

## Role of cAMP modulator supplementations during oocyte *in vitro* maturation in domestic animals



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### ABSTRACT

Cyclic adenosine monophosphate (cAMP) is an important molecule in signal transduction within the cell, functioning as a second cell messenger of gonadotrophin stimulation. The concentration of cAMP in cumulus-oocyte complexes (COCs) is known to be controlled through modulation of its synthesis by adenylyl cyclase (AC) and by degradation through the cyclic nucleotide phosphodiesterase (PDE) enzymes. One of the main obstacles for *in vitro* embryo production is the optimization of reproduction processes that occur in oocyte maturation. The function of cAMP is important in maintaining meiotic arrest in mammalian oocytes. When the oocyte is physically removed from the antral follicle for *in vitro* maturation (IVM), intra-oocyte cAMP concentrations decrease and spontaneous meiotic resumption begins, due to the depletion of inhibitory factors from the follicle. In many studies, relatively greater cAMP concentrations before IVM has been reported to improve oocyte competence, leading to subsequent benefits in embryonic development in different species. There, therefore, has been an increase in oocyte cAMP concentrations with several treatments and different approaches, such as invasive AC, stimulators of AC activity, PDE inhibitors, and cAMP analogs. The aim of this review is to comprehensively evaluate and provide data related to (i) the use of cAMP modulators during IVM and the effects on completion of meiosis and cytoplasmic reorganization, which are required for development of oocytes with the capacity to contribute to fertilization and subsequent embryonic development; and (ii) the main cAMP modulators and the effects when used in oocyte IVM.

### 1. Introduction

Oocyte quality is very important for *in vitro* production (IVP) (Katska-Ksiazkiewicz et al., 2007), and one of the main obstacles is the challenge of mimicking *in vitro* the processes that occur during oocyte maturation *in vivo*. Oocyte maturation is a long and delicate process in which the oocyte acquires the intrinsic capacity to support fertilization and the embryo's initial development until the time of activation of the embryonic genome (Meirelles et al., 2004; Ferreira et al., 2009).

Even with the many advances in IVP, the efficiency of oocyte *in vitro* maturation (IVM) is still less than *in vivo* maturation, which limits its application in assisted reproduction techniques. It is widely accepted that the relatively lesser efficiency of IVM is in part due

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to precocious oocyte meiotic resumption following artificial removal of cumulus-oocyte complexes (COCs) from antral follicles and subsequent culture (Gilchrist and Thompson, 2007). When the oocyte is mechanically removed from the antral follicle for IVP, intra-oocyte cyclic adenosine monophosphate (cAMP) concentrations decrease, and precocious meiotic resumption begins. This process, widely known as spontaneous nuclear maturation, is attributed to the depletion of inhibitory factors from the follicle (Tsafriri and Pomerantz, 1986). Adequate intra-oocyte concentrations of cAMP appear to be required for final oocyte differentiation, such as the chromatin transition and gradual silencing of transcription (Luciano et al., 2011). There can be prevention of spontaneous nuclear maturation by sustaining meiotic arrest with chemical mediators. It is currently difficult to develop techniques to overcome the IVP rates and improve embryo quality. Some researchers suggest that improving IVM might increase the efficacy of the IVP system (Demyda-Peyrás et al., 2013), and several improvements in oocyte competence have been achieved in studies focused on modulating cAMP concentrations during the IVM process (Albuz et al., 2010; Richani et al., 2014; Park et al., 2016).

With this review, the aims are to (i) comprehensively evaluate the use of cAMP modulators on IVM and the effects on the completion of meiosis and cytoplasmic reorganization, which are required for development of a competent oocyte; and (ii) describe the primary cAMP modulators and the effects when used in oocyte IVM. Advances in this field are likely to contribute to an understanding of the mechanisms underlying embryo development, and thus may improve the efficiency of breeding programs.

## 2. Primary processes and challenges of oocyte IVM

In oocyte maturation, the oocyte acquires the intrinsic capacity to progress through the subsequent processes of development. It involves complex and distinct, but associated, mechanisms of nuclear and cytoplasmic maturation (Ferreira et al., 2009). The intercellular communication between cumulus cells (CCs) and the oocyte is essential for oocyte growth because it allows the transfer of nutrients and other small molecules, ensuring that the molecular structures and biochemical pathways develop in oocytes to support early embryo development subsequent to fertilization (Simon et al., 1997; Gilchrist et al., 2004).

There is a cessation of meiotic arrest in mammalian oocytes and resumption of meiotic maturation in response to the pre-ovulatory surge of luteinizing hormone (LH). Germinal vesicle breakdown (GVB) is the first visible change leading to the resumption of meiosis. The GVB is manifested in the condensation of chromatin, appearance of kinetochores (anchorage points between the chromosomes and the microtubules), and dissolution of the nuclear membrane. The cell subsequently enters Metaphase I: the centrioles duplicate and the chromosomes (in diploid pairs), which are now free in the cytoplasm, migrate to the equatorial region (Mermillod and Lannou, 1999). The primary oocyte undergoes meiotic division that leads to formation of two new cells. One of these contains most of the cytoplasm and constitutes the oocyte itself. The other is smaller in size and is expelled into the perivitelline space and becomes the polar corpuscle. The cell is subsequently considered to be a secondary oocyte, and the chromosomes again line up in the center of the spindle, which is a primary characteristic of Metaphase II (Sirard et al., 1992).

The resumption of meiosis depends on several external factors. In mammals, oocyte maturation is induced by the removal of the inhibitory influence of granulosa cells (GC), and the pre-ovulatory LH surge causes the breakdown of gap junctions between the oocyte and the GC in the pre-ovulatory follicle (Eppig, 1991). Although the exact mechanism of oocyte maturation is not clearly understood, cAMP is widely considered to have a very important role in maintaining meiotic arrest in mammalian oocytes. The maintenance of relatively greater concentrations of cAMP in the oocyte is essential for sustaining oocytes in the meiotic-arrested state of development, and the cAMP is produced by the GC and transported to the oocyte via gap junctions (Albuz et al., 2010). Decreases in cAMP concentration allow for the resumption of meiosis, which should not occur until puberty, at which time a pre-ovulatory LH surge release induces a decrease in intra-oocyte cAMP, first by detachment of gap junctions between the oocyte and GC, and subsequently by reduction of cAMP production (Kawamura et al., 2005).

