The Effect of Cilostamide on Gap Junction Communication Dynamics, Chromatin Remodeling, and Competence Acquisition in Pig Oocytes Following Parthenogenetic Activation and Nuclear Transfer

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ABSTRACT

In the pig, the efficiency of in vitro embryo production and somatic cell nuclear transfer (SCNT) procedures remains limited. It has been suggested that prematuration treatments (pre-IVM) based on the prolongation of a patent, bidirectional crosstalk between the oocyte and the cumulus cells through gap junctional communication (GJC), with the maintenance of a proper level of cAMP, could improve the developmental capability of oocytes. The aim of this study was to assess: 1) dose-dependent effects of cilostamide on nuclear maturation kinetics, 2) the relationship between treatments on GJC functionality and large-scale chromatin configuration changes, and 3) the impact of treatments on developmental competence acquisition after parthenogenetic activation (PA) and SCNT. Accordingly, cumulus-oocyte complexes were collected from 3- to 6-mm antral follicles and cultured for 24 h in defined culture medium with or without 1 μM cilostamide. GJC functionality was assessed by Lucifer yellow microinjection, while chromatin configuration was evaluated by fluorescence microscopy after nuclear staining. Cilostamide administration sustained functional coupling for up to 24 h of culture and delayed meiotic resumption, as only 25.6% of cilostamide-treated oocytes reached the pro-metaphase I stage compared to the control (69.7%; P < 0.05). Moreover, progressive chromatin condensation was observed before meiotic resumption based upon G2/M biomarker phosphoprotein epitope acquisition using immunolocalization. Importantly, cilostamide treatment under these conditions improved oocyte developmental competence, as reflected in higher blastocyst quality after both parthenogenetic activation and SCNT.

INTRODUCTION

In addition to its high agricultural value, the pig is an important domestic animal model for biomedical and reproductive research [1]. Pig organs are similar to those of humans in terms of size and physiology, providing a potential source for xenotransplantation [2]. Cloning by means of somatic cell nuclear transfer (SCNT) is the technique used most often to generate genetically modified pigs for xenotransplantation and as models for studying various human diseases [3–6]. However, despite much progress having been made in pig in vitro embryo production (IVP) and SCNT over the last decades, the efficiency of these techniques remains low and further improvements are still required [1, 4, 5, 7].

It is widely agreed that the low efficiencies of pig IVP and SCNT are at least in part due to a suboptimal in vitro maturation (IVM) system that should ultimately produce mature oocytes able to support embryonic development. The challenge of oocyte IVM is, first, to provide a milieu that reflects the naturally changing environment to which the oocyte is exposed and, second, to support the complex cellular changes taking place in the follicular cells and in the oocyte nuclear and cytoplasmic compartments.

One of the problems related to IVM is that oocytes spontaneously resume meiotic maturation when cumulus-oocyte complexes (COCs) are removed from the antral follicles [8] and thus bypass the so-called “oocyte capacitation” that is, in turn, essential for the attainment of a full embryonic developmental competence [9]. Several studies have suggested that extending meiotic arrest in vitro by temporary blockage of spontaneous nuclear maturation, the so-called prematuration culture (PMC), might improve the synchronization between nuclear and cytoplasmic maturational status and ultimately the oocyte competence [10–13] (reviewed in Ref. 14).

Temporary blockage of spontaneous meiotic resumption can be achieved by preventing the intraoocyte drop of cAMP levels, which normally occurs after removal of the COC from the follicle, through the use of inhibitors of phosphodiesterases...
(PDEs), the enzymes that hydrolyze cAMP [15, 16]. One of these compounds is cilostamide, which specifically inhibits the type 3 PDE (PDE3), which is the major active cAMP-PDE in porcine oocytes [17]. While, in some mammals, PDE3 is an oocyte-specific enzyme [18], in the pig this PDE is also expressed in cumulus cells [19].

Interestingly, it has been shown that the use of cilostamide can prolong the functional coupling between oocytes and cumulus cells mediated by gap junction-mediated communication (GJC) during in vitro culture (IVC) of bovine, mouse, and human oocytes [20–22]. This is important, since bidirectional communications between oocyte and cumulus cells are essential to ensure a proper oocyte differentiation and meiotic maturation. Furthermore, it has been recently shown in the bovine model that the maintenance of a proper functional coupling between oocyte and cumulus cells seems to be crucial in sustaining an orderly remodeling of large-scale chromatin configuration [22], which is, in turn, indicative of the oocyte differentiation and metabolic state [23–27] (reviewed in Refs. 28, 29).

On the basis of these observations, this study aimed to test the utility of a prematuration treatment using cilostamide by evaluating pig oocyte quality for various biotechnological applications, including parthenogenesis and SCNT. With this objective in mind, we assessed: 1) dose-dependent effects of cilostamide on the kinetics and the degree of nuclear maturation, 2) the effect of the treatment on GIC functionality and on the process of large-scale chromatin configuration changes, and 3) the effect of the treatment on developmental competence acquisition after parthenogenetic activation (PA) and SCNT.

