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Oocyte Meiotic Resumption under High Surveillance

François J. Richard

Abstract

Germinal vesicle breakdown (GVBD) is the hallmark of oocyte meiotic resumption. It occurs under minimal stimulation during in vitro maturation (IVM). Several factors have been described to be involved in the inhibition of oocyte meiotic resumption such as purine derivatives. This study was assessing whether adenosinergic and guanosinergic systems are functional and participating in the inhibition of oocyte maturation. The objectives of the present study were to evaluate the effect of two purines, adenosine (ADO) and guanosine (GUO), on in vitro oocyte meiotic resumption, cumulus cell expansion, and gap junction communication. Both ADO and GUO significantly inhibited GVBD oocytes. The inhibitory effect lasted 24 hours and was reversible for meiotic resumption and cumulus cell expansion. Both ADO and GUO increased gap junction communication in cumulus cells. Equine chorionic gonadotropin (eCG) and the adenylyl cyclase stimulator, forskolin (FK), were both supportive of ADO and GUO inhibitory effect. The results are suggesting both adenosinergic and guanosinergic systems efficient in inhibiting oocyte meiotic resumption. The use of these two systems as part of a pre-IVM culture period would be a novel strategy to explore in order to improve oocyte developmental competence.

Keywords: oocyte, purine, adenosine, guanosine, meiotic resumption

1. Introduction

Oocyte meiosis begins during fetal development in large animals such as in cow, sow, and ewe. Once the sub-phases of the first prophase are completed, the oocyte meiosis stops at the dictyate stage. At this point, crossing over is a past event and chromatin is accessible for transcription. This G2/M phase transition of the cell cycle characterizes mammalian oocytes. The female gamete bears 4n chromosomes as long as the ovulatory LH peak generates its effect on the preovulatory follicle to induce oocyte meiotic resumption. Dysregulation of the oocyte cell cycle induced by c-MOS proto-oncogene after gene null mutation caused parthenogenic development of the oocyte and explained female mouse infertility [1]. This phenotype illustrates how important it is to appropriately control oocyte meiotic resumption.

Oocyte meiotic resumption is a highly important physiological event for species survival since it refers to a successful reproduction by appropriately preparing the female gamete. This unique cell division has to occur at the right time and imply high surveillance. From an evolutionary point of view, the number of female gametes produced went from a large number, such as in frog, to a small number in mammals. Although several thousands of oocytes are found in the ovaries, only a

small percentage is ovulated, and even less are fertilized. Considering this selective restriction, a framework of the meiotic resumption process has developed. Throughout the evolutionary process, mechanisms have been added to ensure a very precise control of this crucial event related to species survival, which is the final phase of gamete preparation for fertilization. Interestingly, the role of EGF-like peptides fully fits with this notion. It is well-known that the LH peak induces oocyte meiotic resumption in the preovulatory follicles. However, the EGF-like peptides are also active participants in the ovulatory process, meiotic resumption, and cumulus cell expansion, clearly supporting an add-up to the LH surge. Going back to the control of oocyte meiotic resumption, although the contribution of cAMP and cGMP is well described, it is obvious that other mechanisms may still be involved and be discovered.

It has long been known that adenosine (ADO) is a molecule playing an important role in various physiological systems such as in the central nervous system and cardiac function [2]. On the other hand, there are very few studies on the role of ADO in mammalian ovarian follicle. ADO is known to act on specific receptors, to cross plasma membrane using transporters, and to be generated from functional catabolism by extracellular enzymes, a system called adenosinergic [3]. On the other hand, guanosine (GUO) has not been as popular in research. However, in recent years a new interest on GUO has revealed its importance in the effect of ADO on the functioning of the central nervous system [4]. Although no specific receptor for GUO has been yet identified, its physiological impact leaves no doubt. GUO has neuroprotective effects, it diminishes the apoptotic effects observed in Parkinson's disease, and it also has a protective role during a challenge with glutamate, during mitochondrial stress, and during ischemia [4–6]. Because GUO can also be generated by a functional catabolism using extracellular enzymes, these results support the existence of a so-called guanosinergic system.

ADO has also been identified in the follicular fluid with several other purine derivatives. Among these derivatives, hypoxanthine is the compound that has attracted the most studies in last three decades. In mice, ADO improves the inhibitory effect of hypoxanthine on the resumption of meiosis but has no effect when used alone, even at a dose of 5 mM [7]. In the rat, the effect of ADO is also minimal [8]. In cattle, ADO used at 200 μM slowed meiotic resumption [9]. There is one study reporting that it has not been able to measure GUO in follicular fluid [10]. In contrast, GUO showed a very potent effect on the inhibition of meiotic resumption in mice [10] and rat [8].

This study is proposing to assess whether an adenosinergic and guanosinergic system are functional and participating in the inhibition of oocyte meiotic resumption. Specifically, the research presented here aims to study the involvement of ADO and GUO in the physiology of the ovarian follicle by targeting their effect on in vitro meiotic resumption using swine as the animal model.

2. Material and methods

2.1 Chemicals

Unless otherwise stated, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The adenylyl cyclase activator, forskolin (FK), was prepared as a millimolar stock solution and stored at −20°C as already described [11]. ADO and GUO were prepared from the stock powder directly in the culture medium on the day of the experiment. 8-Bromoadenosine (8-BrADO) and 8-Bromoguanosine (8-BrGUO) were dissolved in DMSO, and a DMSO control was run simultaneously.

The chemicals were added to the maturation medium a few hours prior to the addition of the oocytes.

2.2 Ovary collections

As previously described, prepubertal gilt ovaries were collected from a local slaughterhouse [12]. In brief, they were placed in saline (0.9% NaCl containing antibiotics and antimycotics, 100,000 IU/L penicillin G, 100 mg/L streptomycin, 250 μg/L amphotericin B) and kept at 37°C. They were rinsed once in a fresh saline solution having antibiotics and antimycotics at 37°C.

