kinase precursor (prokinase) that is synthesized at each of the subsequent cell cycles. So for it is not clear whether the guanosine receptor

present or not in the ooycte membrane, interestingly, from this study, it is revealed that guanosine can modulate the cGMP concentration in the oocytes cytoplasm, also.

There has been strong interest in elucidating the cellular signals that modulated the maturation induction or inhibition. Some of the signal systems examined in previous study includes maturation regulation factors and a cascade of kinases Mos, MEK and MAP (Abrieu et al., 2001; Choi et al., 1991; Doree et al., 1983; Ferrel 1999; Verlhac et al., 1996). Also, it has been reported recently that the reactive oxygen species and Rho A (Cheon et al., 2000) and the AMP-activated protein kinase (Downs et al., 2002) have a role in oocyte maturation of mouse.

It has been demonstrated that cGMP can delay spontaneous maturation in rat oocytes (Tornell, 1990a, b), and that the cAMP : cGMP ratio increases before oocyte maturation (Hubbard and Terranova, 1982). cGMP may inhibit a step leading to GVBD that is common to both spontaneous and FF-MAD-induced maturation (Faerge, 2001). In addition, during cGMP mediated ANP inhibition of GVBD, the inhibition was persistent over the time period studied (Tornell, 1990b). Type 3 phosphodiesterase inhibitors inhibit the meiotic resumption of the bovine oocyte (Mayes and Sirard, 2002). The role of cGMP was more cleared with this study; cGMP concentration decreased almost 2 times at the time of GVBD (until 1.5 h) but it increased at 3 h to almost the initial level in control group. In adenosine-treated oocytes, [cGMP] increased until 1.5 h, decreased until 3 h, and increased again until 6 h. These patterns coincide with the GVBD rate in adenosine treated group. Interestingly, guanosine stimulated increase of cGMP concentration about 5 times within 3 h. At those time periods, most of the oocytes were GV intact. From these results, cGMP concentration in the oocyte cytoplasm is a key facor in the inhibition mechanism of GVBD.

Most interesting finding is that inhibitory effects of the guanosin were reversible; once the guanosine-treated oocytes were cultured in the guanosine free medium, maturation occurred just as in the control. Put together, it is suggested that guanosine suppress the GVBD, it is mediated with cGMP.

The generation of cAMP and cGMP leads to a complex interplay of cyclases and cyclic nucleotide-dependent kinases and phosphodiesterases that alter the levels of cAMP and cGMP and their relative affinities for PKA and PKG. cGMP enhances that the affinity of cAMP for cGMP. Its ability to cross-activate this kinase is greatly enhanced, endowing PKG with the major role in cellular responses (Francis and Corbin, 1999). However, the inability of cGMP-dependent protein kinases to translocate to the nucleus is responsible for the differing abilities of cAMP-dependent protein kinases and cGMP-dependent protein kinases to activate CREdependent gene transcription, and nuclear redistribution of cGMP-dependent protein kinases is not required for NO/cGMP regulation of gene transcription (Collins and Uhler, 1999, Gudi et al., 1996). Recently, NO has been suggested as a key regulator of the signal transduction cascade that controls the progression from M I to M II or GVBD (Cheon et al., 2000; Jablonka-Shariff and Olson, 1998). Put together, that the inhibitory effects of guanosine may result in increase cGMP concentration and cross talk with other signal molecules.

In summary, these results provide evidence these purines can modulate the cyclic nucleotide concentration in immature oocytes without cumulus. Adenosine has not much effect on GVBD suppression but guanosine avidly suppressed GVBD. However guanosines effect is reversible and dependent on concentration. Guanosine strongly stimulated the cGMP accumulation within 3 h post culture. Based on these results, it is proposed that guanosine is a suppressor of GVBD and its effects mediated by cytoplasmic cGMP. Further studies will be required to confirm and delineate the pathways and metabolites involved. By no means do these results discount the possibility of additional external effects of guanosine on the oocyte regulated by gene expression or other physiological event.

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