MATERIALS AND METHODS

COC Collection and Culture

All chemicals and reagents were purchased from Sigma Chemical Company, unless otherwise stated. Porcine ovaries were recovered at a local abattoir from pubertal females and transported to the laboratory at 30–33°C within 2 h. All the subsequent procedures were performed between 35°C and 38°C, unless otherwise specified. COCs were isolated from early antral follicles (EAFs; diameter 0.5–2 mm) by dissecting individual follicles with a scalpel, or from medium antral follicles (MAFs; diameter 3–6 mm) by aspiration with a 19-gauge needle mounted on a vacuum pump (Cook-IVF, Brisbane, QLD, Australia) with a pressure of ~28 mm Hg. Collection medium was TCM199 supplemented with 20 mM Hepes, 1790 IU/ml of heparin, and 0.4% bovine serum albumin (BSA) fraction V (M199D). Where needed and according to the experimental design, COCs from MAFs were collected in 500 μM 3-isobutyryl-1-methylxanthine (IBMX) supplemented medium to avoid cAMP content drop during COC recovery [10, 12, 30]. COCs were examined under a stereomicroscope, and only those with intact, compact cumulus investments during COC recovery [10, 12, 30]. COCs were either subcultured every 4–6 days or expanded and frozen in DMEMþSOF) [34]. The oocytes with the first polar body extruded were selected and evaluated by florescence microscopy as previously described [12].

PA and SCNT

At the end of the culture period, COCs were freed of their surrounding cumulus cells and washed twice in Heps-buffered synthetic oviduct fluid (H-SOF) [35]. The oocytes with the first polar body extruded were selected and treated for PA or for SCNT procedures.

Matured oocytes were activated as described previously [35]. Briefly, oocytes were washed in 0.3 M mannitol solution, containing 1 mM CaCl2 and activated by double direct current pulses of 1.2 kV/cm for 30 μsec applied in the same medium. After a brief wash in H-SOF, the oocytes were transferred into the chemically assisted activation medium, which was PZM-3 medium [36] supplemented with 5 μg/ml cytochalasin B. After 4 h of incubation, PA embryos were washed twice in H-SOF and transferred to 400 μM PZM3 IVC medium. Embryo cleavage and blastocyst development were observed at Days 2 and 7, respectively.

In order to generate SCNT embryos, porcine adult fibroblasts were obtained through culture of minced tissue from ear or tail biopsies, as previously described [35]. Briefly, fibroblasts were cultured in medium Dulbecco modified Eagle medium (DMEM) + TCM199 (1:1) with 10% FCS in 5% CO2 and 5% nitrogen. Donor cells were induced into quiescence by serum starvation (0.5% FCS) for 2–3 days prior to nuclear transfer. The cells were prepared by trypsinization 30 min before nuclear transfer and then washed, pelleted by centrifugation, and resuspended in H-SOF supplemented with 10% FCS, to serve as nuclear donors. The zona pellucida of the matured oocytes was digested by 0.5% pronase solution for a few minutes. Zona-free oocytes were stained with Hoechst 33342 and subjected to 5 μg/ml cytochalasin B treatment. The manipulation of a dezonated oocyte was performed with a blunt, nonbeveled pipette. In order to localize the metaphase plate, oocytes were briefly exposed to ultraviolet light. Cytoplasts were individually washed for few seconds in 300 μM nitrophenamagglutinin P in PBS and then quickly dropped over a single
donor cell [37]. After pairing donor cell and recipient cytoplasm, the couples were subjected to electrofusion. After two 30-μsec direct current impulses of 1.2 Kv/cm, the constructs were transferred to H-SOF medium supplemented with HEPES [36]. After fusion, the reconstructed SCNT embryos were subjected to activation as previously described for parthenogenetic development. As for the PA embryos, the SCNT embryos were washed twice in H-SOF supplemented with 10% FCS and transferred in IVF medium. IVF was carried out under 5% CO₂ and 5% O₂ in humidified air at 38.5°C. Embryos were positioned in a well of well system [38]. The well was covered with 400 μl IVF-SOF [34]. Cleavage was assessed 48 h after activation, whereas the blastocyst rate (BL) was recorded on Day 7 (Day 0 was the day of activation). During embryo culture, half of the medium was renewed on Day 4 with fresh culture medium. On Day 7, the blastocysts were fixed in 60% methanol in DPBS overnight at 4°C and stained with 1 μg/ml Hoechst 33342 for cell number evaluation.

