Cilostamide and forskolin maintain gap junction function of incubated dog follicles

Running head: cAMP modulators sustained gap junction function

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Highlights

- Supplementation of cilostamide and/or forskolin enhances cAMP production from dog secondary follicles, upregulates genes associated with gap junction communication and oocyte growth as well as improves TZP functionality.

1 **Abstract**

2 Disruption of the communication between the oocyte and granulosa cells is one of the major

3 causes of poor development of in vitro grown follicles and oocytes. The present study investigated

4 the effect of two cAMP modulators, cilostamide and forskolin on in vitro growth of isolated dog

5 secondary follicles and enclosed oocytes, communication between the gamete and surrounding

6 granulosa cells, expression of *GJA1* and *GDF9*, as well as cAMP level. Secondary follicles were

7 incubated with cilostamide or forskolin alone or a combination of 20 µM cilostamide + 1 µM

8 forskolin, and the diameter of the incubated follicles and enclosed oocytes assessed every 72 h.

9 Gap junction activity, transzonal projection (TZP) density, *GJA1* and *GDF9* expression and cAMP

10 level were assessed on Days 6 and 12. Neither cilostamide nor forskolin alone enhanced in vitro

11 growth of dog follicles and the enclosed oocytes ($P > 0.05$). However, these two cAMP modulators

12 dose dependently sustained gap junction activity and stimulated cAMP production compared with

13 the non-supplemented control. Cilostamide at the high dosage (20 µM) also upregulated *GJA1*

14 expression. The combination of cilostamide and forskolin supported oocyte growth during the first

15 9 days and upregulated *GJA1* and *GDF9* expression at Day 12 of in vitro culture. This combination

16 treatment also sustained gap junction activity, cAMP production and increased TZP function

17 (calcein intensity: TZP density ratio). The findings indicated that a combination of cilostamide and

18 forskolin supported growth and survival of oocytes enclosed within cultured follicles by sustaining

19 cAMP production and gap junction activity.

20 **Keyword:** dog, oocyte, follicle, Cilostamide, Forskolin

21

22 **Introduction**

23 Research on in vitro culture of dog ovarian follicles has increased during the past decade.

24 Attempts have focused on expanding knowledge of reproductive biology and translating the

25 information to endangered canid species [1], with several studies evaluating the influences of

26 gonadotropin and growth factor supplementation [2-4], follicle isolation methods [1], and physical

27 microenvironment [5, 6] on in vitro growth of isolated preantral follicles. Nevertheless, the ability

28 to grow dog preantral follicles to obtain healthy oocytes in vitro is still limited. Our laboratory has

29 previously shown that, as in vitro culture progress, incubated oocytes lose the typical 'dark'

30 cytoplasm and become lighter in color [7]. Furthermore, evaluation of the nucleus of these oocytes

31 has demonstrated disruption of nuclear chromatin or complete loss of chromatin material which

32 are the indications of cell degeneration. A study in bovine follicles also has demonstrated the loss

33 of gap junction in incubated follicles after 4 days of culture [7]. Collectively, the inability to maintain

34 oocytes' viability during in vitro culture of dog preantral follicles is likely due to the disruption of

35 the bi-directional communication between the gamete and the surrounding granulosa cells.

36 In ovarian follicles, gap junctions are formed during early oogenesis by the penetration of

37 cytoplasmic process (TZPs) of granulosa cells into oocyte cytoplasm to transport nutrients and

38 metabolic precursors to the gamete [8]. Cyclic adenosine monophosphate (cAMP) is a small

39 molecular weight molecule synthesized in the oocyte by constitutively active G-protein coupled

40 receptor type-3 (GPR3) [9]. This molecule is also supplied to the oocyte by cumulus cells through

41 the gap junction [10]. It has been established that cAMP regulates the growth and survival of the

42 follicle as well as the differentiation of its enclosed oocyte [11, 12]. cAMP modulating agents (e.g.,

43 phosphodiesterase [PDE] inhibitor and adenylyl cyclase activator) have been used in several

44 species such as bovine [13], porcine [14], murine [15, 16], and human [17, 18] to stimulate

45 intracellular cAMP levels of oocytes. Increased cAMP level prevents the loss of gap junction-

46 mediated communication and enhances gamete developmental competence as well as regulates

47 chromatin remodeling and transcriptional silencing [10]. Cilostamide inhibits PDE type 3, which is

48 the major active cAMP-PDE in mammalian oocytes [19, 20]. Incubating human ovarian cortices

49 with cilostamide increases cAMP production and supports the growth and survival of the enclosed

50   follicles [17]. Forskolin is an adenylyl cyclase activator which has been shown to maintain meiotic

51   arrest at the GV stage by increasing intraoocyte cAMP in the rat [21], mouse [22], cow [23] and

52   pig [24].