The cAMP molecule is important in signal transduction within the cell, functioning as a second cell messenger of gonadotropin stimulation. Concentrations of cAMP are controlled through modulation of its synthesis by adenylyl cyclase (AC), an enzyme ATP-pyrophosphatase lyase, which converts adenosine triphosphate (ATP) into cAMP and pyrophosphate (Mehmann et al., 2002; Bilo-deau-Goessels, 2011) in the oocyte (Kuyt et al., 1988) and the GC (Thomas et al., 2004a, 2004b). The degradation of cAMP degradation occurs as a result of the activation of cyclic nucleotide phosphodiesterase (PDE) enzymes within the cumulus-oocyte complexes (COCs) which promote the hydrolysis of cAMP. Relatively greater concentrations of cAMP maintain meiotic arrest by suppressing the activation of maturation promoting factor (MPF), while relatively lesser cAMP concentrations induce MPF activation, resulting in GVB and resumption of meiosis (Mermillod et al., 2000; Bilodeau-Goeseels, 2011).

The progression of meiosis characterizes nuclear oocyte maturation but does not by itself ensure further embryonic development; additional cytoplasm maturation is also necessary which includes structural and molecular changes in the oocyte from the germinal vesicle (GV) stage to the end of Metaphase II (Ferreira et al., 2009). Cytoplasmic maturation can be defined as the set of processes whereby the oocyte gains the capacity to participate in fertilization and to support initial embryonic development (Anguita et al., 2007). It is subdivided into three main stages that are: 1) redistribution of cytoplasmic organelles, including mitochondria (Adona et al., 2008) and cortical granules (Hytel et al., 1997), among others; 2) dynamic reorganization of the cytoskeletal filaments, including the development of the microtubule network structures close to the condensed DNA (Can et al., 2003); and 3) molecular maturation (Ferreira et al., 2009), which occurs at the final stage of cytoplasmic maturation and involves the transcription, storage, and processing of mRNA to prepare the oocyte for post-fertilization development (Sirard, 2001). As meiosis proceeds and chromosomes segregate, the organelles reorganize in the cytoplasm, transcription ceases, the stored mRNA is partially utilized, and the protein phosphorylation pattern changes (Ferreira et al., 2009). Given the established metabolic synergy between oocytes and somatic cells, it is reasonable to consider the physiological implications of this synergy on the cytoskeletal dynamics, spindle assembly, and cortical remodeling.

The use of IVP techniques is hindered by the difficulty of reproducing *in vitro* the developmental changes that occur *in vivo* in oocytes. All molecular and biochemical functions that occur in the oocyte until maturation must be controlled if a viable female gamete is to develop for fertilization and subsequent embryo development is to occur (Wrenzycki and Stinshoff, 2013). Oocyte IVM is not yet fully efficient to the extent of providing many competent oocytes that have the capacity to support the development of a viable embryo, given that the process of maturation itself involves complex mechanisms that are not completely established. Although immature oocytes can resume meiosis *in vitro* after being removed from antral follicles (Edwards, 1965), cytoplasmic maturation apparently occurs asynchronously with nuclear maturation (Huang et al., 1999) due to precocious oocyte meiotic resumption. This is probably the main factor responsible for the lesser rates of embryo production when oocytes are matured *in vitro*. For this reason, the IVM stage has received attention from researchers with the aim to improve IVP results (Demyda-Peyrás et al., 2013, Parrish, 2014).

### 3. cAMP modulators

The oocyte can independently synthesize cAMP (Mehlmann et al., 2002), but the major source of intra-oocyte cAMP is the somatic cells surrounding the oocyte. There is an electrophysiological syncytium between the oocyte, cumulus, and GC (Bornslaeger and Schultz, 1985). Variation in the intra-oocyte concentration of cAMP can modulate the time of resumption of meiosis with relatively greater intracellular concentrations sustaining the oocyte meiotic arrest at the GV stage by activating cAMP-dependent protein kinase (PKA), which in turn maintains the inactive form of MPF, another key regulator of the meiotic cell cycle (Bornslaeger et al., 1986; Spaulding, 1993; Francis and Corbin, 1994).

The MPF, a complex of Cdc2 and cyclin B, is negatively regulated by the phosphorylation of the highly conserved Thr14 and Tyr15 residues of Cdc2. The inhibitory phosphorylations are catalyzed by the Wee1 kinases, whereas dephosphorylation of these residues is dependent on the Cdc25 phosphatases (Lew and Kornbluth, 1996). The PKA regulates the activity of both Cdc25 phosphatases and Wee1 kinases (Han et al., 2005; Zhang et al., 2008; Pirino et al., 2009; Oh et al., 2010). Intra-oocyte cAMP concentrations decrease as a pre-requisite for the consequent dephosphorylation of PKA, activation of MPF, and meiotic resumption.

Relatively greater cAMP concentrations before IVM improve oocyte competence and subsequent embryonic development (Nogueira et al., 2003; Richani et al., 2014; Li et al., 2016; Park et al., 2016), and these findings are consistent among multiple species: humans (Shu et al., 2008), mice (Albuz et al., 2010), cattle (Luciano et al., 1999; Albuz et al., 2010), sheep (Rose et al., 2013), and pigs (Funahashi et al., 1997). Even with these benefits, the time of initiation of modulator supplementation is very important for its effectiveness during the IVM stage. The cAMP content of the oocyte of cattle, for example, decreases approximately 50% within 30 minutes of collection, indicating that treatment with modulators after 30 minutes post-oocyte collection resulted in a lesser modulator efficacy (Luciano et al., 2004).

An interesting feature is that the exogenous modulation of cAMP can evoke a stimulatory or inhibitory response in germ and somatic cells. In fact, disruption of the junctional pathways is not essential for meiotic resumption, but intercellular and metabolic coupling does determine developmental competence (de Loos et al., 1991). Furthermore, there is an effect of cAMP concentrations in COCs with an apparent stimulation of a prolonged somatic and germinal intercellular communication, resulting in a greater developmental capacity after fertilization. Oocyte cAMP concentrations increase with several different treatments, including cAMP analogues, invasive AC, stimulators of AC activity, and PDE inhibitors (Fig.1.). In this review, there is a description of the main modulators used in IVM to increase cAMP concentrations for the temporary maintenance of meiotic arrest, establishing the synchrony between cytoplasmic and nuclear maturation.