2.3 Maturation medium

Oocytes were matured in BSA-free North Carolina State University 23 (NCSU) medium [13] supplemented with 25 μM β-mercaptoethanol (Bio-Rad, Hercules, CA, USA), 0.1 mg/mL cysteine, 10% (v/v) porcine follicular fluid (PFF), and gonadotropins (2.5 IU/well for hCG [APL, Ayerst Laboratories Inc., Philadelphia, PA, USA] and 2.5 IU/well for eCG [Folligon, Intervet, Whitby, ON, Canada]) [12]. PFF was collected from follicles of 2–6 mm in diameter. After centrifugation (1500×g, 30 minutes), the supernatant was filtered (0.8 and 0.45 μm) and stored at −20°C until used [11].

2.4 Recovery of cumulus-oocyte complexes (COC)

Cumulus-oocyte complexes were collected from follicles of 2–6 mm in diameter. They were aspirated with a 10-mL syringe and an 18G needle [11]. The follicular contents were pooled in 50-mL conical tubes (Falcon, Franklin Lakes, NJ, USA). The pellet was washed twice with HEPES-buffered Tyrode's medium containing 0.01% (w/v) polyvinyl alcohol (PVA-TLH) [14]. The COC were recovered under a stereomicroscope and transferred to a petri dish containing PVA-TLH. The COC were washed three times with PVA-TLH and then subjected to their respective treatments. Groups of 20–30 COC were placed in the wells of four-well multi-dishes (Nunc, Roskilde, Denmark) containing 500 μL of maturation medium. The COC were cultured at 38.5°C, 5% CO_2 in 95% air atmosphere with 100% humidity.

2.5 Selecting COC and denuding oocytes

The criteria of selection were COC with a minimum of three layers of clear and compact cumulus cells which surrounded the oocyte [12]. Those with dark, pyknotic, or expanded cumulus cells, and those containing oocytes with a very clear cytoplasm or of small diameter were rejected. The oocytes were denuded of their cumulus cells by drawing several times the COC into a pipette using PVA-TLH. Once denuded, the oocytes were rinsed in PVA-TLH, and those with a homogeneous cytoplasm were selected.

2.6 Assessment of oocyte nuclear maturation stage

The oocyte nuclear maturation stage was evaluated following a 48-hour fixation period in a solution of ethanol and acetic acid (3:1). Using a phase contrast microscope at 100 and 400× magnification immediately after staining with 1% aceto-orcein [15] allows us to assess oocyte nuclear maturation stage. Those having a nuclear membrane were considered at the germinal vesicle (GV) stage, whereas

those without a nuclear membrane were considered to have resumed meiosis. The oocytes were considered mature when they were in anaphase I, telophase I, and metaphase II.

2.7 Cumulus-cumulus gap-FRAP assay to measure gap junction communications (GJC)

After 4 hours of in vitro culture, COC were loaded with calcein-AM (39,69-di(O-acetyl)-29,79-bis(N,N-bis(carboxymethyl) amino methyl)-fluorescein) and tetra(acetoxymethyl ester) (Molecular Probes C-3100) in IVM medium containing 0.1 mg/mL PVA [16]. After 20 minutes at 38.5°C, the live COC were mounted on glass slides in the PVA-containing IVM medium. Fluorescence recovery after photobleaching (FRAP) assays were conducted using Nikon Eclipse TE2000-E inverted confocal microscope. The bleaching was performed for 5 minutes using laser pulses on a limited region of cumulus cells observed at a magnification of 90×. The COC were photographed at 60× before bleaching and every 3 minutes thereafter for 12 minutes. Fluorescence intensity was quantified using ImageJ software (National Institutes of Health, USA). A relative fluorescence value was achieved by dividing the raw fluorescence measurement in the bleached area by the mean fluorescence in two adjacent regions. This value was further divided by the fluorescence value of a region at the opposite end of the COC to correct for unintended bleaching caused by the laser excitation.

2.8 Statistical analyses

All values are presented with their corresponding SEM, and the number of replicates is indicated for each experiment (at least three). The data were analyzed by one-way ANOVA using GraphPad Prism v7.02 for Windows (GraphPad Software, San Diego, CA, USA). When ANOVA indicated a significant effect of treatment ($P < 0.05$), individual treatment differences were compared using Bonferroni's multiple comparison post-hoc test. Significant effects were identified by * or different letters.
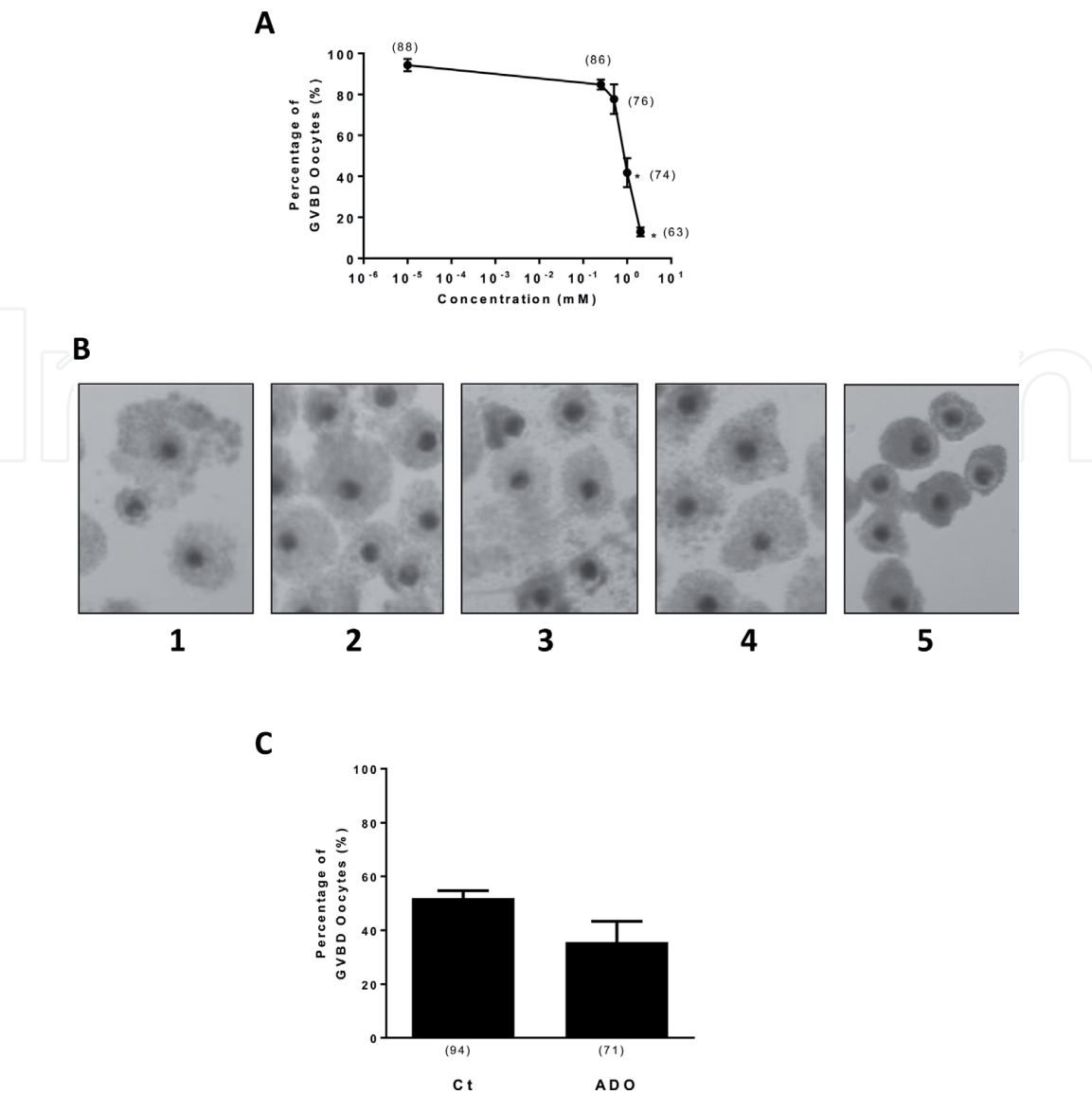
3. Results

