SWINE EMBRYO DEVELOPMENT AND LIPID CONTENT AFTER MATURATION WITH FORSKOLIN

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1. INTRODUCTION

In vitro embryo production (IVP) has lower embryo production rates in pigs (APPELTANT *et al.*, 2016), especially when compared to bovine (KIKUCHI *et al.*, 2016). The first step to successful IVP is to provide the oocyte with a favourable environment, able to support both cytoplasmatic and nuclear maturation (ZHANG *et al.*, 2012), since disharmony between these events is a major obstacle during IVP (PRATES; NUNES; PEREIRA, 2014). Also, an excessive accumulation of lipid droplets (LD) within the cytoplasm of both oocytes and embryos has a negative impact on IVP development rates and biotechniques such as cryopreservation, cloning and transgene (GAJDA, 2009).

Follicular cells produce cyclic adenosine monophosphate (cAMP), transferring it to the oocyte and keeping Maturation Promoting Factor (MPF) inactive. Thus, spontaneous maturation occurs when oocytes are removed from their follicles, leading to meiosis resumption (CONTI; FRANCIOSI, 2018). The addition of Forskolin (FSK), a cAMP modulator, has improved oocyte maturation by keeping MPF inactive until the synchronization of cytoplasmic and nuclear maturation, and provided lipid reduction through lipolysis in a time-dependent manner (PRATES; NUNES; PEREIRA, 2014), however, only when added since the beginning of the maturation procedure.

Therefore, our aim is to test if the addition of FSK during the last 22 h of *in vitro* maturation could result in better maturation synchrony, cytoplasmatic lipid consumption and development of porcine embryos.

2. METODOLOGY

Ovaries collected at local abattoir were transported to the laboratory in saline solution (0,9% NaCl) at 30°C to 35°C. With a vacuum pump (10 - 15 mL/min) attached to a 19 G needle, follicles with 3-6 mm diameter were aspirated and their *cumulus oophorus*-oocyte complexes (COCs) were searched under stereomicroscopy and subsequently placed on TCM-HEPES medium (TCM-199 with 0.1% PVA, 2.0mM $C_3H_4O_3$ and 2.5mM $NaHCO_3$) for stabilization. Oocytes with homogenous cytoplasm and at least three layers of *cumulus oophorus* cells were selected and washed again in TCM-HEPES before maturation.

Thirty to forty COCs were matured per well in 4-well plates (Nunc, Roskilde, Denmark) containing 400 µL of media, in two treatments: 1) Control (TCM-199 supplemented with 0.1% PVA and 2.5 mM $NaHCO_3$, 0.57 mM cysteine, 0.91 mM $C_3H_3NaO_3$, 0.001 g/mL EGF, 0.05 g/mL streptomycin, 0.065 g/mL penicillin and

25% pFF); 2) FSK (Control + 10 µM FSK). All treatments were matured for 44 h at 38.5°C in 5% CO_2 atmosphere with saturated humidity, with gonadotrophins (20 µg/µL FSH, 5 µg/µL LH) for the first 22 h of maturation. Forskolin was prepared as reported by FU *et al.*, (2011).

After maturation, COCs were denuded by vortex for 5 min. Denuded oocytes were parthenogenetically activated as previously described (CHE; LALONDE; BORDIGNON, 2007) and potential parthenotes were cultured in 60 µL droplets of PZM (+) (YOSHIOKA *et al.*, 2002) under mineral oil. Cleavage rates were evaluated at 24 h and 48 h and blastocyst rates at the 7th day. At day 5, feeding was performed by changing 50% of the drop, with the addition of 10% estrus mare serum (EMS). Six replicates were conducted.

At day 7, embryos were fixed in 4% paraformaldehyde for 30 min, washed and kept in PBS until staining. A total of 55 embryos were stained with 7.5 µg/mL Hoechst 33342 for 10 min to evaluate the number of cells and with 1 µg/mL Nile Red overnight (GENICOT *et al.*, 2005) to evaluate the lipid content.

After staining, embryos were washed in PBS and placed on slides containing a single Mowiol droplet covered with coverslips. For lipid measurements, as previously described by BARCELÓ-FIMBRES & SEIDEL (2011), images were obtained by epifluorescence microscopy (Nikon 80i) using a G2A filter with a 1920 x 1080 resolution and 80ms exposure and fluorescence intensity was determined by selecting only the cytoplasm of embryos with Image J free-hand drawing tool, where the values were adjusted by Corrected Total Cell Fluorescence (CTCF): CTCF = Integrated Density - (Area of selected cell X Mean fluorescence of background readings) (FITZPATRICK, 2014).