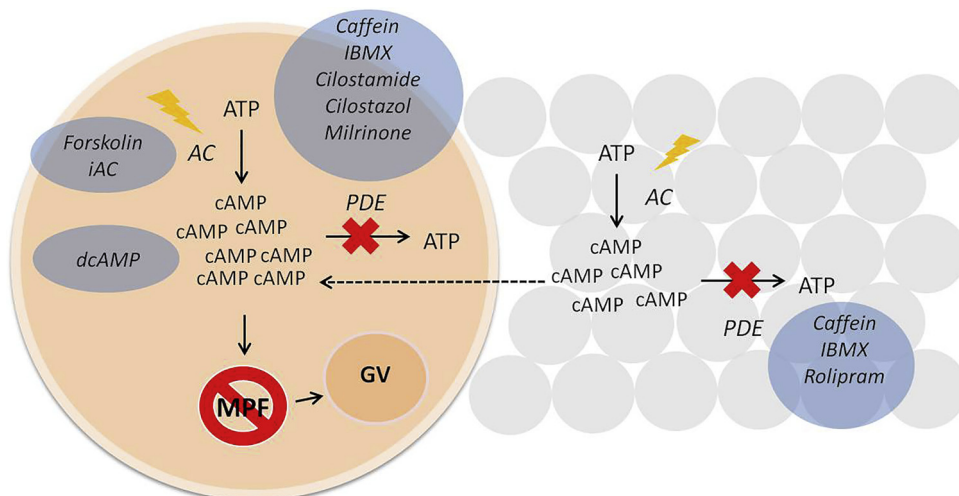


Fig. 1. Schematic illustration of different action pathways of the main cAMP modulators used in IVM (see text for abbreviations).

### 3.1. Dibutyl cAMP (dcAMP)

The dcAMP molecule is a membrane permeable analog of cAMP that mimics the action of endogenous cAMP. For this reason, it can be used as an effective meiosis inhibitor to maintain meiotic arrest and enhance meiotic competence, mainly in pig oocytes (Bagg et al., 2006; Kim et al., 2008; Nascimento et al., 2010). In comparison with cAMP, dcAMP has a lipophilic nature and is thus more effective at maintaining relatively greater intra-oocyte concentrations of cAMP when used with intact cells because it has greater permeability and resistance to hydrolysis by PDEs (Henion et al., 1967; Swislocki, 1970).

Supplementation with dcAMP during the first 20 hours of IVM can provide effective support for meiotic progression and developmental competence of pig oocytes (Akaki et al., 2009), improve the fertilization process contributing to embryo production, and promote pre-implantation embryo development (Funahashi et al., 1997; Bagg et al., 2006). Bagg et al. (2006) reported that the effects of dcAMP treatment on cAMP accumulation and oocyte competence were greater in oocytes derived from pre-pubertal gilts than sexually mature sows, suggesting a cAMP deficiency in oocytes of immature gilts.

Appeltant et al. (2015) concluded that dcAMP supplementation results in the yield of a greater percentage of normally fertilized zygotes after fertilization of oocytes that were IVM, decreasing the polyspermy rate. Treatment with dcAMP and porcine follicular fluid results in a synergistic effect of these two factors in regulation of fertilization by reducing the polyspermy rate after *in vitro* fertilization (IVF) and improving the rate of blastocyst formation, resulting in a greater total number of cells per blastocyst when compared with dcAMP supplementation alone (Nascimento et al., 2010).

There is a relationship between nuclear and cytoplasmic maturation. For this reason, it is possible that the improvement associated with inhibiting polyspermy by dcAMP addition induces a synchronized nuclear and cytoplasmic maturation, allowing for the adequate migration of the cortical granules and subsequently leading to less polyspermy. Also, the addition of dcAMP prolongs the maintenance of gap junctional communication (GJC) between oocytes and CCs (Flagg-Newton et al., 1981; Sun et al., 2001).

Supplementation with dcAMP also leads to improvement in values for embryo quality variables such as structural integrity, mitochondrial membrane potential, and apoptosis in IVF embryos (Kim et al., 2008). Sugimura et al. (2010) reported that there are no effects of dcAMP treatment on the proportion of pig oocytes that undergo nuclear maturation. Supplementation with dcAMP results in an increased blastocyst apoptosis and greater glutathione (GSH) concentrations, which are related to cytoplasmic maturation (de Matos et al., 2002d; Gasparrini et al., 2003). Furthermore, this treatment leads to protection of oocytes from reactive oxygen species (ROS; Yoshida et al., 1993). Park and Yu (2013) speculated that it might be unrealistic to expect to observe a constant, precise effect of dbcAMP in undefined media, and for this reason, it is important to perform more studies in a chemically defined medium.

### 3.2. Invasive adenylate cyclase (iAC)

The iAC is a bacterial toxin produced by *Bordetella pertussis* that can be internalized by mammalian cells (Confer et al., 1984) and if this occurs there is inhibition of spontaneous oocyte maturation as a result of increased cAMP concentrations within the host cell as a result of the pool of ATP (Hanski and Farfel, 1985).

While the physiological modulation of cAMP in COCs results as a consequence of an increase in gonadotropin and growth factor actions through specific receptors, it has been suggested that the COOH-terminal portion of the toxin creates a channel in the membrane through which the NH<sub>2</sub>-terminal fragment is translocated (Rogel and Hanski, 1992), thereby allowing iAC to function without the need for specific receptors. Although the mechanism by which iAC enters the cell is not clear (Otero et al., 1995), iAC apparently exerts its beneficial effect on oocyte maturation by directly inducing the optimal cAMP concentration and bypassing specific receptors for physiological effectors, such as FSH (Luciano et al., 1999).

When iAC is supplemented in the collection medium, the original intracellular concentration of cAMP in COCs is maintained from the time the COCs are removed from the follicle until the beginning of maturation, resulting in optimal subsequent development (Bilodeau et al., 1993; Aktas et al., 1995).

Relatively lesser concentrations of iAC stimulate nuclear maturation in cattle oocytes, while relatively greater concentrations inhibit the resumption of meiosis (Luciano et al., 1999). In addition, the relatively lesser concentrations induce increased cleavage rates following IVF and there is no effect of this treatment on blastocyst development rates compared with those obtained after IVM in a medium supplemented only with serum and gonadotropins. Luciano et al. (2004) reported that there was a greater developmental potential of COCs matured in the presence of a relatively lesser concentration of iAC. This outcome may be due to a moderate increase in oocyte intracellular cAMP, which in turn could result in beneficial effects on developmental competence by delaying the natural inhibition of oocyte-CC communications.

The use of iAC in the maturation medium may offer a significant advantage in the development of efficient methods for IVP. In earlier studies, there were reports of iAC being effective in inhibition of meiosis in rats (Aberdam et al., 1987) and cattle COCs (Aktas et al., 1995) without decreasing the developmental competence of both cumulus-attached and cumulus-free cattle oocytes in a dose-dependent manner by accumulating intercellular cAMP (Aktas et al., 1995). Somfai et al. (2003) also assessed the effects of iAC on pig oocytes; however, results indicated the use of dcAMP was more advantageous for nuclear progression, fertilization, and embryonic development.