In our hands, prior to in vitro maturation, more than 95% of swine oocytes have not started their nuclear maturation, that is, their meiotic resumption (<5% of GVDB oocytes), and cumulus cells have not expanded yet [12].

3.1 Adenosine inhibits oocyte meiotic resumption

The first experiment is aiming to assess ADO on inhibition of oocyte GVBD. When COC are cultured for 24 hours in control treatment, more than 94.3 ± 3.0% of the oocytes were in GVBD, that is, oocyte meiotic resumption took place (**Figure 1A**). The inhibitory effect of ADO was dose-dependent with only 13.9 ± 2.1% in GVBD after an exposure to ADO at 2.0 mM for 24 hours (**Figure 1A**). The EC$_{50}$ of ADO on the percentage of GVBD oocyte is calculated as already described [11] and determined to be 1.3 mM. In addition, at 2.0 mM ADO inhibited cumulus cell expansion (**Figure 1B5**) compared with the control (**Figure 1B1**). When cultured for 24 hours as oocytes denuded of their cumulus cells (DO), ADO (2.0 mM) did not significantly inhibit oocyte GVBD compared with the control conditions (**Figure 1C**). These results support an inhibitory effect of ADO on both oocyte meiotic resumption and cumulus expansion when cultured as COC but not as DO.
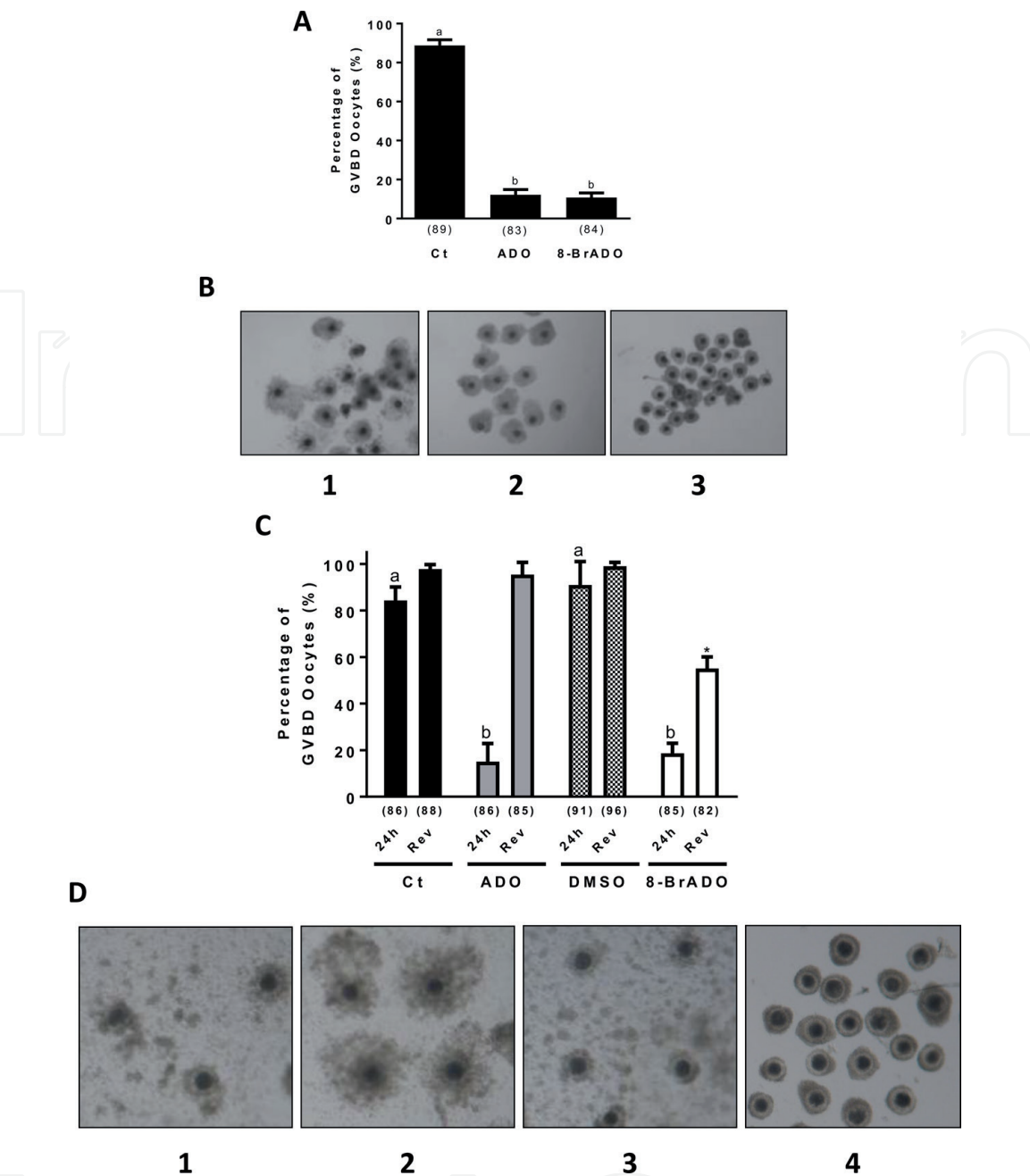
Figure 1.
Dose-response of ADO on both (A) the percentage of GVBD oocyte and (B) cumulus cell expansion. (C) Effect of ADO (2.0 mM) on the percentage of denuded oocytes in GVBD. Data are presented as the mean ± SEM of a minimum of three replicates. The number of oocytes used is illustrated in parentheses. Statistically significant effect of the treatment at P < 0.05 is shown by *. In (B) treatments were (1) Ct, (2) ADO 0.25 mM, (3) ADO 0.5 mM, (4) ADO 1.0 mM, and (5) ADO 2.0 mM.