53   To date, there have been no studies on the impact of cAMP modulating agents on in vitro growth

54   of dog follicles. Therefore, the objectives of the present study were to investigate the effects of

55   cilostamide and forskolin on 1) in vitro growth of dog secondary follicles and the resident oocytes,

56   2) communication between the gamete and surrounding somatic cells,3) the expression of gap

57   junction protein-1 (*GJA1*) and oocyte derived growth factor, growth differentiation factor 9 (*GDF9*),

58   and 4) cAMP production. We hypothesized that cilostamide and/or forkolin stimulate cAMP

59   production and sustain gap junction mediated communication, that in turn promote in vitro

60   development of dog preantral follicles and the enclosed oocyte.

61

62   **Materials and methods**

63   All chemicals used in this study were purchased from Sigma Aldrich, St Louis, MO, USA, unless

64   stated otherwise.

65   2.1 Collection of ovaries and isolation of dog preantral follicles

66   Ovaries were collected opportunistically from dogs (age, 5 mo – 4 yr) undergoing routine

67   ovariohysterectomy at local spay clinics and transported at 4°C to the laboratory within 1 to 5 h

68   after surgery. Cortices were dissected from the ovaries and incubated in collection medium

69   (Eagle's αMEM; Irvine Scientific, Santa Ana, CA, USA) containing 2 mM L-glutamine, 40 IU/ml

70   penicillin, 40 µg/ml streptomycin, 1mM HEPES, 3 mg/ml BSA, and 3 mg/ml collagenase for 40

71   minutes at 38.5°C. At the end of the incubation, the collagenase reaction was stopped by

72   adding collection medium containing 20% fetal bovine serum (1:1), and the digested cortices

73   were vortexed vigorously for 1 min. Secondary follicles (preantral) were recovered from the

74 suspension and follicles that remained partially dissociated from the cortices were mechanically

75 dissected from the tissue using 25 G needles. Isolated follicles with normal morphology (round,

76 homogenously dark centralized oocyte, intact basement membrane [5]) were selected under

77 stereomicroscope (Nikon SMZ-2T, Tokyo, Japan) and stained with 50 µg/ml neutral red at

78 38.5°C for 20 min to assess follicle viability [25] and those which appeared morphologically

79 normal and viable (neutral red positive) were used for the study.

80 2.2 In vitro culture of secondary follicles

81 Isolated secondary follicles were encapsulated in 0.5% alginate hydrogel (Pronova UP MVG;

82 Novamatrix, Sandvika, Norway) as previously described [5] and cultured in 500 µl of basic culture

83 medium (Eagle's αMEM with 2 mM L-glutamine, 50 IU/ml penicillin G sodium, 50 µg/ml

84 streptomycin sulfate, 1 mg/ml BSA, 4.2 µg/ml human insulin, 3.8 µg/ml human transferrin, and 5

85 ng/ml sodium selenite, and 50 µg/ml ascorbic acid) supplemented with 1 µg/ml recombinant

86 porcine FSH (Folltropin-V; Bioniche Animal Health, Belleville, Ontario, Canada) and randomly

87 assigned into one of the following treatments: cilostamide (1, 10 and 20 µM in dimethyl sulfoxide

88 [DMSO]) alone, forskolin (1, 10 and 20 µM in DMSO) alone, or 20 µM cilostamide + 1 µM forskolin.

89 Follicles cultured without the cAMP modulator were served as non-supplemented controls.

90 Follicles cultured in basic culture medium with DMSO (0.3% v/v) served as vehicle controls.

91 Follicles in all treatments were cultured in a humidified atmosphere of 5% CO_2 in air for 12 days.

92 Medium was half-changed every 3 days throughout culture period.

93 2.3 Assessment of follicle and oocyte growth

94 The mean diameter of each follicle/oocyte was recorded on Day 0, 3, 6, 9 and 12 using an inverted

95 microscope)Leitz DMIL, Leica Microsystem, Buffalo Grove, Illinois, USA(equipped with an ocular

96 micrometer. Each follicle was sized from the outer layer of somatic cells, with the measurements

97 including the widest diameter and perpendicular width to the initial assessment. The mean of

98   these two metrics was calculated and reported as diameter. Oocyte size, excluding the zona

99   pellucida, was evaluated using the same method. The growth rate was calculated as the

100   difference in diameter (μm) assessed at Day 0 using the below formula. Nine culture replications

101   were performed in this experiment.

102    $$Relative\ growth\ at\ days\ (3,6,9,12) = [\frac{follicle\ or\ oocyte\ diameter\ at\ days\ (3,6,9,12)}{follicle\ or\ oocyte\ diameter\ at\ day\ 0}] - 1$$

103   2.4 Assessment of granulosa cell and oocyte communication

104   The communication between granulosa cells and the enclosed oocyte was assessed on Day 6

105   (N=100) and 12 (N=83) of in vitro culture using calcein-AM staining as previously described [18].