### 3.3. Forskolin (FK)

The FK molecule is a diterpene that can be isolated from the roots of an Indian plant, *Coleus forskohli*. It is an AC activator,

stimulating the production of cAMP (Thomas et al., 2004b), and has been studied extensively because it regulates cAMP concentrations of oocytes in many species, including mice (Urner et al., 1983), cattle (Albuz et al., 2010), rats (Dekel et al., 1984), and swine (Racowsky, 1985). Also, FK has been widely used in association with PDE inhibitors as a strategy to suppress meiotic resumption (Thomas et al., 2004a, 2004b; Zeng et al., 2014; Li et al., 2016; Park et al., 2016; Dall'Acqua et al., 2017). Treatment of COCs with FK prolongs the maintenance of GJC in cattle (Thomas et al., 2004a, 2004b), and short-term culture with FK before maturation (pre-IVM) inhibits spontaneous resumption of meiosis and induces an increase in CC layers, embryo cleavage, and blastocyst formation in swine (Park et al., 2016).

The FK molecule has a lipolytic action and is used as a lipid modulator because the activation of AC induces the activation of cAMP-dependent protein kinases, which activate intracellular lipases via phosphorylation, increasing intracellular  $\beta$ -oxidation (Vaughan et al., 1964; Seamon et al., 1981; Prates et al., 2013). In many species, this function is associated with an improvement in oocyte and embryo cryotolerance (Paschoal et al., 2012; Gomis et al., 2013; Sanches et al., 2013). Lipid modulators are substances that can reduce or modify the intracellular lipid content, and FK treatment may induce an enhanced modification in intracellular lipids in a dose- and time-dependent manner (Prates et al., 2013). Pig oocytes cultured with FK for extended periods (24 or 42 hours) and vitrified pig blastocysts cultured with FK (24 hours) had a lesser lipid content and greater survival rate after warming (Men et al., 2006; Fu et al., 2011). By contrast, short term treatment with FK 2 hours before maturation (pre-IVM) did not induce a reduction in the lipid content in cattle blastocysts (Leal et al., 2018), indicating that treatment time with FK was not sufficient to ensure the lipolytic action, at least not to an extent that could be identified when using the technique utilized in this study.

Among cAMP modulators, FK is one of the most frequently described (alone and in combination) and its use has been associated with a variety of results: increased oocyte cAMP concentrations (Park et al., 2016), maintenance of meiotic arrest (Albuz et al., 2010; Leal et al., 2018), improvement in oocyte transport conditions (Dall'Acqua et al., 2017), beneficial effects on embryonic development (Albuz et al., 2010; Park et al., 2016), increased intra-oocyte GSH and decreased intra-oocyte  $H_2O_2$ , suggesting a mechanism that contributes to the improvement of oocyte developmental competence (Li et al., 2016).

### 3.4. PDE nonspecific inhibitors

The PDEs belong to a complex and diverse superfamily of at least 11 structurally related, highly regulated, and functionally distinct gene families (PDE1–PDE11), which differ in primary structures, affinities for cAMP and cGMP, responses to specific effectors, sensitivities to specific inhibitors, and regulatory mechanisms (Francis et al., 2011). Methylxanthines, a family of PDE inhibitors, can be used to sustain oocyte cAMP concentrations in cells (Jiang et al., 1984; Minelli and Bellezza, 2011). Members of this family, such as caffeine, IBMX, and theophylline, prevent GVB completely in rodents and inhibit GVB entirely/partially in larger animals (Sirard and First, 1988; Lonergan et al., 2003). In most studies, there is use of xanthines/methylxanthines as PDE inhibitors that maintain the oocyte intracellular concentrations of cAMP so that meiotic arrest is sustained (Albuz et al., 2010; Fathi et al., 2014; Bernal-Ulloa et al., 2016; Santiquet et al., 2017; Fathi et al., 2018).

#### 3.4.1. Hypoxanthine

Hypoxanthine is a naturally occurring cAMP-PDE inhibitor described in some studies decades ago as a cAMP modulator used in IVM. It is a component of follicular fluid in swine and mice (Downs et al., 1985; Eppig et al., 1985), and is a compound that functions as an intercellular communicator between oocytes and the surrounding GC *in vitro* (Eppig and Downs, 1987). Hypoxanthine facilitates these effects because it inhibits cAMP PDE activity, resulting in increased cAMP concentrations (Downs et al., 1989) that maintain meiosis arrest. In fact, hypoxanthine and adenosine, which is also present in mouse follicular fluid, interacts synergistically to prevent spontaneous maturation in culture (Eppig et al., 1985). The effectiveness of the purine/hypoxanthine combination in sustaining the relatively greater cAMP concentrations indicates these compounds could maintain meiotic arrest *in vitro* by preventing the catabolism of cAMP. It has been suggested that purines maintain the concentrations of cAMP by other mechanisms as well (Down et al., 1989), indicating the importance of these compounds for the maintenance of mouse oocytes in the GV stage *in vivo* (Downs and Eppig, 1987).

Bovine follicular fluid does not contain concentrations of hypoxanthine comparable to that in other animals (Eppig and Downs, 1988). Oocytes of cattle recovered from follicles and cultured in serum-free, hypoxanthine-supplemented medium have a sustained meiotic arrest at the GV stage (Senbon and Miyano, 2002). Its presence in the culture medium is thought to result in maintenance of cAMP concentrations in GC by inhibiting cAMP-PDE, maintaining the communication between the oocyte and surrounding GC of *in vitro*-developed cattle oocytes, and inhibiting the resumption of meiosis in the oocytes through this association.

Harada et al. (1997) reported that the number of oocytes with GC attached increased when COCs of cattle with pieces of parietal granulosa were cultured in hypoxanthine-supplemented medium. In this study, it was not clear why hypoxanthine promoted a continued association between oocytes and GC, but it is now known that IVM with inclusion of cAMP modulators results in an increased communication between the oocyte and CCs because the modulators maintain the integrity of gap junctions that are associated with maintenance of meiotic block. This communication also provides essential metabolic support for oocyte growth, maturation, and embryo development after fertilization (Albuz et al., 2010; Luciano et al., 2011).