3.2 Cell-permeable analog of adenosine (8-BrADO) inhibits GVBD

The following experiment is performed to assess a membrane-permeable analog of ADO, 8-BrADO (mimicking the intracellular effect). When COC are cultured for 24 hours, 12.2 ± 2.7% of the oocytes were in GVBD when treated with 2.0 mM ADO and 15.9 ± 0.8% when treated with 2.0 mM 8-BrADO (**Figure 2A**). These two treatments are significantly different to the control treatment (88.7 ± 2.9%; **Figure 2A**). The inhibition of cumulus cell expansion is also observed in both treatments (**Figure 2B2** and **B3**). These results support that the inhibitory effect of ADO may be mimicked by the cell-permeable analog of adenosine, 8-BrADO.

3.3 Reversibility of GVBD inhibition

To assess the reversibility, the test compounds (ADO and 8-BrADO) are washed out, and the COC are cultured under control conditions for a second 24-hour
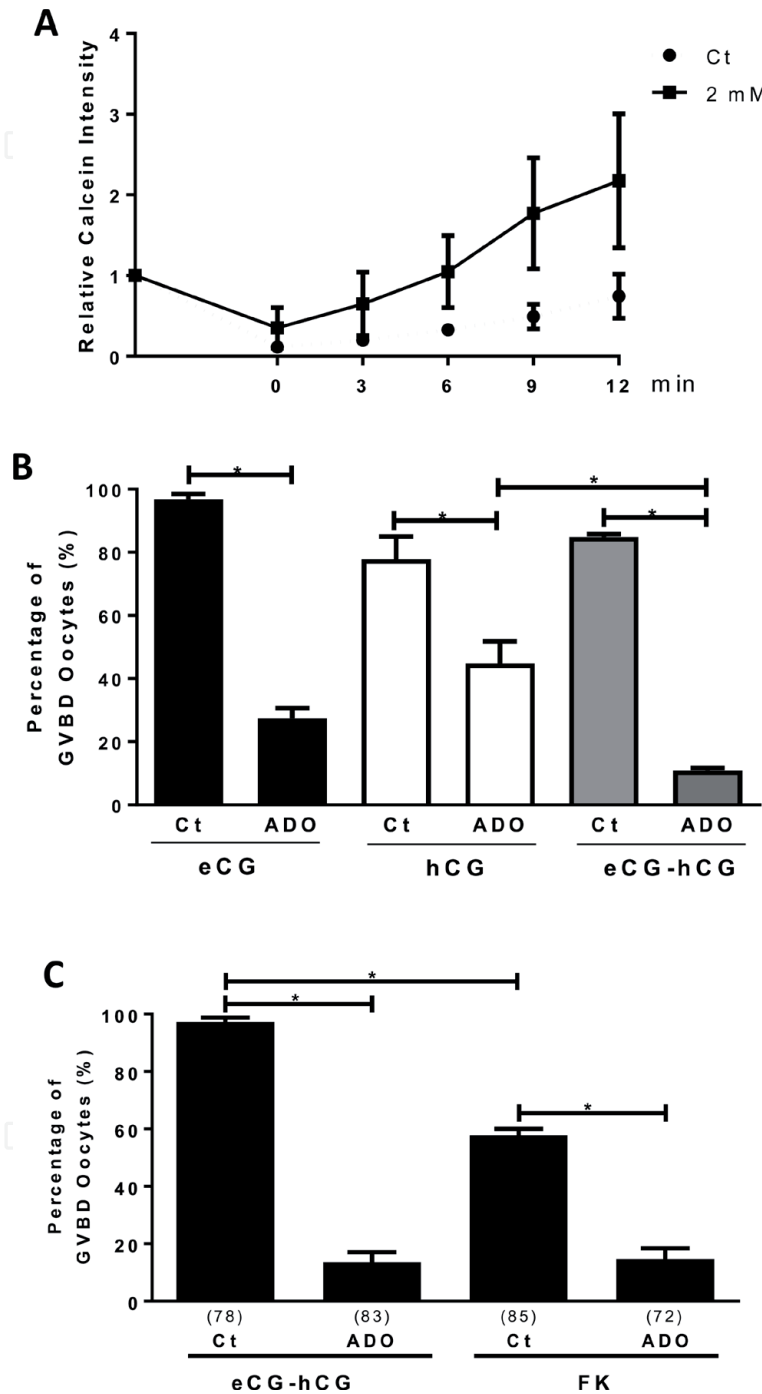
Figure 2.
Comparison of the effect of ADO (2.0 mM) with the cell-permeable analog 8-BrADO (2.0 mM) on both the percentage of GVBD oocytes and cumulus cell expansion after (A–B) 24 hours and (C–D) reversibility of 24 hours (Rev). DMSO treatment at 0.1% is the control for 8-BrADO. (C) Reversibility: COC were first cultured for 24 hours according to treatment and then cultured for a second 24 hours in control culture medium (Ct) to assess the reversibility of the inhibition of oocyte meiotic resumption. Data are expressed as the mean ±SEM of a minimum of three replicates. The number of oocytes used is illustrated in parentheses. Statistically significant effect of treatment at P < 0.05 is shown by different letters and *. In (B), treatments were (1) Ct, (2) ADO, and (3) 8-BrADO. In (D) treatments were (1) Ct, (2) ADO, (3) DMSO, and (4) 8-BrADO.