106   In brief, follicles were mechanically isolated from alginate beads and stained with 5 μM calcein-

107   AM (non-fluorescent, Invitrogen, Carlsbad, CA, USA) for 12 minutes to allow the acetoxymethyl

108   group in calcein-AM to permeate into the cytoplasm of granulosa cells, but not the oocyte. Follicles

109   were subsequently washed in the culture medium and incubated for 30 min to allow non-specific

110   endogenous esterase in the granulosa cells to cleave the lipophilic acetoxymethyl groups and

111   producing calcein (flurorescent with negatively charged molecule that is unable to leak out of the

112   cells across plasma membrane, but able to pass between the cumulus cells and oocyte via gap

113   junctions [26]). At the end of the incubation, the follicles were mounted on microscopic slides and

114   assessed for fluorescent emission of calcein under a fluorescence microscope. The fluorescent

115   intensity in the oocyte cytoplasm was quantified using ImageJ software (National Institute of

116   Health, Bethesda, MD, USA). Follicles (N=10) stained with calcein-AM but were not subjected to

117   the 30 min incubation in calcein-free medium were served as a negative control.

118   2.5 RNA extraction

119   Total RNA was extracted from a group of follicles on Days 6 and 12 (n=6-9/treatment/replicate)

120   using absolutely RNA nanoprep kit (Agilent technologies, Santa Clara, CA, USA) following the

121   manufacturer's instructions. Extracted RNA was treated by RapidOut DNA removal kit (Thermo-

122   Scientific, Carlsbad, CA, USA) to remove genomic DNA contamination. The quantity and purity

123   of extracted RNA was assessed using a Nanodrop-One spectrophotometer (Thermo- Scientific,

124   Carlsbad, CA, USA).

125   2.6 Quantification analysis of *GDF9* and *GJA1* expression

126   Complementary DNA was synthesized from mRNA (2.3 ng from each sample) using Transcriptor

127   High Fidelity cDNA synthesis kit (Roche, Basel, Switzerland) according to the manufacturer's

128   instructions. The product was stored at -20⁰C until qPCR analysis. The gene-specific primers

129   were described in Table 1. The relative expression of all genes was normalized to an endogenous

130   control gene, *β-actin*. Each PCR reaction (total volume 20 μl) consisted of 2 μl DNA and 18 μl of

131   reaction mixture that contained 10 μl of FastStart Essential DNA green master (Roche, Basel,

132   Switzerland), 1 μl each of 10 μM forward and reverse primers of each genes, and 6 μl of nuclease

133   free water. The reactions were performed with the following settings 95⁰C for 10 min, followed by

134   45 cycles of 95⁰C for 10 sec, specific annealing temperature (Table 1) for 30 sec, and 72⁰C for 10

135   sec. All amplifications were performed in triplicate using LightCycler® 96 (Roche, Basel,

136   Switzerland). Reactions with nuclease free water were performed in parallel as a negative control.

137   Primer efficiency was assessed in each gene by serial dilution of cDNA. Calculation of mRNA

138   expression levels was performed by the comparative Ct method using the amplification efficiency

139   of each gene as a correction factor. Relative quantitations were reported as n-times difference in

140   relation to the non-supplemented control group assessed either Day 6 or Day12 [27].

141

142   2.7 Transzonal projections (TZPs) analysis by confocal microscopy

143   At the termination of culture, cumulus oocyte complexes (COCs) were mechanically

144   recovered from incubated follicles then fixed in 4% paraformaldehyde for 12 hours. Fixed COCs

145 were then stored in wash buffer (0.2% azide, 2% normal goat serum, 1% bovine serum albumin,

146 0.1 M glycine, and 0.1% Triton X-100) at 4ºC until analysis [28]. COCs (N = 136) were stained for

147 actin using Alexa Fluor 488 Rhodamine Phalloidin (Invitrogen, at 1:100 dilution in wash buffer)

148 and for chromatin with 1 µg/ml (2.2 µM) Hoechst 33342 (in a 90% glycerol, 10% PBS solution) for

149 at least 30 min at room temperature. No more than four oocytes per treatment group were

150 mounted on a microscopic slide with GVA Aqueous Mounting Solution (Genemed

151 Biotechnologies, San Francisco, CA, USA) then imaged at 100x with an LSM 510 laser scanning

152 confocal microscope (Carl Zeiss, Germany) with a 488 nm krypton/argon laser and ultraviolet

153 light. Z-stack images of each COC were taken and TZPs were pseudo-colored using ImageJ

154 software (NIH, Bethesda, MD, USA). The z-stack image of an oocyte at its widest diameter was

155 used for TZP assessment. The circumference of the oocyte was measured in this slice, and TZPs

156 were counted and density calculated per 1 µm oocyte circumference.

157

158 2.8 cAMP assay

159 Cyclic AMP levels were assessed in spent culture media on Days 6 and 12 day using direct

160 immunoassay kit obtained from Abcam (ab65355; Cambridge, MA, USA). Following assay

161 validation for sample type, test samples were diluted 1:4 using with 0.1 M HCl, and all assays

162 were performed as directed by the manufacturer protocol.