#### 3.4.2. Isobutyl-1-methylxanthine (IBMX)

As a nonspecific inhibitor, IBMX blocks PDE activity in the oocyte and CCs; it can inhibit every PDE subtype except PDE8 (Sasseville et al., 2006), thereby preventing catabolism of cAMP in the oocyte. The IBMX inhibitor of PDE activity has been widely used in oocytes during IVM in different species in several studies (Albuz et al., 2010; Rose et al., 2013; Zeng et al., 2014; Appeltant et al., 2015; Santiquet et al., 2017). The cAMP concentrations decrease in oocytes treated with IBMX within 2 hours of inhibitor

removal from the media, at which point meiosis resumes in the oocytes (Minelli and Bellezza, 2011).

The PDE inhibitory activity of IBMX does not affect the expression of LH receptors in CCs surrounding the oocytes, whereas the binding of LH to its receptor induces a further increase in cAMP concentrations, progesterone production, and acceleration of meiotic progression to the metaphase I stage. Results of a detailed study of the effects of IBMX on the oocyte meiotic block (Barretto et al., 2007) indicate IBMX prevents the resumption of meiosis by maintaining the relatively greater cAMP concentrations in the oocyte. In IVF experiments, the synergistic actions of IBMX with FSH and LH synchronizes the oocytes, causing a greater blastocyst rate development in oocytes matured in these conditions. Shimada et al. (2003a, b) suggested that the treatment of oocytes with FSH and IBMX induces an increase in LH receptors in the plasma membrane of CCs, maintains the oocytes at the GV stage, and is a beneficial procedure to obtain *in-vitro*-matured oocytes with greater developmental competence as compared with CCs not treated with IBMX and FSH.

Short-term culture with IBMX and dbcAMP before the maturation of oocytes increased the capacity of oocytes to mature and contribute to embryo production in cattle (Huang et al., 2013) and swine (Funahashi et al., 1997). The supplementation with IBMX and PDE3 inhibitors also maintained the GJC that regulates oocyte RNA synthesis through a cAMP-mediated mechanism that results from actions in both the oocytes and CCs (Alam et al., 2018). Inclusion of IBMX with iAC in the COC collection medium has significant effects on subsequent oocyte development as indicated by Luciano et al. (1999). Appeltant et al. (2015) observed that the use of IBMX during oocyte collection did not induce meiotic arrest in pig oocytes which is inconsistent with the effects in mice and cattle, where IBMX addition increased oocyte cAMP concentrations during collection, inhibited GVB, and also improved the rate of blastocyst development (Bornslaeger et al., 1986; Luciano et al., 1999; Albuz et al., 2010).

#### 3.4.3. Caffeine (1,3,7-trimethylxanthine)

Caffeine is a plant-derived alkaloid and the first identified nonspecific PDE inhibitor. It functions as a nonselective competitive PDE inhibitor (Jiang et al., 1984) within the COC (Bernal-Ulloa et al., 2016). In oocytes, treatment with caffeine induces an increase in intracellular cAMP concentrations by binding to PDE enzymes, such as PDE-3 in the oocytes and PDE-4 and PDE-5 in the CCs, and thereby increases cAMP-dependent protein kinase activity, which in turn inhibits meiotic resumption (Kren et al., 2004; Miao et al., 2007).

The positive effect of caffeine supplementation during IVM on the development of oocytes following IVF has been reported in several species. In dromedary camel oocytes, caffeine supplementation during the last 6 hours of IVM can improve nuclear maturation and subsequent preimplantation development (Fathi et al., 2014). In dogs, caffeine treatment for 24 hours during IVM improves the total number of cleaved embryos following IVF (Fathi et al., 2018). Furthermore, in cattle, the use of caffeine prior to IVM also delayed meiosis progression and improved blastocyst survival after vitrification (Bernal-Ulloa et al., 2016).

Results of some studies indicate caffeine supplementation during IVM of mouse oocytes can sustain mitochondrial function, which is important for ATP production and subsequent maintenance of homeostasis during fertilization and embryo development (Dumollard et al., 2004; Zhang et al., 2011). In other studies, caffeine treatments resulted in a lesser rate of polyspermy in camels and sheep after IVF (Maalouf et al., 2009; Fathi et al., 2014). In sheep oocytes, caffeine increases MPF and mitogen-activated protein kinase activities, prevents age-related changes, and increases cell numbers in blastocysts produced by somatic cell nuclear transfer (Lee and Campbell, 2006, 2008). In experiments of nuclear remodeling of somatic cell nuclear transfer embryos, subsequent development, and DNA methylation patterns, caffeine increases the rates of premature chromosome condensation and blastocyst formation, and reduces the apoptotic cell index. This finding suggests that caffeine affects a type of nuclear remodeling that can affect *in vitro* development and the methylation status of nuclear transfer in relation to nuclear reprogramming (Kwon et al., 2008).

Although caffeine is widely used, its effects on oocyte maturation and subsequent embryo development have been inconsistent and species-dependent. In mice, caffeine treatment during IVM has a negative effect on oocyte nuclear maturation (Miao et al., 2007). In sheep, however, there is apparently a positive effect of caffeine supplementation during IVM on the developmental potential of oocytes after IVF (Maalouf et al., 2009).

#### 3.4.4. Dipyridamole

Dipyridamole is also a non-specific inhibitor that can modulate the activity of PDE 7, 8, 10, and 11 (Sasseville et al., 2009). Indeed, dipyridamole has been described and used as a potent inhibitor of PDE8 (Soderling et al., 1998).

Sasseville et al. (2009) provided evidence that PDE8 is an important factor for controlling cAMP concentrations in the ovarian follicle as an important functional PDE in CCs and in the oocyte, and demonstrated that inhibition of PDE8 in cattle COCs with dipyridamole which results in an increase in cAMP concentrations in the oocyte and delays meiosis. Furthermore, Santiquet et al. (2017) explained that could not be use of dipyridamole to consistently maintain meiotic arrest in the conditions used in the studies that were conducted.

The assumption was that this inhibition might negatively affect essential CC functions, such as gap-junction communication, cell signaling, or the supply of metabolites for oocyte or cumulus expansion, because although its use increases the cAMP concentrations in COCs and delays oocyte nuclear maturation, it does not improve oocyte developmental capacity. In another study, there was a lesser cleavage and blastocyst development rate after treatment with dipyridamole, suggesting that although PDE-8 is the predominant PDE in CCs. Its continuous inhibition by dipyridamole in this study during oocyte maturation did not promote oocyte cytoplasmic maturation and developmental competence (Sasseville et al., 2009).