**Immunofluorescence Staining**

Indirect immunofluorescence was carried out to evaluate 1) chromatin condensation by monitoring phosphorylation of histone H3 at Ser28 (HE-S28) and 2) the patterns of mitosis-associated ser/Thr-phospho-proteins (MPM2) by using the monoclonal antibody MPM2. This antibody is known to detect epitopes of many relevant substrates for M-phase-specific kinases and is a specific biomarker for the G2/M cell cycle transition [39–43].

Oocytes were simultaneously fixed and extracted in a microtubule-stabilizing buffer containing proteases and phosphatases inhibitors, as described elsewhere [40, 42, 43] and then incubated overnight with primary antibody at 4°C. The primary antibodies used were rabbit polyclonal anti-phospho-histone H3 (Ser28; dilution, 1:100; Merk Millipore, Temecula, CA) or mouse monoclonal anti-phospho-Ser/Thr-Pro antibody, MPM2 (dilution 1:100; Merck Millipore). After extensive washing in 0.1% Triton, 0.1% Tween, and 1% BSA, oocytes were incubated for 1 h at room temperature with conjugated secondary antibodies that were TRITC-conjugated AffiniPure Donkey Anti-Rabbit (dilution 1:100; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) or AlexaFluor-488-labeled donkey anti-mouse (dilution 1:500; Invitrogen, Life Technologies, Carlsbad, CA). Samples were extensively washed again and finally mounted on slides in the antifade medium, Vectashield (Vector Laboratories) supplemented with 1 μg/ml 4′,6-diamidino-2-phenylindole. In each experiment, negative controls were performed by omitting the primary antibodies, and did not reveal any staining. Samples were analyzed on an epifluorescence microscope (Eclipse E600; Nikon Corp., Japan) equipped with a 40× objective and a digital camera (DS-Fi2; Nikon Corp., Japan). For the analysis of phosphorylation status of histone H3-S28, immune fluorescent staining was also conducted on paraformaldehyde-fixed oocytes, as previously described [32].

**Analysis of MPM2 staining** was conducted by capturing images, using identical exposure times and gain settings, at the focal plane containing the GV chromosomes and at the equatorial focal plane, in order to assess fluorescence intensity (FI) of the nucleus and cytoplasm respectively. Finally, mean FI of each GV was calculated using NIH ImageJ software. To determine the cytoplasmic FI, mean FI in five different areas of the cytoplasm was calculated using NIH ImageJ and averaged.

**Statistical Analysis**

All the experiments were repeated three to five times. Observations from all the experiments were pooled. Statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA). Differences in maturation, cleavage, and BLs, as well as differences in the percentages of COCs with open GCJ and of oocytes with different chromatin configurations among the experimental groups, were analyzed by Fisher exact test. FL of MPM2, oocyte diameter, and blastocyst cell number are expressed as means ± SE. In these cases, statistical significance was tested using one-way ANOVA followed by Tukey multiple comparison test or by the Student t-test when means of two groups were compared. Values of P < 0.05 were considered significant.

**RESULTS**

**Dose-Response and Reversibility of Cilostamide Treatment**

The first set of experiments aimed at determining the treatment that could prevent GV breakdown (GVBD) in porcine oocytes under our experimental condition. COCs were cultured in dIVM medium (control) or dIVM medium supplemented with 0.1 or 1 μM cilostamide for 24, 36, or 48 h, and oocyte nuclear maturation was assessed by lacmoid staining after acetic fixation. Oocytes were classified as: GV when the nuclear membrane was detectable independently of the chromatin organization within the nuclear envelope; intermediate when the chromosomes were arranged in prometaphase, metaphase I plate, anaphase I, or telophase I; and mature when the chromosomes were arranged in a metaphase II plate with the first polar body extruded. The effect of the prematuration treatment on oocyte maturation status. COCs were incubated in the presence of 1 μM cilostamide for 24 h. The maturation status was assessed after culturing the COCs for a further 20 h in dIVM medium (see main text). A total of 146 oocytes were used in these experiments. Data were analyzed by Fisher exact test. Asterisks (single and double) indicate significant differences between classes (P < 0.05).

**FIG. 1.** A) Dose-response effect of cilostamide on oocyte meiotic resumption. COCs were cultured in media supplemented with increasing concentrations of cilostamide (0, 0.1, and 1 μM) for varying periods of time (24, 36, and 48 h). A total of 960 oocytes were used in these experiments. Oocytes where judged to be: intermediate when the chromosomes were arranged in prometaphase, metaphase I plate, anaphase I, or telophase I; and mature when the chromosomes were arranged in a metaphase II plate with the first polar body extruded. B) The effect of the prematuration treatment on oocyte maturation status. COCs were incubated in the presence of 1 μM cilostamide for 24 h. The maturation status was assessed after culturing the COCs for a further 20 h in dIVM medium (see main text). A total of 146 oocytes were used in these experiments. Data were analyzed by Fisher exact test. Asterisks (single and double) indicate significant differences between classes (P < 0.05).
Chromatin configuration distribution at the time of oocyte isolation from early and medium antral follicles.