period. The ADO-treated oocytes resumed meiosis showing no statistical difference with the control treatment, whereas a significant lower percentage of GVBD oocytes is observed following treatment with 8-BrADO (**Figure 2C**). Cumulus cell expansion is compromised by 8-BrADO (**Figure 2D4**), supporting an impairment of oocyte maturation (nuclear maturation and cumulus cell expansion) when using the halogenate compound. However, cumulus cells are expanding following reversibility treatment of the ADO-treated COC (**Figure 2D2**), supporting the reversibility of the ADO inhibitory effect. The appropriate controls are presented in **Figure 2D1** and **D3**. These data are supportive of an adenosinergic system involved in the control of oocyte meiotic resumption.

3.4 Gap junction communications measured by cumulus-cumulus gap-FRAP assay

Since gap junction communications are highly regulated during in vitro maturation [17, 18], the aim of the following experiment was to measure the impact of
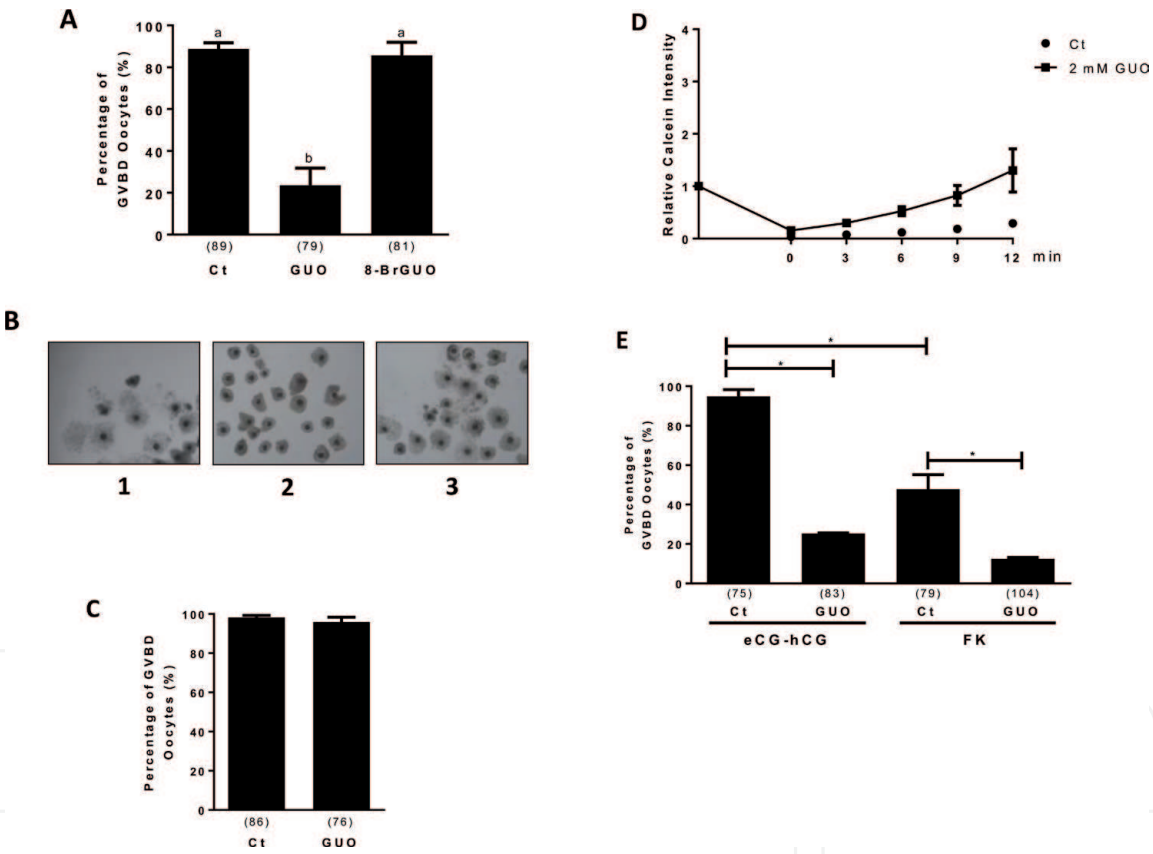

Figure 3.
Effect of ADO (A) on gap junction communication, (B) according to hormonal supplementation, and (C) according to FK supplementation. (A) The effect of ADO (2.0 mM) on gap junction communications in between cumulus cells measured by fluorescent recovery after photobleaching after 4 hours of in vitro culture. The COC were prepared using calcein-AM as fluorescent probe. The data were plotted as relative intensity and presented as the mean ±SEM of a minimum of three replicates. ADO-treated cumulus cells recover significantly more fluorescence than the control cumulus cells (P < 0.05). (B) The effect of ADO according to hormonal supplementation on the percentage of GVBD oocyte after 24 hours of in vitro maturation. ADO (2.0 mM) was added either with eCG or hCG or both eCG and hCG. Data are expressed as the mean ± SEM of a minimum of three replicates. Statistically significant effect of the treatment at P < 0.05 is shown by *. (C) The effect of ADO (2.0 mM) in the presence of either eCG and hCG or the adenylyl cyclase activator forskolin (FK, 1.1 µM), for 24 hours on the percentage of GVBD oocyte. Data are expressed as the mean ± SEM of a minimum of three replicates. The number of oocytes used is illustrated in parentheses. Statistically significant effect of the treatment at P < 0.05 is shown by *.