Dipyridamole also inhibits the uptake of adenosine by oocytes, preventing an increase in ATP as induced by adenosine in mice. It, however, has no significant effect on the adenosine inhibition of maturation, suggesting that adenosine is not required to enter the cell (Salustri et al., 1988). In association with FK and IBMX, dipyridamole has a role, not only in oocyte competence when COCs are

cultured *in vitro*, but also in the inhibition of oocyte nuclear maturation. It can regulate cell metabolism, intracellular signaling, and cumulus expansion through modulation of PKA activity, which controls these functions and coordinates calcium signaling (Khan et al., 2015).

### 3.5. PDE3-specific inhibitors

The PDE3 gene has greater relative expression in the oocyte and this expression is responsible for the breakdown and concomitant inactivation of cAMP and cGMP, which are implicated in the regulation of oocyte meiotic maturation (Sasseville et al., 2006; Gilchrist et al., 2016). Inhibition of oocyte-specific PDE3 results in the sustaining of intra-oocyte cAMP concentrations, thus, maintaining oocytes in the arrested state at the GV stage, therefore, the function of the cAMP metabolic pathway is sustained in the surrounding CCs (Richard, 2007). Meiosis can be arrested by adding a PDE3 inhibitor to the medium either *in vivo* (Wiersma et al., 1998) or *in vitro* in cattle (Mayes and Sirard, 2002; Thomas et al., 2002), mice (Tsafriri and Pomerantz, 1986; Coticchio et al., 2004), and macaque (Jensen et al., 2002) oocytes.

#### 3.5.1. Cilostamide (CIL)

Effects of CIL treatment on *in vitro* developmental potential has been assessed in humans (Shu et al., 2008; Vanhoutte et al., 2009), cattle (Albuz et al., 2010; Luciano et al., 2011), sheep (Rose et al., 2013), and mouse (Nogueira et al., 2003; Vanhoutte et al., 2008; Jee et al., 2009; Albuz et al., 2010) oocytes, with outcomes varying by species, time of COC incubation, combination with other agents, and experimental approach. Furthermore, CIL treatment has a beneficial role in pig oocyte cumulus expansion, embryo cleavage, and blastocyst formation after IVM supplementation (Lee et al., 2017).

Treatment with CIL during IVM promotes a functional coupling between the oocyte and CCs by maintaining the function of GJC in cattle oocytes (Dieci et al., 2013), and this benefit occurs in combination with administration of other PDE inhibitors (Alam et al., 2018). Regarding associations between cAMP modulators, a partial improvement in the COC transport conditions was also observed when oocytes were transported in IVM-like medium supplemented with IBMX and FSK, and then with IVM with CIL (Dall'Acqua et al., 2017). Treatment with FSK and CIL pre-IVM resulted in more expanded CC layers and had positive effects on the developmental competence of oocytes in swine, possibly by improving cytoplasmic maturation (Park et al., 2016). In general, IVM studies have revealed that CIL has a reversible action in the regulation of meiotic progression, but long-term treatment of rat oocytes can cause irreversible changes (Tsafriri et al., 1996).

#### 3.5.2. Milrinone (MIL)

In conditions in which cAMP synthesis is stimulated, there is intra-oocyte accumulation of MIL but CC cAMP concentrations do not increase (Thomas et al., 2002). Results from studies, however, indicate CIL is more effective than MIL at maintaining meiotic arrest in cattle oocytes (Tsafriri et al., 1996).

The inhibitory effect of MIL on spontaneous meiotic resumption is either a direct result of the attenuation in GJC disruption, which may alter the capacity of an oocyte to undergo cytoplasmic maturation and to improve oocyte developmental potential, or a separate phenomenon attributable to the increase of oocyte cAMP (Thomas et al., 2004a, 2004b).

According to Naruse et al. (2012), treatment with MIL delays the time of polar body extrusion without impairing oocyte competence, improves the frequency of development to the blastocyst stage, and increases the yield of cloned embryos. Alam et al. (2018) reported that the inhibition of PDE3 with CIL and MIL results in the sustaining of meiotic arrest of cattle oocytes and retention of GJC functions of CCs for 5 days of growth culture, thereby contributing to the full acquisition of oocyte competence.

Treatment with MIL did not result in additional extra benefits when added to the IVM transporting medium (Dall'Acqua et al., 2017). Conversely, supplementation during IVM was important in many species. For example, this treatment inhibited meiotic resumption in rodent oocytes, improved developmental competence in both sheep (Wang et al., 2016) and cattle (Thomas et al., 2004a, 2004b) oocytes, and resulted in maintenance of pig oocytes in the GV stage (Gruppen et al., 2006). Different results between species may be due to differences in PDE3 activity and cAMP content. Treatment with several cAMP modulators, such as MIL, CIL, and IBMX, results in various effects on oocyte maturation and progression of embryonic development to the blastocyst stage, depending on the concentrations and/or time of treatment. The cAMP modulators, however, may negatively regulate oocyte meiosis if improper concentrations are used and/or oocytes are treated for too long (Racowsky, 1985).

#### 3.5.3. Cilostazol (CLZ)

The CLZ compound is an FDA-approved PDE3 inhibitor and a cAMP modulator recently used for IVM (Elahi et al., 2016; Xiong et al., 2017). The PDE3A compound is a member of the PDE family and is present in the oocytes of mice (Shitsukawa et al., 2001), cattle (Sasseville et al., 2006), humans, and swine (Gruppen et al., 2006). Cilostazol is a PDE3A inhibitor with the capacity to increase oocyte concentrations of cAMP by inhibiting its degradation (Elahi et al., 2016). Recently, Li et al. (2012) reported that there was a reversible effect of CLZ on the resumption of meiosis and blastocyst formation in oocytes, as well as on full-term development of fertilized embryos in mice, leading to the inference that CLZ affects oocyte meiotic resumption in a dose-dependent manner. There was also a similar response with use of CIL, a PDE3 inhibitor, in pig and macaque oocytes (Jensen et al., 2002). Interestingly, results indicate there is an association with pregnancy block in naturally estrous cyclic mice (Taiyeb-Albarzanchi et al., 2013), resulting in ovulation of oocytes that are in the GV or metaphase I developmental stages depending on dose, frequency, and time of administration (Taiyeb et al., 2014).