**Table 1.** Chromatin configuration distribution at the time of oocyte isolation from early and medium antral follicles.

<table>
<thead>
<tr>
<th>Follicle</th>
<th>N</th>
<th>FC (%)</th>
<th>SCd (%)</th>
<th>SCn (%)</th>
<th>GVI (%)</th>
<th>GVII–GVIV (%)</th>
<th>Pro-MI–MI (%)</th>
<th>Deg (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAF</td>
<td>91</td>
<td>44 (48.4)</td>
<td>8 (8.8)</td>
<td>11 (12.1)</td>
<td>9 (9.9)</td>
<td>3 (3.3)</td>
<td>0 (0.0)</td>
<td>16 (17.6)</td>
</tr>
<tr>
<td>MAF</td>
<td>245</td>
<td>9 (3.7)</td>
<td>23 (9.4)</td>
<td>87 (35.5)</td>
<td>71 (29.0)</td>
<td>20 (8.2)</td>
<td>0 (0.0)</td>
<td>35 (14.3)</td>
</tr>
</tbody>
</table>

*a,b* Different letters within columns indicate significant differences (Fisher exact test, *P* < 0.05).

* Deg, degenerated.

Effect of Cilostamide Treatment on Chromatin Configuration Remodeling and Meiotic Progression

Before assessing the effect of cilostamide treatment on the process of chromatin configuration remodeling and meiotic progression, preliminary studies were conducted to identify discrete categories of prophase I arrested oocytes. To this end, we assessed chromatin configuration changes at the time of isolation from the follicles and compared our observations with the plethora of classifications present in the literature. Chromatin configuration was assessed in living porcine oocytes soon after the isolation from EAFs (0.5 to <2 mm in diameter) and MAFs (3–6 mm in diameter) by Hoechst 33342 staining and fluorescence microscopy analysis. Our observations confirmed, to a large extent, data from Bui et al. [32] and Hirao et al. [44]. As shown in Figure 3A, the filamentous chromatin (FC) configuration was characterized by the presence of a diffuse filamentous pattern of chromatin distributed in the whole nuclear area. The stringy chromatin (SC) configuration was characterized by an increased level of condensation of the chromatin that became clearly thicker and organized into clumps. Within the SC class, a further distinction was based on the presence or absence of a ring of condensed chromatin around the nucleolus; thus the configuration in which clumped chromatin was distributed throughout the GV was designated as SC distributed (SCd), whereas the configuration in which both clumped chromatin throughout the nucleoplasm and a rim of condensed chromatin surrounding the nucleolus were detected was termed SC with nucleolar rim (SCn). Finally, in the GVI configuration, all the condensed chromatin mass was organized around the nucleolus, as previously described by Motlik and Fulka [31]. That the SCd and SCn configurations are intermediated states of chromatin condensation between the FC and GVI configurations was confirmed by the oocyte diameter analysis, which showed that change of chromatin configuration from FC to GVI was accompanied by a significant increase of oocyte diameter (*P* < 0.05; Fig. 3B). Mean diameter of FC oocytes from EAF and of SCd, SCn, and GVI oocytes from MAF was 110.8 ± 1.2, 118.4 ± 0.8, 119.4 ± 0.4, and 121.3 ± 0.4 μm, respectively. Moreover, as shown in Table 1, the distribution analysis of the above-described patterns in EAFs and MAFs showed that, at the time of collection, the majority of oocytes isolated from with 1 μM cilostamide for 24 h was adopted in all the following experiments.

Effect of Cilostamide Treatment on GJC Between Oocytes and Surrounding Cumulus Cells

This set of experiments was designed to investigate the effect 1 μM cilostamide treatment on the persistence of a functional intercellular coupling between the oocyte and the surrounding cumulus cells. GJCs functionality was assessed at the time of collection and after 12, 18, and 24 h of culture in dIVM medium supplemented with 0 (control) or 1 μM cilostamide. At the time of collection, the injection of LY in the oocytes resulted in an immediate spread of the dye into neighboring corona radiata cells in 79.8% of COCs, whereas 5.1% and 15.2% of COCs showed a pattern of partially and completely closed communications, respectively (Fig. 2). As shown in Figure 2B, at 18 h of culture, we observed a substantial drop in GJC functionality in the control group. In contrast, GJCs functionality was maintained for up to 24 h of culture in the cilostamide-treated group. Importantly, when different times were compared, the percentage of COCs with open GJCs in the cilostamide-treated group after 24 h of culture did not differ significantly to that observed at the time of collection (*P* = 0.129).