ADO treatment on gap junction communications measured by cumulus-cumulus gap-FRAP assay using calcein-AM as already described [12, 16]. Gap junction communication in cumulus cells was assessed after a 4-hour incubation in the presence of ADO (2.0 mM). The results show that gap junction communications between cumulus cells is increased following ADO treatment compared with the control treatment (**Figure 3A**). These results support that ADO maintains functional gap junctional communications between cumulus cells.

3.5 The contribution of eCG in adenosine treatment

The goal of the following experiment was to assess the contribution of gonadotropins in ADO-treated COC. The effect of ADO in inhibiting GVBD oocytes is observed in the presence of either eCG or hCG, or both in combination (**Figure 3B**). Comparison of ADO treatment supplemented with these hormones revealed no significant difference between eCG and the combination of eCG and hCG. However, a significant decrease in the percentage of GVBD was measured
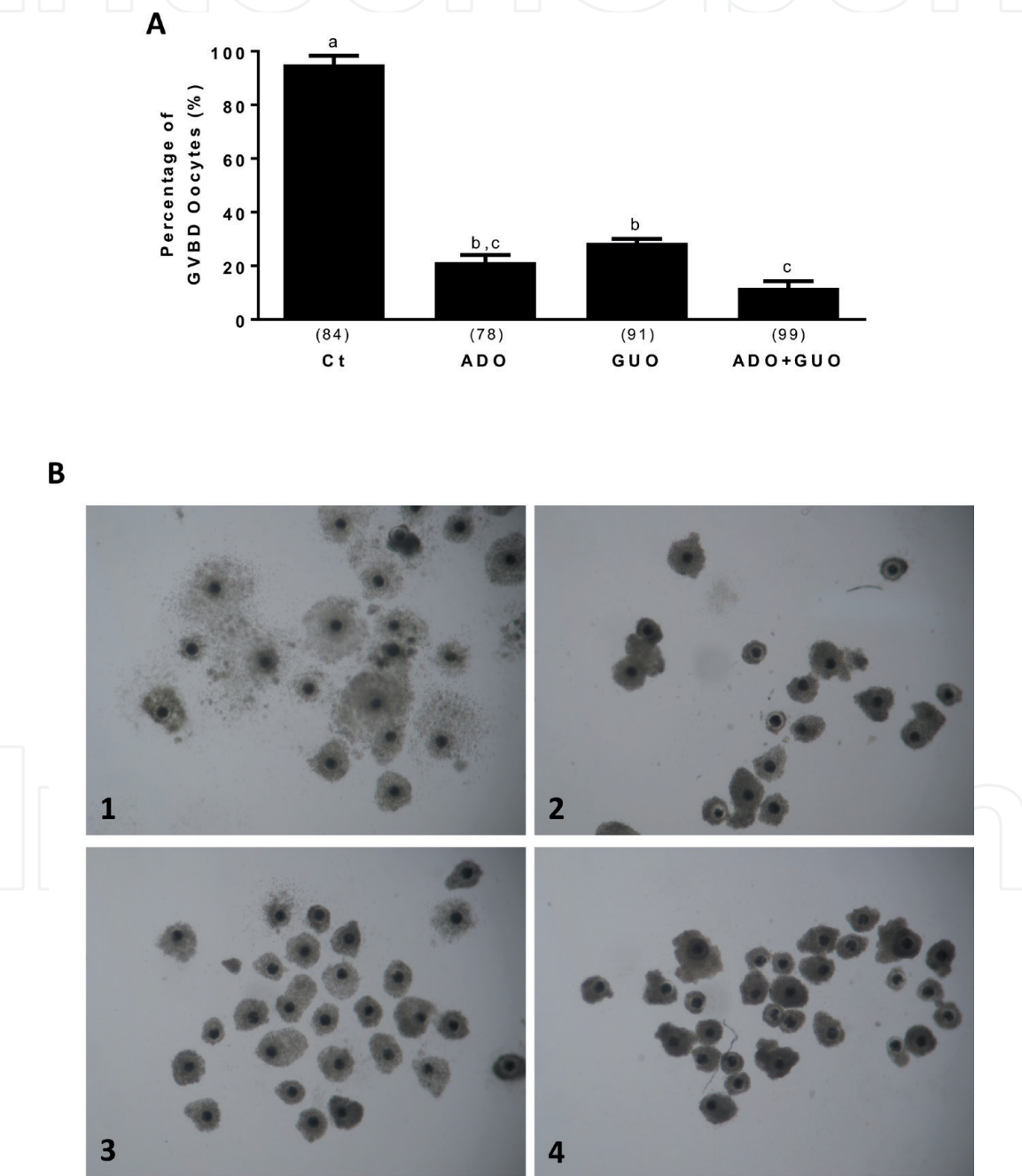
Figure 4.
*The effect of GUO (A) on the percentage of GVBD oocytes, (B) on cumulus cell expansion, (C) on reversibility, (D) on gap junction communication, and (E) according to FK supplementation. (A–B) Comparison of the effect of GUO (2.0 mM) and 8-BrGUO (2.0 mM) after 24 hours. (C) Reversibility: COC were first cultured for 24 hours in the presence of 2.0 mM of GUO and then cultured for a second 24 hours in control culture medium (Ct) to assess the reversibility of the inhibition of oocyte meiotic resumption. Data are expressed as the mean ± SEM of a minimum of three replicates. The number of oocytes used is illustrated in parentheses. Statistically significant effect of the treatment at P < 0.05 is shown by different letters. (D) The effect of GUO (2.0 mM) on gap junction communications in between cumulus cells measured by fluorescent recovery after photobleaching after 4 hours of in vitro culture. The data were plotted as relative intensity and presented as the mean ± SEM of a minimum of three replicates. GUO-treated cumulus cells recover significantly more fluorescence than the control cumulus cells (P < 0.05). (E) The effect of GUO (2.0 mM) in the presence of either eCG and hCG or the adenylyl cyclase activator forskolin (FK, 1.1 μM), for 24 hours on the percentage of GVBD oocyte. Data are expressed as the mean ± SEM of a minimum of three replicates. The number of oocytes used is illustrated in parentheses. Statistically significant effect of the treatment at P < 0.05 is shown by *. In (B), treatments were (1) Ct, (2) GUO, and (3) 8-BrGUO.*

between hCG alone and the combination of hCG and eCG (**Figure 3B**), showing the significant contribution of eCG to ADO-inhibiting GVBD, yet supporting an adenosinergic system.