3. RESULTS AND DISCUSSION

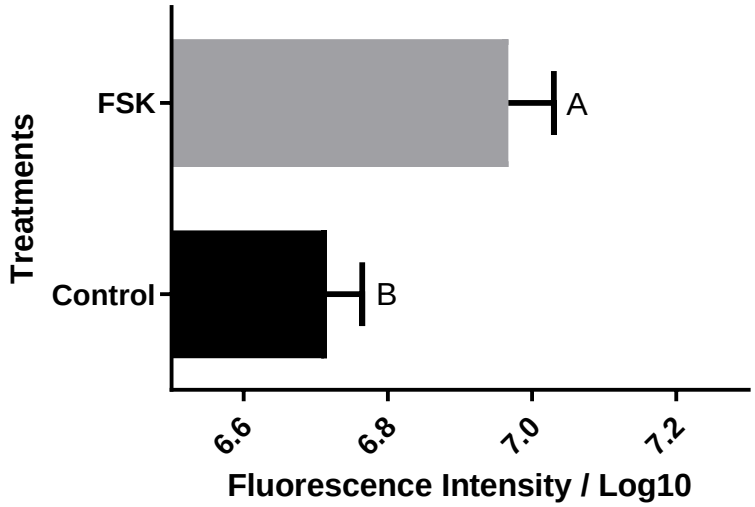
As demonstrated in Table 1, oocytes matured in the presence of Forskolin had similar cleavage and blastocyst development rates, and number of blastomeres compared to the control ($P < 0.05$), demonstrating that embryo development and quality was not affected by the treatment. Regarding lipid content, Figure 1 demonstrates that the control had greater lipid consumption ($P < 0.05$). Here, we added Forskolin for comparisons since this cAMP modulator influences meiotic arrest and leads to a better synchrony between nuclear and cytoplasmic maturation. Fu et al. (2011) treated swine oocytes with FSK for the entire maturation period and also for the second half, describing that both the lipid content and the blastocyst formation were reduced, when oocytes were exposed to FSK for 42 h, possibly due to alterations in the cytoplasmic maturation. In oocytes treated with FSK during the second half, lipid content was reduced, and embryo development was not compromised (FU et al., 2011), which is, in part, similar to our results.

Table 1 - Embryo development rates and embryo cell count of structures derived from pig oocytes matured in vitro in the presence of Forskolin (FSK)

	Treatments	
	Control	FSK
Cleavage 24h (%)	52.1 (162/311)	48.8 (158/324)
Cleavage 48h (%)	76.5 (238/311)	70.4 (228/324)
Blastocyst (%)	38.6 (120/311)	31.8 (103/324)
Embryo Cells (n)	57.8 ± 4.7	53.9 ± 6.7

Control - TCM-IVM - TCM-199 supplemented with 0.1% PVA and 2.5 mM NaHCO3, 0.57 mM cysteine, 0.91 mM C3H3NaO3, 0.001 g/mL EGF, 0.05 g/mL streptomycin, 0.065 g/mL penicillin, with gonadotrophins (20 µg/µL FSH, 5 µg/µL LH) for the first 22 h of maturation
FSK - Forskolin (TCM-IVM + 10 µM FSK during the last 22 h) (Fu et al, 2011).

Figure 1 - Fluorescence intensity of lipid droplets (Log10) of embryos stained with Nile Red after maturation in Control and Forskolin (FSK). A,B Mean frequencies ± SEM with different superscripts differ by at least p <0.005



Control (n = 41) - TCM-IVM - TCM-199 supplemented with 0.1% PVA and 2.5 mM $NaHCO_3$, 0.57 mM cysteine, 0.91 mM $C_3H_3NaO_3$, 0.001 g/mL EGF, 0.05 g/mL streptomycin, 0.065 g/mL penicillin, with gonadotrophins (20 µg/µL FSH, 5 µg/µL LH) for the first 22 h of maturation;
FSK (n = 33) - Forskolin (TCM-IVM + 10 µM FSK during the last 22 h) (Fu et al, 2011)

4. CONCLUSIONS

Oocyte maturation with Forskolin during the last 22 h does not impact initial cleavage and embryo quality, although there was no reduction in the lipid content.

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