![Image](https://www.biolreprod.org)
TABLE 2. Effect of 1 μM cilostamide treatment on chromatin configuration remodeling and meiotic progression.*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>FC (%)</th>
<th>SCd (%)</th>
<th>SCn (%)</th>
<th>GVI (%)</th>
<th>GVII–GVIV (%)</th>
<th>Pro-MI–MI (%)</th>
<th>Deg (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18 h CTRL</td>
<td>80</td>
<td>0 (0.0)</td>
<td>1 (1.3)</td>
<td>6 (7.5)</td>
<td>26 (32.5)</td>
<td>31 (38.8)</td>
<td>14 (17.5)</td>
<td>2 (2.5)</td>
</tr>
<tr>
<td>18 h CILO</td>
<td>80</td>
<td>0 (0.0)</td>
<td>5 (6.1)</td>
<td>14 (17.1)</td>
<td>26 (31.7)</td>
<td>24 (29.3)</td>
<td>8 (9.8)</td>
<td>5 (6.1)</td>
</tr>
<tr>
<td>24 h CTRL</td>
<td>89</td>
<td>0 (0.0)</td>
<td>5 (5.6)</td>
<td>1 (1.1)</td>
<td>8 (9.0)</td>
<td>12 (13.5)</td>
<td>62 (69.7)</td>
<td>1 (1.1)</td>
</tr>
<tr>
<td>24 h CILO</td>
<td>86</td>
<td>0 (0.0)</td>
<td>4 (4.7)</td>
<td>6 (7.0)</td>
<td>21 (24.4)</td>
<td>29 (33.7)</td>
<td>22 (25.6)</td>
<td>4 (4.7)</td>
</tr>
</tbody>
</table>

*a,b Different letters within columns indicate significant differences (Fisher exact test, P < 0.05).
* CILO, cilostamide; CTRL, control; Deg, degenerated.

Effect of Cilostamide Treatment on Embryonic Developmental Competence

In order to assess the effect of cilostamide treatment on the embryonic developmental competence of porcine oocytes, COCs were cultured for 44 h in dIVM medium (control) or for 24 h in dIVM medium in the presence of 1 μM cilostamide. After the culture period, matured oocytes were treated for PA or SCNT procedures. As shown in Table 3, cleavage rate after PA of cilostamide-treated oocytes did not differ significantly from the control group. Moreover, no differences were observed between treatments in the percentage of PA embryos that developed to the blastocyst stage after 7 days of culture. Interestingly, however, the frequency of expanded blastocyst in the cilostamide-treated group was significantly higher than that of the control group.

Effect of Cilostamide Treatment on Chromatin Configuration Remodeling and Pig Oocyte Competence

EAFs were characterized by an FC pattern. This configuration significantly decreased in oocytes isolated from MAFs (P < 0.05). No differences were observed in the percentage of oocytes with the SCd configuration between the two follicular classes, while the frequencies of SCn and GVI oocytes were significantly higher in MAFs than in EAFs. Few of the oocytes collected from MAFs showed early signs of GVBD (GVII–GVIV stages; see below), as already reported [44].

After 18 h of culture in dIVM medium, we observed an increase of the oocytes that showed typical features of early GVBD (Table 2), as previously described by Motlik and Fulka [31], and oocytes were classified as GVII–IV stages accordingly (Fig. 4). Some of these oocytes were also present at the time of collection, as previously reported [44]. In these oocytes, signs of condensation and individualization of filamentous bivalents were detectable. Moreover, in some oocytes, chromosomes were arranged in pro-metaphase I (pro-MI) and metaphase I (MI) plate (Fig. 4).

To further confirm that SC and GVII–GVIV configurations were different, and more precisely that GVII–GVIV are typical configurations that occur between removal of the COC from the follicle (i.e., when spontaneous meiotic resumption occurs) and GVBD, we conducted immunofluorescent staining of H3-S28 and MPM2 at the time of collection and after 24 h of culture, as these have been indicated as markers of meiotic progression [32, 39–43, 49]. Under our conditions, H3 started to be phosphorylated at pro-MI, while no differences were observed between SC, GVI, and GVII–GVIV stages (Fig. 5).

On the other hand, analysis of MPM2 staining revealed a progressive increase of signal intensity, first in the nuclear and then in the cytoplasmic compartments from SC to pro-MI stages (Fig. 6). Importantly, nuclear FI of GVII–GVIV oocytes was significantly higher than that of SC and GVI oocytes, confirming that GVII–GVIV configurations temporarily follow the GVI stage (Fig. 6C).

Having established the patterns of chromatin configuration changes before and during culture, we were finally able to evaluate the effect of cilostamide treatment on the process of chromatin configuration changes and meiotic progression. COCs were cultured for 18 and 24 h in dIVM medium supplemented with 0 (control) or 1 μM cilostamide. At the end of the culture periods, oocytes were freed of cumulus cells, stained with Hoechst 33 342, and analyzed. As shown in Table 2, after 24 h of culture, significantly fewer oocytes progressed to the pro-MI–MI stages in the cilostamide-treated group when compared to the control group. Accordingly, the percentages of GVI and GVII–GVIV oocytes were significantly higher in the cilostamide-treated group, indicating that meiotic resumption had been delayed by the treatment.