163

164 2.9 Experimental design

165 Secondary follicles (N = 466) were isolated from ovarian cortices and cultured with various

166 concentrations of cilostamide or forskolin alone (1, 10, 20 µM) or a combination of 20 µM

167 cilostamide + 1 µM forskolin for 6 or 12 days to determine relative growth of follicle and oocyte

168 and calcein intensity. The diameter of incubated follicles and resident oocytes were assessed

169 every 72 h and at the end of incubation)Day 12). Based on the results of in vitro follicle and oocyte

170   growth as well as calcein intensity, incubated follicles from non-supplemented control, 20 μM

171   cilostamide, 1 μM forskolin and a combination of 20 μM cilostamide + 1 μM forskolin treatments

172   were further evaluated for mRNA expression of *GJA1* and *GDF9*, cAMP levels on Days 6, and 12

173   and TZPs density at the end of incubation (Day 12).

174

175   2.10 Statistical analysis

176   All data were reported as mean ± SEM. All data were tested for normality using the Shapiro Wilk

177   normality test and variance homogeneity using the Barlett's test. Differences in mean percentages

178   of follicle and oocyte growth, calcein-AM intensity, and TZP density among treatments were

179   analyzed using one way ANOVA followed by a Dunn's multiple comparison test. Differences in

180   mRNA expression were evaluated using a one-way ANOVA followed by a Tukey's multiple

181   comparison test. All statistical analyses were performed using Graphpad Prism version 5.0 for

182   Windows)GraphPad Software, La Jolla, CA, USA(. Differences with a value of $P < 0.05$ were

183   considered statistically significant.

184

185   **Results**

186   Exposure of dog follicles to cAMP modulators (cilostamide or forskolin) did not enhance follicle

187   growth compared to the non-supplemented control. With a few exceptions, overall relative growth

188   of incubated follicles was not different ($P > 0.05$) among treatments (Fig. 1a). At Day 3 of

189   incubation, relative growth of follicles incubated with the high dosage (20 μM) of cilostamide or

190   forskolin was lower ($P < 0.05$) than the non-supplemented control and 1 μM forskolin and

191   cilostamide + forskolin treatments with those cultured in other conditions exhibited intermediate

192   growth. The detrimental effect of the high dosage of cilostamide continued through the culture

193   period as follicles in this treatment exhibited the smallest growth rate ($P < 0.05$) at Day 12

194 compared with other conditions. The growth of follicles incubated in the presence of both cAMP

195 modulators reached the peak level at Day 9, then declined to similar levels as those cultured in

196 20 µM cilostamide.

197 With the exception of the combination treatment, the diameter of oocytes decreased during in

198 vitro incubation. The oocytes in the combination treatment slightly increased in size after 9 days

199 of in vitro culture. However, the benefit of the combination treatment was not observed at Day 12

200 (Fig. 1b). To assess gap junction activity, we quantified the transfer of calcein-AM dye from

201 granulosa cells into the enclosed oocyte. Low calcein intensity was observed in follicles treated

202 with 10 µM forskolin compared with the non-supplemented control and 10 µM cilostamide

203 (4.97±3.45 vs 23.43±5.61 and 21.83±6.83, respectively, Fig. 2). On Day 12, cilostamide dose

204 dependently supported gap junction activity, as calcein intensity was higher ($P < 0.05$) in oocytes

205 incubated in 20 µM cilostamide than those cultured in the lower concentrations (Fig. 2). Although

206 the intensity of calcein dye was lower in oocytes incubated in 1 µM forskolin than 20 µM

207 cilostamide treatment, the level did not significantly ($P > 0.05$) differ from those incubated in the

208 higher forskolin doses (Fig. 2).

209 Although on the presence of cAMP modulators did not impact *GJA1* and *GDF9* expression on

210 Day 6, *GJA1* was upregulated in Day 12 follicles treated with 20 µM cilostamide and the

211 combination treatment, and the expression of *GDF9* was upregulated in the combination

212 treatment (Fig. 3).

213 TZP density significantly decreased after in vitro culture (Fig. 4F). cAMP modulator treatments

214 did not sustain TZP density as the TZP numbers significantly ($P <0.05$) decreased after in vitro

215 culture compared to fresh follicles. Furthermore, the density of TZP in the combination treatment

216 was lower than that of 1 µM forskolin (0.08±0.01 vs 0.12±0.01, Fig. 4F). We further determined

217 the functionality in communication (calcein intensity) per number of connections (TZP density) on

218 Day 12 follicles. The ratio of calcein intensity per TZP density was highest in the combination

219   treatment (1148.3) compared to control (673.25), 20 µM cilostamide (789.12), and 1 µM forskolin

220   (442.07).