3.6 Adenylyl cyclase activator (forskolin)

Since eCG contributes to ADO inhibitory effect, the following experiment was to evaluate the involvement of cAMP while using the adenylyl cyclase activator, forskolin. On itself, forskolin is significantly decreasing the percentage of GVBD oocytes (**Figure 3C**). ADO was significantly inhibiting GVBD both in presence of eCG-hCG and forskolin (**Figure 3C**). However, forskolin was not significantly

Figure 5.
The effect of supplementing both ADO and GUO (2.0 mM each) on (A) the percentage of oocyte meiotic resumption and (B) cumulus cell expansion. Data are expressed as the mean ± SEM of a minimum of three replicates. The number of oocytes used is illustrated in parentheses. Statistically significant effect of the treatment at $P < 0.05$ is shown by different letters. (B) The treatments were (1) Ct, (2) ADO 2.0 mM, (3) GUO 2.0 mM, (4) ADO 2.0 mM and GUO 2.0 mM.

changing the percentage of GVBD compared with eCG-hCG (**Figure 3C**). The results are proposing the contribution of cAMP to ADO-inhibiting GVBD.

3.7 Guanosine inhibits oocyte meiotic resumption

High-performance liquid chromatography was used to detect the presence of GUO in porcine follicular fluid from follicles of 2–6 mm in diameter (data not shown). Since GUO was detected in PFF, we undertook to assess whether GUO could play a role in inhibiting GVBD. **Figure 4** shows the significant effect of GUO in inhibiting both GVBD and cumulus expansion after 24 hours, highlighting the contribution of GUO in oocyte maturation. However, the membrane-permeable analog 8-BrGUO did not significantly inhibit GVBD (**Figure 4A**). Cumulus cell expansion was observed (**Figure 4B3**), supporting the inefficacy of 8-BrGUO in inhibiting oocyte GVBD and cumulus cell expansion. The reversibility treatment showed that GUO-treated COC resumed meiosis without any statistical significance compared with the control treatment (**Figure 4C**), supporting the reversibility of GUO inhibitory effect on GVBD. As ADO, GUO was also significantly increasing gap junction communication as measured by gap-FRAP assay (**Figure 4D**). Finally, forskolin was not significantly improving the inhibitory effect of GUO compared to eCG-hCG supplementation as measured on the percentage of GVBD oocytes (**Figure 4E**). GUO was also reversibly inhibiting oocyte GVBD, thus supporting a guanosinergic system.

3.8 The effect of using both ADO and GUO

The final experiment evaluated the effect of supplementing both ADO and GUO at 2.0 mM each on GVBD oocytes using COC (**Figure 5A**). The results showed that ADO, GUO, and both ADO and GUO were all significantly decreasing GVDB percentage compared with the control treatment. Although a significant effect of adding ADO with GUO was observed when compared with GUO (**Figure 5A**), the impact on the percentage went from 28.6 ± 1.4% for GUO to 11.8 ± 2.5% for ADO and GUO. Cumulus cell expansion was inhibited by ADO (**Figure 5B2**), GUO (**Figure 5B3**), and the combination of ADO and GUO (**Figure 5B4**) when compared with the control (**Figure 5B1**).

4. Discussion

The present study showed that both ADO and GUO are efficient in reversibly inhibiting swine oocyte GVBD (**Figures 1** and **4**). Cumulus cell expansion is significantly inhibited after 24 hours in presence to either ADO or GUO (**Figures 1** and **4**), supporting that these two purine nucleosides inhibit oocyte maturation with low GVBD percentages and no cumulus cell expansion. Both ADO and GUO also increased GJC in cumulus cells (**Figures 3** and **4**). eCG and FK are both supporting the two purines' inhibitory effect (**Figures 3** and **4**). The data suggest that the inhibitory effect of these two purines on GVBD strengthens the involvement of both adenosinergic and guanosinergic systems in meiotic resumption.

Several years ago it was clearly shown that hypoxanthine, a purine derivative, was an important component of a low molecular weight fraction from porcine follicular fluid and efficient at inhibiting oocyte GVBD [19]. It was also reported that this fraction did not exclusively contain hypoxanthine [10]. Although hypoxanthine was efficient in the mouse and rat, the question was still not fully assessed regarding the efficacy of ADO and GUO on swine COC with respect to the inhibition of oocyte meiotic resumption.

From a broader perspective, it is known that ADO is involved in several biological functions such as nucleotide biosynthesis and cellular energy metabolism [19]. The cellular uptake of ADO played by two classes of nucleotide transporters (SLC28 and SLC29) regulates these biological functions. Inside the cell, ADO is rapidly metabolized and either converted to inosine or adenosine monophosphate through adenosine deaminase or adenosine kinase, respectively [20]. Alternatively, extracellular ADO may serve as a signaling molecule, which activates adenosine receptors (ADORA) on the cell membrane surface. Four different seven-transmembrane domain receptors have been described [21]. It has been reported that a low dose of ADO (0.2 mM) produced a transient delay in bovine oocyte GVBD [9]. After 21 hours in culture, neither ADO nor hypoxanthine resulted in an efficient inhibition of oocyte GVBD [9]. In the mouse, 4.0 mM of hypoxanthine was clearly efficient at inhibiting oocyte GVBD, while the use of ADO by itself did not produce a significant inhibition [10]. However, the efficacy was enhanced when ADO was used in combination with hypoxanthine [7]. This was also observed when ADO was used together with FSH [22], forskolin [23], or cAMP analogs such as 8-bromo-cAMP [8]. Although these results have been provided to support that adenosine uptake and metabolism contribute to the inhibition of GVBD [24], ample evidence shows that functional adenosine receptors are present on ovarian cells [25]. The measured concentration of ADO in murine follicular fluid was between 0.38 and 0.68 mM [7]. In the present study, the inhibitory effect on GVBD was dose-dependent with an IC_{50} of 1.3 mM (**Figure 1**). Using 2.0 mM, ADO was efficient at reversibly inhibiting swine oocyte GVBD and cumulus cell expansion (**Figure 1**).