Effect of Cilostamide Treatment on Embryonic Developmental Competence

In order to assess the effect of cilostamide treatment on the embryonic developmental competence of porcine oocytes, COCs were cultured for 44 h in dIVM medium (control) or for 24 h in dIVM medium in the presence of 1 μM cilostamide, and then washed and cultured for an additional 20 h in dIVM medium. After the culture period, matured oocytes were treated for PA or SCNT procedures. As shown in Table 3, cleavage rate after PA of cilostamide-treated oocytes did not differ significantly from the control group. Moreover, no differences were observed between treatments in the percentage of PA embryos that developed to the blastocyst stage after 7 days of culture. Interestingly, however, the frequency of expanded blastocyst in the cilostamide-treated group was significantly higher than that of the control group.

FIG. 3.  A) Fluorescent images after Hoechst 33 342 labeling of porcine oocytes at the time of collection with FC (a), SCd (b), SCn (c), and GVI (d) configuration.  B) GV chromatin configuration in relation to oocyte diameter at the time of collection. A total of 228 oocytes were used in these experiments. Number of oocytes analyzed in each group is indicated in the graph. Mean diameter values, for each GV category, are expressed as means ± SE. Data were analyzed by ANOVA followed by the Tukey multiple comparison test. Superscript letters indicate significant differences between groups (P < 0.05).
cilostamide-treated group was significantly higher when compared to the control group, whereas there was no significant effect of cilostamide treatment on PA blastocyst cell number.

As shown in Table 4, the cleavage and BLs, as well as the frequency of expanded blastocyst after SCNT treatment, were similar in the control and cilostamide-treated groups. However, the mean cell numbers per blastocyst were significantly higher in the cilostamide-treated group when compared to the control group.

**DISCUSSION**

The present study demonstrates that inhibition of PDE activity during COC recovery by IBMX, and during the following 24 h of culture with cilostamide in defined serum-free medium, is able to delay meiotic resumption while prolonging functional coupling between oocytes and cumulus cells; importantly, these conditions improved the developmental capability of pig oocytes. The increased oocyte developmental competence is reflected in the enhancement of blastocyst quality based upon expansion capability after PA (cytoplasmic competence), and an increased cell number per blastocyst after SCNT, compared with control groups. The immediate implication of these findings is that improved quality of transferred embryos would materially enhance the overall efficiency of SCNT, which is currently low, with the average yield of live births per embryo transferred being no more than 1%–5% [4, 6, 50–53]. This hypothesis is currently under investigation in our laboratories. Nevertheless, to the best of our knowledge, this is the first study reporting the use of cilostamide to improve pig SCNT efficiency.

Several cultural strategies have been proposed to improve oocyte quality in different mammals (for a review see Refs. 14, 54). Many of these strategies, often referred to as “premature culture,” are based on the temporary blockage of oocyte spontaneous meiotic resumption by modulating intraoocyte cAMP content with different agents, such as cAMP analogs or PDE inhibitors [12, 16, 20–22, 55–67]. Our results on the effect of the use of PDE inhibitors on meiotic resumption are largely supportive of previous data in prepubertal gilts in which cilostamide or other PDE3 inhibitors, such as milrinone, have been used [17, 67, 68]. Effects of cilostamide treatments on in vitro developmental potential have been tested in human [20, 21, 63], cow [22, 64], sheep [65], and mouse [56, 62, 64, 69], with different outcomes depending on the species, the time of COC incubation with cilostamide, the combination with other

**FIG. 4.** Representative fluorescent images after Hoechst 33342 labeling of porcine oocytes with GVII, GVIII, GVIV, pro-MI, and MI configurations after meiotic resumption.

**TABLE 3.** Effect of 1 μM cilostamide treatment on embryonic developmental competence after parthenogenetic activation.*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>Cleaved (%)</th>
<th>Blastocyst (%)</th>
<th>Expanded blastocyst of total blastocyst (%)</th>
<th>Mean cell number per blastocyst ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTRL</td>
<td>150</td>
<td>132 (88.0)</td>
<td>77 (51.3)</td>
<td>35 (45.5) ^a</td>
<td>23 ± 1.21</td>
</tr>
<tr>
<td>CILO</td>
<td>142</td>
<td>120 (84.5)</td>
<td>59 (41.5)</td>
<td>38 (64.4) ^b</td>
<td>26 ± 1.44</td>
</tr>
</tbody>
</table>

^a,b Different letters within columns indicate significant differences (Fisher exact test, P < 0.05)

* CILO, cilostamide; CTRL, control.