221   Extending the culture period to 12 days significantly decreased cAMP concentration compared to

222   the Day 6 level in the non-supplemented control (Fig. 5). However, the presence of 20 µM

223   cilostamine or cilostamine + forskolin combination sustained ($P < 0.05$) cAMP levels during in vitro

224   culture. Although cAMP level of 1 µM forskolin treatment was low ($P < 0.05$) at Day 6, extending

225   the culture period to 12 days increased cAMP concentration to the same level as that observed

226   in other cAMP modulator treatments. Finally, supplementation of either cilostamide or forskolin

227   alone, but not the combination increased cAMP level on Day 12 compared to that observed on

228   Day 6 (0.75±0.01 and 0.72±0.06 vs 0.70±0.02 and 0.53±0.07 pmol/ml, $P < 0.05$, Fig. 5).

229   **Discussion**

230   Bi-directional communication between the oocyte and its companion somatic cells through gap

231   junctions is essential for folliculogenesis [29]. Disruption of the bidirectional communication has

232   been suggested as one of the major causes of subpar development of incubated follicles and the

233   resident oocyte. A previous study from our group has shown the cytoplasm of dog oocytes exhibits

234   pale appearance and gamete's chromatin becomes degenerated as in vitro incubation progress

235   [30]. Activation of cAMP has been shown to promote gap junction assembly [31]. In the present

236   study, we investigated the effect of two cAMP modulators, cilostamide and forskolin on in vitro

237   growth of dog follicles and oocytes as well as the communication between the companion somatic

238   cells and the gamete. We discovered that both cilostamide and forskolin supported cAMP

239   production during in vitro culture but supplementing a culture medium with either cilostamide or

240   forskolin alone failed to enhance in vitro growth of isolated secondary follicles. Incubating dog

241   secondary follicles in 20 µM cilostamide + 1 µM forskolin maintained cAMP production, as well as

242   sustained the survival and stimulated growth of the enclosed oocytes likely by supporting the gap

243   junction activities during in vitro culture.

244   cAMP is a second messenger that is involved in a variety of cellular processes including regulating

245   gap junction protein assembly [31]. Previous studies have examined the impact of cAMP or its

246   modulators on cumulus cell-oocyte communication and developmental competence of the

247   gamete [18, 19, 26, 32]. It is known that cAMP maintains meiotic arrest in mammalian oocytes.

248   Decreased intra- oocyte cAMP levels trigger oocyte maturation [18]. To date, few studies have

249   investigated the impact of cAMP on in vitro follicle and oocyte growth. Dibutyryl cAMP has been

250   shown to promote mouse follicle growth and survival by increasing expression of kit ligand in

251   granulosa cells [33, 34]. In the human, 8-Bromo-cAMP has been shown to promote the transition

252   of primordial follicles enclosed within the ovarian cortex to secondary follicles during 14 in vitro

253   culture [17]. However, the mechanism by which this cAMP analogue supporting in vitro follicle

254   growth was not investigated.

255   In the present study, we examined the impact of a PDE3 inhibitor, cilostamine and an adenylyl

256   cyclase activator, forskolin on cAMP production, gap junction function and in vitro folliculogenesis.

257   We demonstrated that cilostamine and forskolin stimulated cAMP production and dose

258   dependently enhanced gap junction activity. However, the presence of one of these two cAMP

259   modulators alone did not promote in vitro growth of dog secondary follicles. The dose-dependent

260   effect of cilostamide and forskolin on calcein dye transfer observed in the present study was

261   similar to that previously observed in the rat cumulus-oocyte complex [35]. In the rat study, the

262   positive effect of PDE3 inhibitors on gap junction function was short-lived, as prolonged exposure

263   of cumulus-oocytes complexes to these compounds reduced gap junction activity. Furthermore,

264   Nogueira et al [36] also have shown that long term exposure (12 days) of mouse follicles to

265   cilostamide did not alter somatic cell proliferation, differentiation or follicle survival. Therefore, the

266   lack of stimulating effect of cilostamide and forskolin on in vitro growth of dog follicles and oocytes

267   may be due to the inability of these compounds to sustain gap junction activity during a long-term

268   culture as the functionality in communication was lower than the combination treatment.

269   Although the combination of a high dosage (20 μM) of cilostamide + a low (1 μM) forskolin did not

270   stimulate in vitro follicle growth, this treatment was able to sustain oocyte survival and modestly

271   promote gamete growth. This finding is supported by the finding that cilostamide + forskolin

272   treatment upregulated mRNA expression of *GDF9*, a gene that encode oocyte-secreting growth

273   factor. The beneficial effect of the combination treatment on the survival and growth of the oocyte

274   was probably linked to increase accumulation of intracellular cAMP level that in turn supported

275   gap junction function and communication between the gamete and surrounding granulosa cells.

276   Our findings that cilostamide + forskolin treatment upregulated *GJA1* expression and follicles

277   incubated with this treatment exhibited a higher calcein intensity and calcein intensity: TZP density

278   ratio than other in other treatments supported this assertion.

279   **Conclusion**

280   In conclusion, this is the first report on the effect of cAMP modulators (cilostamide and/or forskolin)

281   on in vitro development of dog pre-antral follicles and oocytes. Supplementation of cilostamide

282   and/or forskolin enhanced cAMP production from dog pre-antral follicles and upregulated gene

283   associated with gap junction communication and oocyte growth and improved TZP functionality.

284   The findings supported our hypothesis that the inability to maintain the viability and promote

285   growth of dog oocytes enclosed within incubated preantral follicles is, in part, due to the disruption

286   in communication between the gamete and surrounding somatic cells. The findings will assist in

287   the development of an in vitro microenvironment that supports survival and growth of preantral

288   dog follicles and oocytes.