Although hypoxanthine and ADO have been measured in PFF [10], GUO has never been reported. In the present study, GUO has been found in PFF from 2 to 6 mm diameter follicles (data not shown). While limited information is available in the literature regarding GUO, it has been described as having important functions as an intercellular messenger especially in the central nervous system [4]. The mechanism underlying the neuroprotective properties of GUO is still not fully understood. One working hypothesis is that GUO may exert its biological effect by synchronizing distinct signaling pathways that may be related to the activation of purinergic receptor and specific G-protein binding sites [26–28]. In this study, 2.0 mM of GUO is reversibly inhibiting GVBD and cumulus cell expansion (**Figure 5**). In the mouse, GUO (1.0 mM) was reported to inhibit GVBD, while the same concentration of either hypoxanthine or ADO was inefficient [10]. In rats, the reported order of potency of these nucleosides was GUO > hypoxanthine > ADO [8]. In the mouse, there is an assumption based on a synergistic effect of ADO and GUO on the inhibition oocyte GVBD [7]. In the present study, the combination at 2.0 mM significantly increased the effect of GUO (**Figure 5**). Thus, the inhibitory effect of the combination of both ADO and GUO are somewhat additive according to the concentration used. Although GUO is proposed to activate a specific G-protein-coupled receptor with the involvement of P1 receptor [29], it has been recently reported that GUO functions as an extracellular signaling molecule without the need for GUO receptors [30]. In vascular smooth muscle cells, extracellular GUO regulated extracellular ADO [30]. This proposed GUO-ADO mechanism further regulated cell proliferation in vitro [31] and decreased inflammation in vivo [32], supporting the additive inhibitory effect observed on oocyte GVBD.

We recently demonstrated the regulation of gap junction communications between cumulus cells during in vitro maturation [16–18]. An increase in gap junction communication was evident after 4 hours of in vitro culture [17, 18]. This observation provides an indication of how cumulus cells respond to the treatment and not only looking at cumulus cell expansion. In the present study, the effect of both treatments, ADO and GUO, was to increase gap junction communications

compared with the control (**Figures 3** and **4**). Since gap junction communications are known to play a primordial role in oocyte maturation [33, 34], this result supports a clear impact of the treatments on cumulus cell functions that may be beneficial for the oocyte.

This study also provides evidence of hormonal supplementation impacting the inhibition of GVBD oocytes by ADO (**Figure 3**). This ADO-mediated inhibition of GVBD oocytes is improved according to the supplementation. Although it is not the purpose of this study to understand how this effect is transduced into the cells, eCG clearly improved the effect of ADO in the presence of hCG. This FSH-type stimulation seemed to be sufficient since the effect of eCG alone was not significantly different from that of eCG and hCG (**Figure 3**). As it is well-known that cumulus cells have an efficient response to FSH [35, 36], the inhibitory effect of ADO is significantly increased by FSH. This effect has been reported in the rat where the percentage of GVBD oocyte treated with ADO was decreased in the presence of FSH [22]. In addition, the FSH-induced granulosa cell differentiation was reduced by ADO [37], supporting the involvement of ADO in FSH response.

Although different forms of adenylyl cyclase have been characterized in oocytes and in cumulus cells [38], forskolin, a known adenylyl cyclase activator, makes a significant contribution to the inhibition of spontaneous maturation in several species as observed for rat [39], bovine [40], and porcine [11] oocytes. In the present study, forskolin inhibited oocyte GVBD while treated with ADO (**Figure 3C**). Similar results were obtained in the presence of GUO (**Figure 4E**). These results support that constant stimulation of adenylyl cyclase, which increases the intracellular concentration of cAMP [40], promoted the inhibitory effects of ADO and GUO. In this regard, the results are proposing the contribution of cAMP to both ADO- and GUO-inhibiting GVBD.

The effect of the two purines goes well with the current working model of inhibition of oocyte GVBD involving C-type natriuretic peptide (CNP) as an oocyte-meiosis-inhibiting peptide [41, 42]. CNP is involved in inhibiting oocyte GVBD [41, 43, 44]. CNP produced by granulosa cells is a ligand for NPR2, a member of guanylyl cyclase receptor family. NPR2 stimulation by CNP increased intracellular concentration of cGMP, inhibited oocyte phosphodiesterase type 3A, and thus maintained high intra-oocyte concentration of cAMP. The contribution of both purine nucleosides supports adenosinergic and guanosinergic system in the inhibition of oocyte meiotic resumption.

5. Conclusion

In conclusion, this study puts forward the contribution of ADO and GUO as inhibitory for oocyte GVBD in vitro suggesting that both adenosinergic and guanosinergic systems are efficient in inhibiting oocyte meiotic resumption. The use of these two systems as part of a pre-IVM culture period would be a novel strategy to explore in order to improve oocyte developmental competence.

Finally, it should be emphasized that the signaling involved in oocyte meiotic resumption may be modulated through the contribution of different pathways. The adenosinergic and guanosinergic systems of which we have presented the contribution illustrate this situation for meiotic resumption. It is also to be expected that other studies will pave the way for additional contributions. For example, a preliminary study from our lab revealed that, as demonstrated in bovine [45], porcine theca cells secreted efficient factors involved in oocyte meiotic resumption. Without knowing these secreted elements, this result highlights that oocyte meiotic resumption is under the control of the ovarian follicular cells. Meiotic resumption is thus under high surveillance!

Acknowledgements

Conflict of interest

The author has no conflict of interest to declare.

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