Treatment pre-IVM with CLZ increases intra-oocyte GSH concentrations (Zeng et al., 2014; Xiong et al., 2017). The

supplementation of CLZ during IVM is effective at preventing meiotic resumption and maintaining relatively greater concentrations of cAMP and GSH in yak oocytes, and there is subsequent improved preimplantation development following *in vitro* culture. This treatment during the early stages of IVM also improved the developmental competence of pig oocytes by influencing the intra-oocyte cAMP concentration and meiotic progression, thereby creating a greater coordination between nuclear and cytoplasmic maturation *in vitro* (Elahi et al., 2016). Intra-oocyte GSH content appears to be an important factor for oocyte cytoplasmic maturation and normal embryonic *in vitro* development in swine and cattle (Sakatani et al., 2007). Hence, its accumulation in oocytes during maturation positively affects fertilization and subsequent developmental competence (de Matos and Furnus, 2000).

Also, treatment during IVM with CLZ resulted in a more central distribution of cortical granules in oocytes in the GV stage of development compared with control oocytes in the same developmental stage, and increased intra-oocyte cAMP concentrations in mice. There appeared to be a synchronization of nuclear and cytoplasmic maturation as a result of CLZ treatment during IVM of oocytes in the GV developmental stage. The immature oocytes had lesser rates of meiotic aneuploidy, and there was a beneficial effect on fertilization rates and developmental competence (Taiyeb et al., 2017).

### 3.6. Rolipram

Rolipram is a selective PDE4 inhibitor that interferes with the PDE4 catalytic action through interactions with two types of sites (reviewed by Francis et al., 2011), preventing cAMP degradation and resulting in intracellular cAMP accumulation. With conditions in which cAMP synthesis is stimulated, Rolipram treatment increases cAMP concentrations in GC but not oocytes. The cAMP production by mural GC were consistent with those of CCs, indicating that the follicular somatic cells contain the Rolipram-sensitive type 4 PDEs (Thomas et al., 2002). The beneficial effects of using Rolipram, however, frequently occur when there is treatment in association with other modulators (Thomas et al., 2002; Thomas et al., 2004b).

Specific PDE subtypes localize differentially within the two compartments of the follicle: type 3 PDE to the oocyte and type 4 PDE to the GC (Thomas et al., 2002). The use of selective PDE inhibitors, such as MIL and CIL (PDE3 inhibitor), and Rolipram (PDE4 inhibitor) can enhance the understanding of the differential regulation of cAMP concentrations within the oocyte and somatic (cumulus) cell compartments of the follicle. This type of study represents a powerful experimental technique to ascertain the functions of cAMP at different concentrations in the two follicular compartments and may prove to be useful in the elucidation of precise mechanisms regulating mammalian oocyte maturation.

A 3-hour treatment with Rolipram during IVM attenuated the GJC disruption, though to a lesser extent than with MIL treatment (Thomas et al., 2004a, 2004b). According to Thomas et al. (2002), treatment with MIL, but not Rolipram, prevented the resumption of spontaneous meiotic maturation and increased intra-oocyte cAMP concentrations in cultured, denuded oocytes. Rolipram treatment alone, even at the largest dose, did not maintain any denuded oocytes at the GV stage. By contrast, Rolipram had no effect on the oocyte but did increase the mural granulosa and CC cAMP production. Other studies verified the efficacy of PDE3 inhibitors to inhibit the spontaneous resumption of meiosis (Albuz et al., 2010; Naruse et al., 2012), but this efficacy did not extend to PDE 4 inhibitors (Jensen et al., 2002).

### 3.7. Notable associations between modulators

The supplementation with cAMP modulators on IVM inhibits early nuclear maturation, improving oocyte quality by maintaining the integrity of gap junctions and subsequently increasing the physical association and communication between the oocyte and CCs (Albuz et al., 2010). This supplementation during IVM has also resulted in increased COC oxygen consumption and greater oocyte oxidative metabolism (Zeng et al., 2014), an initial increase and subsequent decrease in Epidermal Growth Factor (EGF) pathway genes (Richani et al., 2014), steroidogenesis (Jamnongjit et al., 2005), and intracellular signaling (Ghayor et al., 2009), all of which are implicated in oocyte quality. In turn, oocyte quality is directly associated with an increase in early embryonic developmental competence (Katska-Ksiazkiewicz et al., 2007). Based on the improvement associated with the use of these substances (Table 1), in some studies there has been different modulators used during IVM to optimize this approach.

#### 3.7.1. FK and IBMX

Together, treatment with FK and IBMX results in a sustaining of intra-oocyte cAMP and reversible meiotic arrest in sheep (Rose et al., 2013; Buell et al., 2015), cattle (Thomas et al., 2002; Ezoie et al., 2015; Farghaly et al., 2015), and mouse (Albuz et al., 2010) oocytes. The choice of FK and IBMX is based on a two-way pathway that increases oocyte cAMP through a dual mechanism: 1) stimulation of AC by FK to produce cAMP and 2) inhibition of PDE activity by IBMX to prevent cAMP degradation.

Treatment before IVM with FK and IBMX resulted in an increase in rates of cumulus expansion, fertilization, cleavage, blastocyst formation, and blastocyst hatching. These treatments in combination with FSH during IVM inhibit the resumption of meiosis and enhances oxidative metabolism and ATP production (Zeng et al., 2014). Supplementation of FK and IBMX in transport medium delays the resumption of meiosis during transport for 6 hours, as the percentage of GV oocytes from these treatments is greater than in those oocytes transported without meiosis blockers or cultured throughout the period within an incubator (Dall'Acqua et al., 2017).

The use of these modulators can prolong maintenance of the GJC (Albuz et al., 2010) and tends to improve the developmental competence after IVF of vitrified-warmed GV oocytes (Ezoie et al., 2015). Furthermore, Monteiro et al. (2017) reported that a short-term culture with FK and IBMX applied before vitrification also resulted in an improvement of oocyte cryotolerance, as indicated by an increase in the integrity of cytoskeletal actin filaments, which have an important role in meiosis progression.



