**Introduction**

*In vitro* maturation (IVM) is an important process in the *in vitro* production of embryos. It has been recently shown that three cytokines (FGF2, LIF, IGF1) have increased the efficiency of *in vitro* maturation, blastocyst production and *in vivo* development in pig (Yuan et al., PNAS 2017, 114(29): E5796-E5804). IVM in medium supplemented with cytokines doubled the blastocyst rate and quadrupled the litter size when transferred. We have investigated whether this medium will have similar effects in the bovine model. Cytokine supplemented medium will be referred to as "FLI medium".

**Objectives**

- To assess the effect of FLI medium on IVM in bovine SCNT and IVF in vitro development
- To assess the effect of FLI medium on oocyte and blastocyst quality
- To assess the effect of FLI medium on bovine SCNT pregnancy and birth rate

**Methods**

**Oocyte Maturation** Cumulus oocyte complexes were matured for 21 hours in either our standard maturation medium (TCM-199 [Gibco], containing 10% FBS, 0.5 μg/mL FSH, 5 μg/mL LH, and 100 U/mL penicillin/streptomycin) or maturation medium supplemented with 20 ng/mL human LIF, 20 ng/mL human IGF1 and 40 ng/mL human FGF2.

**GSH/ROS Staining** MII oocytes were incubated in 20 μM CellTracker Blue CMF2HC or 10 μM H2DCFDA to measure GSH and ROS levels, respectively. Fluorescence was observed under ultraviolet light and measured using ImageJ software.

**Somatic Cell Nuclear Transfer (SCNT)** After IVM, the first polar body and metaphase plate were removed from MII oocytes. Donor cells were placed in the perivitelline space and fused with ooplasts in 0.28 M sorbitol fusion medium (0.1 mM calcium, 0.5 mM magnesium, 0.5 mM hepes, 1 g/mL BSA) by a single pulse of 1.75 kV/cm for 22 microseconds. Fused embryos were activated by exposure to 5 μM ionomycin for 5 minutes, followed by a 4 hour incubation in 2 mM DMAP and 10 μg/mL cycloheximide.

**In Vitro Fertilization** after IVM cumulus oocyte complexes were placed in fertilization medium with frozen thawed sperm as described by (Bavister et al. 1977), with modifications.

**In Vitro Culture** Embryos were cultured in groups of 45 in 50 μL droplets of SOF medium under mineral oil at 38.5°C with 5% CO2. Cleavage and blastocyst rates were assessed at day 2 and day 8, respectively.

**Blastocyst Cell Count** day 8 blastocysts were fixed, all cells stained with Hoechst and trophectoderm cells with CDX-2 and Alexa Fluor 488, a goat anti-mouse IgG secondary antibody. Blastocysts were imaged using a Zeiss fluorescent microscope. Cells were counted using ImageJ software (NIH, Bethesda, MD, USA).

**Embryo Transfer** Day 7 blastocysts were transferred to synchronized recipients. Initial pregnancy was determined by ultrasound on Day 40.

**Results**

**Experimental Design**

**Figure 1.** Chart outlining experimental design

**Figure 2.** Fluorescent images of *in vitro* matured bovine oocytes. (A-B) Oocytes were stained with CellTracker Blue CMF2HC to detect intracellular GSH levels. Relative GSH levels were significantly higher in oocytes matured in control medium (P <0.05). (C-D) Oocytes were stained with 20,70-dichlorodihydro-fluorescein diacetate to detect intracellular ROS levels. Relative ROS levels were also significantly higher in oocytes matured in control medium compared to those matured in FLI medium (P <0.05).

**Figure 3.** Fluorescent images of fixed stained SCNT blastocysts. (A) Blastocyst cell DNA was stained with Hoescht and trophectoderm cells were stained with CDX-2 antibodies. (B) Violin plot of total cell count data. Total cell count was significantly higher in FLI embryos (P <0.05). There was no significant difference in trophectoderm and inner cell mass cell count.

**Table 1.** Effect of FLI medium on oocyte maturation

<table>
<thead>
<tr>
<th></th>
<th>Oocytes (n)</th>
<th>Matured Oocytes (n)</th>
<th>Maturation Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLI Medium</td>
<td>885</td>
<td>709</td>
<td>80.2±2.3%</td>
</tr>
<tr>
<td>Control</td>
<td>822</td>
<td>549</td>
<td>66.8±1.8%</td>
</tr>
</tbody>
</table>

**Table 2.** Effect of FLI medium on IVF embryo development

<table>
<thead>
<tr>
<th></th>
<th>Oocytes (n)</th>
<th>Cleaved Embryos (n)</th>
<th>Cleavage Rate (%)</th>
<th>Blastocysts (n)</th>
<th>Blastocyst Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLI Medium</td>
<td>518</td>
<td>435</td>
<td>84.0±1.3%</td>
<td>155</td>
<td>35.6±2.1%</td>
</tr>
<tr>
<td>Control</td>
<td>551</td>
<td>420</td>
<td>77.7±1.9%</td>
<td>117</td>
<td>27.3±1.9%</td>
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</table>

**Table 3.** Effect of FLI medium on SCNT embryo development

<table>
<thead>
<tr>
<th></th>
<th>Reconstructed Embryos (n)</th>
<th>Cleaved Embryos (n)</th>
<th>Cleavage Rate (%)</th>
<th>Blastocysts (n)</th>
<th>Blastocyst Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLI Medium</td>
<td>446</td>
<td>418</td>
<td>93.9±1.1%</td>
<td>181/446</td>
<td>40.6±5.1%</td>
</tr>
<tr>
<td>Control</td>
<td>300</td>
<td>272</td>
<td>90.9±1.2%</td>
<td>73/300</td>
<td>24.3±2.9%</td>
</tr>
</tbody>
</table>

- Percentages are means ± SEM. 4, 3, 8, and 4 replicates were performed in tables 1-4, respectively. Data were analyzed by One-way ANOVA in Tables 1-3. Data in Table 4 were analyzed using chi-square (the Jamovi Project 2020)
- a, b values within a column with a different superscript are significantly different.

**Summary**

- FLI medium improved bovine IVM rate and oocyte quality as evidenced by the decreased ROS level.
- Oocyte matured in FLI medium exhibited greater blastocyst formation rate following IVF and SCNT. Additionally, total cell number in FLI SCNT blastocysts was greater than in the control group.
- Furthermore, pregnancy rate (Day 40) was also improved in the FLI group. We continue to monitor these pregnancies.

**Future work**

- Measurement of mRNA levels will be performed to identify differentially expressed genes in FLI treated group.
- RNA sequencing will be performed on cumulus cell samples.

**References**