289

290   **Conflicts of interest**

291   The author declares that there is no conflict of interest.

292

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397   **Figure legends**

398   **Figure 1**. Relative growth)mean ± SEM(of (a) secondary follicles and (b) resident oocytes

399   cultured for 12 days in medium supplemented with cilostamide or forskolin alone (0, 1, 10 or 20

400   μM) or 20 μM cilostamide + 1 μM forskolin. [a,b,c] Different superscripts indicate significant

401   differences among treatments within the same culture day)P <0.05(.

402   **Figure 2.** Calcein intensity (mean ± SEM) at Day 6 and 12 in oocytes enclosed within follicles

403   cultured in medium supplemented with cilostamide or forskolin (0, 1, 10 or 20 μM) alone or 20 μM

404   cilostamide + 1 μM forskolin (C20F1). [A,B] indicate significant difference among treatments on day

405   6)P <0.05(. [a,b,c] indicate significant differences among treatments on day 12)P <0.05(.

406   **Figure 3**. Relative expression)mean ± SEM(of genes regulating gap junction communication

407   (*GJA1*) and oocyte growth)*GDF9)* in follicles at Days 6 and 12 of culture in medium without cAMP

408   modulator supplementation (Control), 20 μM cilostamide, 1 μM forskolin, or the combination of

409   cilostamide 20 μM and forskolin 1 μM (C20F1). Bars show average, and [a,b] indicate significant

410   differences among treatments on day 12)P <0.05(.

411   **Figure 4.** TZP staining for actin (Alexa Fluor 488 Rhodamine Phalloidin) and nuclei (Hoechst

412   33342) in fresh COCs (A) and oocyte derived from day 12 cultured follicles in medium

413   supplemented with or without (B) cilostamide 20 μM (C), Forskolin 1 μM (D), or the combination

414   of cilostamide 20 μM and Forskolin 1 μM (E). TZP density (mean ± SEM, F). Asterisks indicate

415   significant levels. * $P ≤ 0.05$, *** $P < 0.001$. Scale bar = 20 μm.

416   **Figure 5.** cAMP concentrations (pmol/ml, mean ± SEM) in spent medium of follicles cultured for

417   6 or 12 d without cAMP modulator supplementation (Control), 20 μM cilostamide, 1 μM forskolin,

418   or the combination of cilostamide 20 μM and Forskolin 1 μM. [A,B] indicate significant differences

419   among treatments on day 6)P <0.05(. [a,b] indicate significant differences among treatments on

420   day 12)P <0.05(. Asterisks indicate significant levels between day 6 and 12 within the same

421   treatment group, * $P \leq 0.05$, *** $P < 0.001$.