**TABLE 4.** Effect of 1 μM cilostamide treatment on embryonic developmental competence after somatic cell nuclear transfer.*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>Cleaved (%)</th>
<th>Blastocyst (%)</th>
<th>Expanded blastocyst of total blastocyst (%)</th>
<th>Mean cell number per blastocyst ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTRL</td>
<td>163</td>
<td>98 (60.1)</td>
<td>27 (16.6)</td>
<td>18 (66.7)</td>
<td>45 ± 3.35 ^a</td>
</tr>
<tr>
<td>CILO</td>
<td>161</td>
<td>84 (52.2)</td>
<td>25 (15.5)</td>
<td>21 (84.0)</td>
<td>58 ± 4.9 ^b</td>
</tr>
</tbody>
</table>

^a,b Different letters within columns indicate significant differences (Fisher exact test, P < 0.05).

* CILO, cilostamide; CTRL, control.
agents, and the experimental approach itself. In some cases, substantial differences exist among treatments, making it difficult to compare their efficacies. However, an important aspect that must be considered is the actual growth and differentiation stage of the immature oocytes subjected to the cilostamide-based treatment, since oocytes with different metabolic properties may require different cultural conditions [29]. Moreover the source of the ovaries needs to be taken into account. In pigs, for example, many studies have used prepubertal gilts [17, 67, 68], while others, including the present study, have used pubertal animals as the source of ovaries.

In general, the aim of the PMC is to allow the oocytes to complete those processes that are interrupted once they are removed from the follicular environment [14, 29] (i.e., the oocyte growth phase, for those oocytes collected from preantral or EAFs, and/or the so called “capacitation” process, that is the acquisition of the cytoplasmic machinery necessary to fully support preimplantation embryo development). These processes normally occur in fully grown oocytes when the follicles approach the preovulatory stage in vivo [9]. Therefore, fully grown oocytes at advanced stages of differentiation would not benefit from a protracted period of IVC [29, 70]. As a consequence, PMC with cilostamide generally improves the yield (% of embryo obtained) of oocytes at early stages of their development [21, 22, 56, 62], while it increases the quality of the embryos obtained from oocytes at more advanced stages of differentiation, collected from midsized antral follicles, without a significant increase in the embryo rate [20, 65, 71]. Accordingly, in the present study, similar outcomes on the quality of SCNT embryos are reported. In fact, oocytes used in this study are to be considered to have been at advanced stages of development. This is demonstrated by the analysis of the chromatin configuration at the time of collection, which is a marker of the oocyte differentiation state [29]. It indeed revealed that this population of oocytes did not contain growing oocytes with the FC configuration, while around 30% of the oocytes had already acquired the GVI configuration, in which the chromatin has reached its most compacted state before spontaneous meiotic resumption at the time of oocyte removal from the follicle (see Ref. 32 and the present study). Therefore, this heterogeneity could account at least in part for the unchanged percentage of embryos obtained from treated oocytes when compared to the control groups. This supports the original data in mice where the authors tested the effect of PDE3 inhibitor Org 9935 on the developmental competence of oocytes that were retrieved 24 h after gonadotropin injection, when the antral follicles were not yet fully developed. Importantly, the proportion of oocytes with condensed chromatin (SN configuration) was lower at 24 h compared with 48 h after eCG priming, and the arrest in vitro allowed the transition from uncondensed (NSN configuration) to SN configuration in the oocyte nucleus, which was associated with a significant increase in embryonic developmental competence [56].

To date, the only cilostamide-based treatment that substantially improved oocyte developmental outcomes, with a very high embryo yield, is the “simulated physiological oocyte maturation,” the so-called “SPOM” system, proposed by R.B. Gilchrist’s group in mouse and cow [64]. In this system, COCs are incubated for 1–2 h with cAMP modulating agents (IBMX and the adenylate cyclase activator Forskolin), followed by an extended IVM phase containing FSH and cilostamide for the entire culture period. As indicated by the authors, starting from the idea that specific PDE subtypes differentially exert their function within the somatic and the germ cell compartments in

FIG. 5. Representative images showing the status of histone H3-S28 phosphorylation (P-H3-S28) in porcine oocyte with different chromatin configurations and during early stages of meiotic maturation. Red, P-H3-S28; blue, DNA. Scale bar = 50 μm. No differences were observed in oocytes fixed either in paraformaldehyde or in microtubule-stabilizing fixative.
rodents [18] and cow [15, 72], the SPOM is specifically designed to target initially both cumulus cell and oocyte PDEs, by using the nonspecific PDE inhibitor IBMX, and then the oocyte PDE only, by using the PDE3-specific inhibitor cilostamide [64]. On the contrary pigs express PDE3 also in the cumulus cells [19]. Therefore, our cultural approach uses a "biphasic" system in which cilostamide is present only during the first 24 h of culture, and where its concentration is reduced by 10–20 fold compared to the that used in bovine systems [22, 64], but that is able, at the same time, to delay meiotic resumption at the same extent obtained with higher concentrations (20 μM) used in previous works in pig [17, 68].