**Table 1**  
Effects of different pharmacological agents used during oocyte *in vitro* maturation to modulate cAMP concentrations in domestic species

Species	Modulator	Modulator Action Pathway	Results	Reference
Cattle	IBMX	PDE Inhibitor	Improved embryo development	Albuz et al., 2010, Huang et al., 2013 Bernal-Ulloa et al., 2016 Luciano et al., 2011, Dieci et al., 2013 Thomas et al., 2004a, 2004b
	Caffeine			
	Cilostamide			
	Milrinone			
	Rolipram			
Pig	Forskolin	Adenylate cyclase stimulator	Improved oocyte competence and embryo development	Albuz et al., 2010, Li et al., 2016 Luciano et al., 1999, Luciano et al., 2004 Appeltant et al., 2015 Dieci et al., 2013, Lee et al., 2017 Elahi et al., 2016 Park et al., 2016 Funahashi et al., 1997, Bagg et al., 2006 Kim et al., 2008, Nascimento et al., 2010 Appeltant et al., 2015 Rose et al., 2013 Rose et al., 2013 Rose et al., 2013 Fathi et al., 2018
	IAC	Invasive adenylate cyclase	Improved oocyte competence	
	IBMX	PDE Inhibitor	Improved oocyte competence and embryo development	
	Cilostamide			
	Cilostazol			
Sheep	Forskolin	Adenylate cyclase stimulator	Improved embryo development	Rose et al., 2013 Maalouf et al., 2009 Rose et al., 2013 Rose et al., 2013 Fathi et al., 2018
	dcAMP	cAMP analog	Improved oocyte competence	
	IBMX	PDE Inhibitor	Improved oocyte competence	
	Caffeine		Improved oocyte competence	
	Cilostamide			
Dog	Forskolin	Adenylate cyclase stimulator	Improved oocyte competence	Rose et al., 2013 Rose et al., 2013
	Caffeine	PDE Inhibitor	Improved embryo development	

Abbreviations: IBMX 3-isobutyl-1-methylxanthine; iAC invasive adenylate cyclase; dbcAMP dibutyryl cAMP; PDE phosphodiesterase

### 3.7.2. SPOM system (FK, IBMX, and CIL)

Simulated physiological oocyte maturation (SPOM) is a two-phase IVM system where there is a 2-hour pre-maturation phase in which medium is supplemented with FK and IBMX, followed by an extended IVM (28 hours) phase with treatments with CIL and a large dose of FSH (Albuz et al., 2010). Because it was established, the IVM-SPOM system has attracted considerable scientific and commercial attention as it has proven to be effective in delaying meiotic progression in several species such as cattle (Bernal-Ulloa et al., 2016), swine, sheep (Rose et al., 2013), and mice (Albuz et al., 2010). The use of the system, however, has resulted in inconsistent results (Guimarães et al., 2015; Leal et al., 2018), and according to its developers, it is not consistently effective when used with different conditions (Gilchrist et al., 2015).

Results of further studies indicate there are benefits of the SPOM system specifically in the pre-IVM treatment alone (Richani et al., 2014; Zeng et al., 2014), which involves treatment with FK and IBMX. This finding led to the concept of SPOM version 2, wherein there is no use of CIL during IVM (Gilchrist et al., 2015). While the beneficial effects of cAMP-modulated pre-IVM treatments on oocyte competence are well accepted, the optimal duration of pre-IVM treatment is unknown. Extending pre-IVM treatment period for cAMP modulators beyond 1 to 2 hours appears to be beneficial for mouse oocyte quality (Richani et al., 2014), and 6 hours of pre-IVM treatment appeared to be optimal for cattle oocytes (Li et al., 2016).

### 3.7.3. PDE Inhibitors

Due to the similarity between PDE subtype sequences across species, it is likely that the presence of other subtypes that are similar in regulation and function should also function similarly in other mammalian species. It follows that rodent oocytes are experimentally more responsive than those of ungulates to cAMP and its analogues, as well as to PDE inhibitors (Schultz, 1991).

In several studies, there has been a combined treatment with nonspecific and specific PDE inhibitors to improve IVM conditions for oocytes (Thomas et al., 2002; Thomas et al., 2004a, 2004b). The aim was to increase intra-oocyte cAMP concentrations by inhibiting its degradation in oocytes and CCs, considering the differential regulation of cAMP concentrations within the oocyte and somatic cell compartments of the follicle. The inhibition of meiotic resumption of fully developed oocytes using PDE inhibitors has been studied extensively *in vitro* (Gilchrist et al., 2016). According to Alam et al. (2018), IBMX, CIL, and MIL treatments induce antrum formation by COCs and maintain meiotic arrest in oocytes in culture, whereas treatment with Rolipram neither promotes antrum formation nor maintains oocyte meiotic arrest. There is maintenance of GJC between oocytes and CCs with IBMX and CIL treatments. Results of studies performed with MIL and Rolipram indicate the inhibitory effect of MIL on spontaneous meiotic resumption is a direct result of the attenuation in GJC loss. Alternatively, this effect may be due to a separate phenomenon attributable to the increase of oocyte cAMP, in which treatment with Rolipram is a significant contributor to the total cAMP content of the oocyte (Thomas et al., 2004a, 2004b).

### 3.8. Further associations

In addition to the association between different modulators for IVM supplementation, results of some studies indicate associations with other substances that may result in the maintenance of meiotic arrest. For example, butyrolactone I treatment efficiently induces a meiotic block probably because it is a potent and specific inhibitor of cyclin-dependent kinases, however, it has few inhibitory effects on other protein kinases such as mitogen-activated protein kinase (Kubelka et al., 2000). Riscovitin is also used for this purpose, and it might induce even more notable changes such as disruption of the integrity of the surrounding CCs, swelling of the

mitochondrial crista, and degeneration of the cortical granules (Lonergan et al., 2003). It, therefore, is important to identify substances that can modulate oocyte cAMP concentrations without adversely affecting the oocyte.

In fact, cAMP and cGMP are key molecules regulating processes in mammalian oocytes, and it is widely known that moderate to high intra-oocyte concentrations of the second messenger cAMP maintain oocyte meiotic arrest (Cho et al., 1974). The maintenance of meiotic arrest, however, has recently been enhanced by the elucidating the important contribution of natriuretic peptides in this process. The C-type natriuretic peptide (CNP), for example, can increase cGMP intracellular concentrations through stimulatory mechanisms in both CCs and the oocyte, and it results in PDE3 inhibition that preserves the relatively greater cAMP concentrations that are present in these cells. The CNP is known to be a peptide meiosis inhibitor *in vitro* (Franciosi et al., 2014), leading to the possibility for associations with other substances that modulate cAMP actions in the future.

#### 4. Conclusions

As evidenced by the current review, cAMP modulators have an important role during IVM, and all approaches to increase its concentrations can result in some improvement in oocyte competence. It is important to emphasize that the interval between the removal of the oocyte from follicle and the commencement of treatment to preserve cAMP concentrations is a very important aspect of the efficacy of treatment and the prolonged treatment with modulators can reduce the effectiveness of these compounds. Also, different doses, exposure time, modulator action pathways, and species response differences to these compounds influence the outcomes. Nevertheless, supplementation with cAMP modulators during IVM certainly has the potential to prolong meiotic arrest, thereby resulting in a recovery of the synchrony between nuclear and cytoplasmic maturation and greatly contributing to the optimization of IVM.

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#### Conflict of interest

The authors declare that they have no conflict of interest.

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