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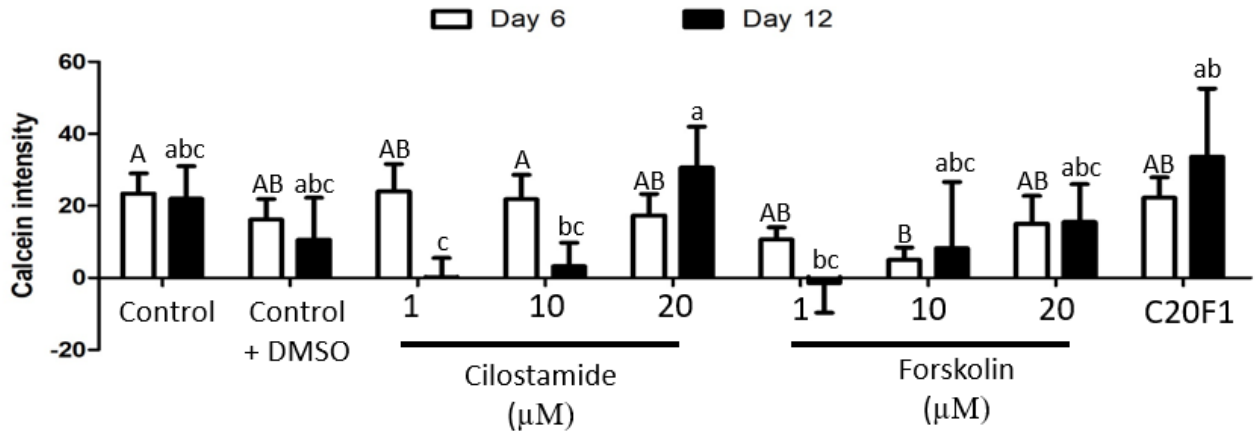
424 **Figure 1**
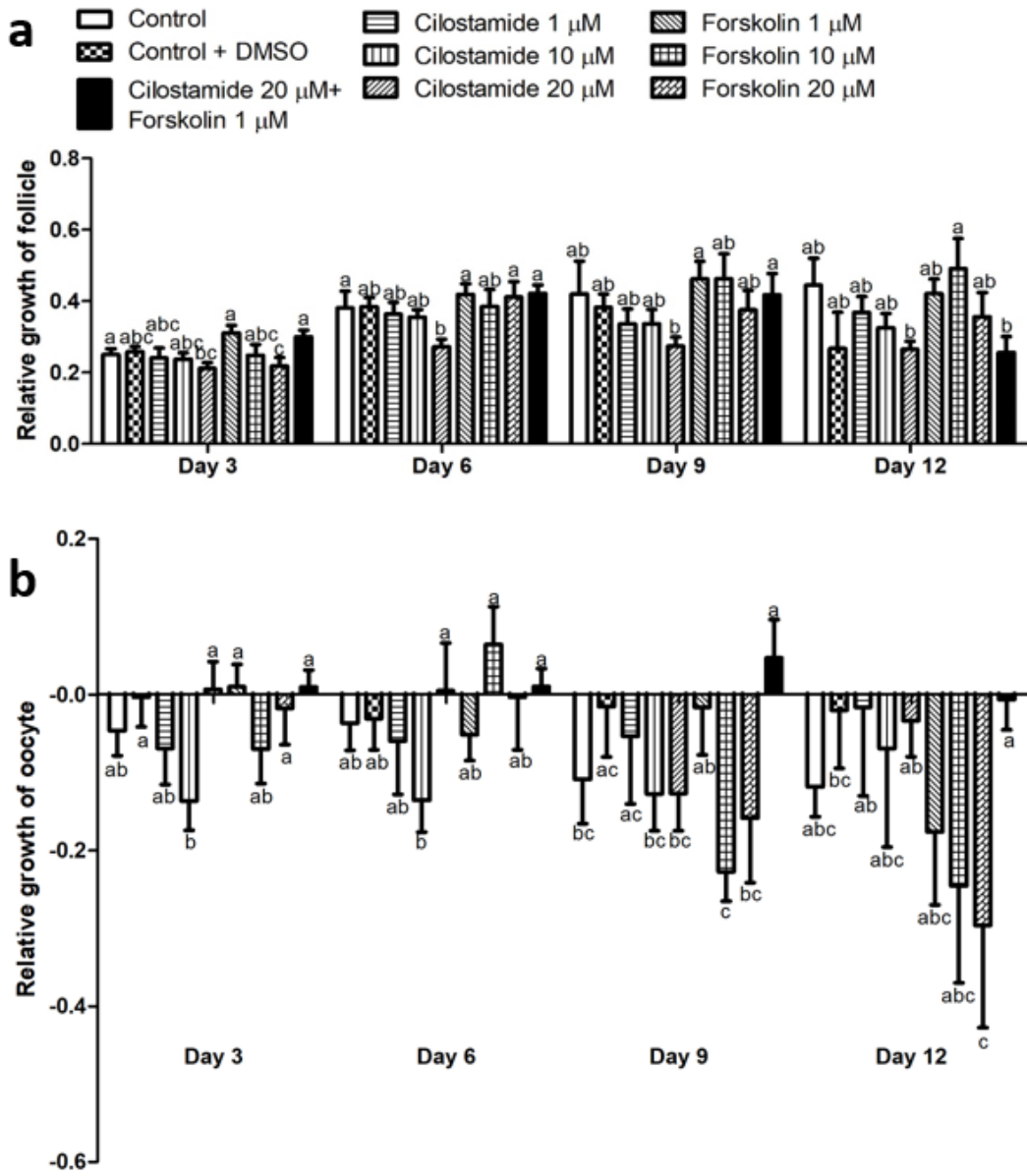
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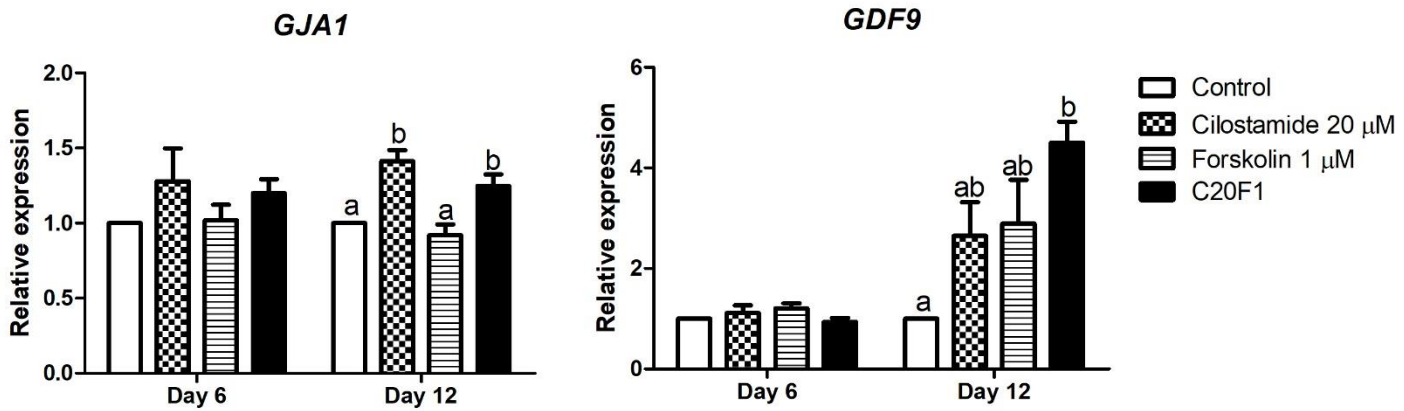
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450   **Figure 2**