FIG. 6. A) Representative images showing the pattern of tyrosine phospho-protein changes (MPM2 staining) in porcine oocyte with different chromatin configurations and during early stages of meiotic maturation. Green, MPM2; blue, DNA. Scale bar = 50 μm. B and C) Histograms indicate the FI of MPM2 signal within the nuclear (B) or cytoplasmic (C) area according to the chromatin configuration changes at the time of collection and during early stages of meiotic maturation. Values are expressed as means ± SE. Data were analyzed with ANOVA followed by the Tukey multiple comparison test. Number of oocytes analyzed in each group is indicated. Asterisk indicates significant differences between classes (P < 0.05).
Importantly, cilostamide treatment in our system promotes the maintenance of a proper functional coupling between oocyte and cumulus cells up to 24 h, confirming previous results in the bovine model [13, 22], which exerts, in turn, a positive effect on oocyte quality. We hypothesize that enhanced coupling during IVC could stimulate the bidirectional transfer of essential molecules for the attainment of full developmental competence. However, further studies are needed to understand the precise nature of these molecules.

Our previous studies in cows suggest that the maintenance of a proper functional oocyte-cumulus cell coupling is essential for an orderly remodeling of chromatin during the G2/M cell cycle transition during IVC [22, 29]. In fact, when bovine COCs from EAFs, in which chromatin is mostly decondensed (GV0; [25, 26]), are cultivated in vitro in a system that promotes the maintenance of a patent bidirectional coupling, the chromatin gradually organizes into a configuration with a higher degree of condensation (GV1), thus acquiring the ability to mature and be fertilized in vitro [22]. In the present study, a direct relationship between the maintenance of GJC and the chromatin remodeling process before meiotic resumption could not be properly determined. More precisely, the synchronization in GV1 configuration could not be directly verified. This is mainly due to the fact that meiosis is delayed by approximately 6 h, but not completely blocked. At 18 and 24 h of culture, oocytes in both control and cilostamide groups already presented signs of meiotic resumption, being in GVII–GVIV stages or having reached pro-MI stages. However, we could only speculate that, at some point before the 18th h of culture, most of the oocytes synchronize at the GV1 stage. On the other hand, since meiotic progression is delayed, chromatin/chromosome rearrangements during the first 24 h of culture are slowed down in the cilostamide group. We cannot exclude that this could account, in part, for the increased quality of these oocytes. However, this hypothesis remains to be rigorously tested.

In this context, it is very important to point out that the present study confirms that GVII–GVIV configurations in pig are typical of oocytes that have already resumed meiosis, even though the nuclear envelope is still detectable and the chromatin is condensing into bivalents that will ultimately become fully condensed metaphase chromosomes. It is worth noting that the expression “GV stage,” commonly used by investigators to indicate the stage at which the oocyte is collected from the ovarian follicle (or even the period when the GV is clearly detectable within the oocyte before GVBD occurs), should not to be confused with the phase of meiotic arrest, since meiotic resumption is initiated well ahead of the disappearance of the nuclear envelope [73]. Therefore, even though GVBD is the first grossly obvious manifestation of meiotic resumption, these two events should not to be considered equivalent [29]. That GVII–GVIV configurations represent early signs of meiotic resumption in pig is indicated by original experiments by Mottlik and Fulka, who indeed proposed this classification [73]. In their work, using aceto-orcein staining, the authors describe GVII–GVIV as those configurations that characterize early stages of in vivo maturation and IVM, and that precede the complete disappearance of the nuclear envelope [73]. This classification was further confirmed by Hirao et al. [44], who, studying oocytes collected from follicles of different diameters, introduced the SC configuration as the one preceding the GV1 stage. Finally immunofluorescence studies by Bui et al. [32] further confirm the above “temporal” stage progression showing that the phosphorylation of histone H3-S28 is first detected in condensing chromosomes at the GVII–GVIV stages [32]. Since chromosome condensation begins at this point, the authors suggest that H3-S28 phosphorylation might be one of the key events initiating meiotic chromosome condensation [32]. Because, under our experimental conditions, H3-S28 phosphorylation was not detected in GVII–GVIV oocytes, we employed another G2/M cell cycle marker to test whether the chromatin configurations observed in GVII–GVIV stages are truly indicative of meiotic resumption. Therefore, SC and GVII–GVIV stages must be carefully evaluated to avoid misinterpretation of experimental data. Moreover, identification of additional unequivocal biomarkers will aid in discerning the events that are critical to establishing oocyte quality during IVM.

In conclusion, the present work demonstrates that delaying the timing of GJC interruption while slowing down early meiotic progression has a long-acting effect on the developmental program of the oocyte. Our results contribute to our understanding of the mechanisms regulating oocyte maturation and the acquisition of developmental competence, and offer intriguing tools for further studies.

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