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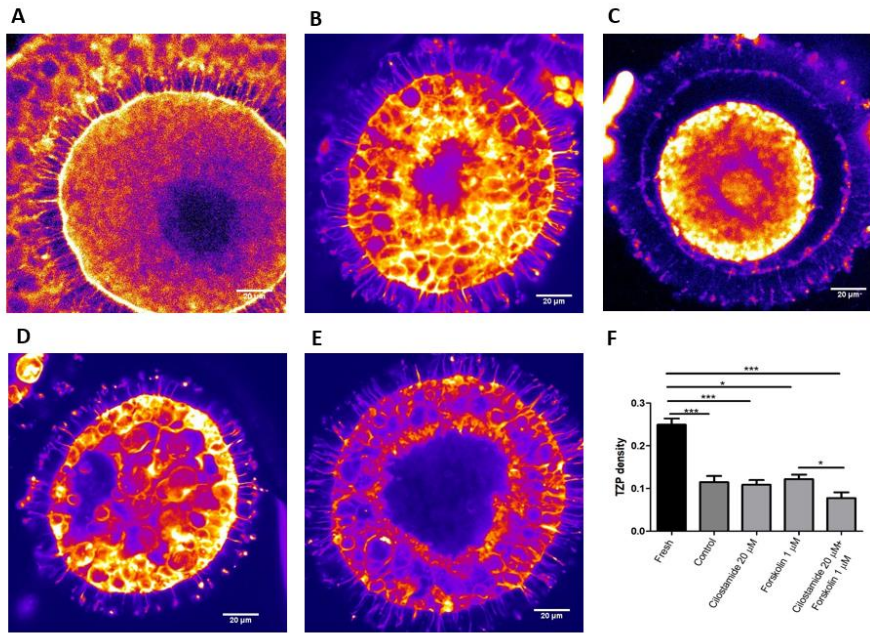
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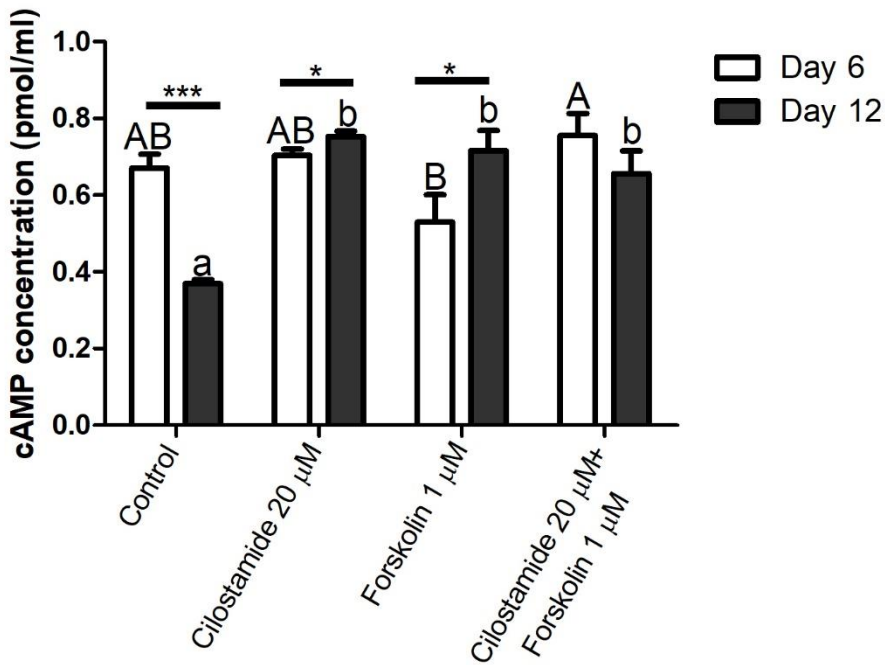

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461   **Figure 4**

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463   **Figure 5**

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477   Table 1: Primers used in this study for qPCR analysis of dog follicles.

Gene	Primer sequences forward (F) and reverse (R)	Accession number	Annealing temperature (°C)	Product length (bp)
β-actin	F: 5'-TCGCTGACAGGATGCAGAAG-3'	XM_845524.1	60	127
	R: 5'-GTGGACAGTGAGGCCAGGAT-3'			
GDF9	F: 5'-CAGAAGGGAGGTCTGTCTGC-3'	NM001168013.1	55	170
	R: 5'-TGTTGGGGGAAAAGAAAGTG-3'			
GJA1	F: 5'-AGAAAGAGGAGGAGCTCAAAGTTG-3'	AY462223	57	71
	R: 5'-TTCAATCTGCTTCAACTGCATGT-3'			

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