Dissertation summary

Improvement of the *in vitro* growth culture system for bovine oocytes derived from early antral follicles: Effect of the duration of culture, oxygen environment, and astaxanthin supplementation on the acquisition of oocyte developmental competence

Madalitso Chelenga

Today, the global utilization of *in vitro*-derived cattle embryos through *in vitro* embryo production (IVP) techniques has increased compared to their *in vivo*-derived counterparts. The bovine ovary contains a large pool of small follicles that are a potential source of oocytes for IVP. However, the majority are lost during their growth and development phase due to atresia. Several *in vitro* growth (IVG) culture systems using oocytes derived from early antral follicles (*i.e.*, measuring between 0.4-1 mm in diameter) have been reported. Still, the developmental competence of the oocytes derived from these culture systems is lower than *in vivo*-grown oocytes. Therefore, improving the current IVG culture systems is necessary to utilize oocytes derived from early antral follicles by IVP. In addition, IVG culture systems can be used as *in vitro* growth models for studying *in vivo* oogenesis in mammals. Therefore, in chapter I attempted to improve the efficiency of the IVG culture systems by shortening the duration of culture to mimic the *in vivo* growth from early antral to the antral follicle stage. On the other hand, chapter II explored the optimal O_2 environment for the 8-day IVG culture system using the GP device with Ax supplementation.

IVG culture of bovine oocyte-cumulus-granulosa cell complexes (OCGCs) is generally carried out for 12 or 14 days using conventional gas-impermeable (GIP) culture

devices. This culture duration may be longer than follicular development from early antral to antral follicle stage, which only takes about 7 days *in vivo*. Furthermore, follicles receive O_2 supply from micro vessels; however, O_2 supply may be limited under the conventional IVG culture conditions. This problem can be mitigated by using the gas permeable (GP) culture device during IVG culture. While the GP culture device increases dissolved O_2 availability in culture media, excessive O_2 concentration during culture may increase reactive oxygen species (ROS) generation by OCGCs, which causes oxidative stress. Oxidative stress during culture can be mitigated by using antioxidant supplementation. Astaxanthin (Ax) is one of the antioxidants popular due to its potent antioxidant activity during oocyte and embryo culture.

The purpose of chapter I was to investigate the effect of increasing dissolved O_2 availability using a GP culture device with or without Ax supplementation on the *in vitro* development bovine OCGCs derived from early antral follicles cultured for 8 days. OCGCs were cultured in GP, GP supplemented with Ax (*i.e.*, GP + Ax), and a conventional GIP device (control) for 8 or 12 days. OCGC viability was significantly higher when cultured for 8 days than 12 days ($p < 0.001$) in all culture conditions, but a significant difference was not observed among groups ($p > 0.05$). Antrum formation rates and granulosa cell numbers on days 8 and 12 of culture were significantly higher in control than in the GP groups regardless of Ax supplementation ($p < 0.05$). Oocyte diameters were similar among day-8 GP + Ax, day-8 control, and day-12 control groups ($p > 0.05$). Nuclear maturation rates of oocytes grown for 8 days were significantly higher in the GP + Ax group than in the control and the GP groups ($p < 0.05$) and similar to oocytes grown for 12 days regardless of the culture conditions ($p > 0.05$). The generation of ROS by OCGCs on day 8 of IVG culture was significantly lower in the GP + Ax group

than in the GP and control groups ($p < 0.05$). After 8 days of culture, IVG oocytes developed into blastocysts, and the cleavage and blastocyst rates were similar in all treatment groups. However, *in vivo*-grown oocytes had significantly higher ($p < 0.05$) cleavage and blastocyst rates than the IVG oocytes in all groups. In summary, this study demonstrates that increased O_2 availability using a GP culture device with Ax supplementation promotes oocyte growth and maturation competence but inhibits proliferation of granulosa cells and antrum formation compared with a conventional GIP culture device. Furthermore, OCGCs can attain developmental competence after only 8 days of IVG culture.

Despite the 20% O_2 environment being used often during IVG culture, *in vivo* studies have established that the ovarian follicular fluid contains only about 1.3-5.5% O_2. Furthermore, previous studies have described the detrimental effects of culture in a constant 20% O_2 environment on the *in vitro* growth of oocytes. On the other hand, many cell types grow well under lower oxygen environments *in vitro.* Therefore, the purpose of chapter II was to determine the effects of a constant low (5-5%) and modulated (5-20%) oxygen environments on the *in vitro* development of bovine OCGCs cultured in the presence or absence of Ax. These OCGCs were cultured in a GP culture device for 8 days. The oxygen concentration was switched from 5% to 20% on day 4 of culture in the oxygen modulated culture conditions. Ax supplementation slightly increased the viability of OCGCs cultured under 5-20% O_2 conditions. E_2 production increased during the IVG culture, whereas P_4 production did not, regardless of the O_2 environment or Ax supplementation. IVG oocytes cultured in the 5-20% O_2 environment with Ax supplementation showed the highest nuclear maturation rate among all experimental groups, similar to *in vivo*-grown oocytes. The development of blastocysts was promoted

by the 5-5% O_2 environment regardless of Ax supplementation; however, Ax supplementation under 5-20% O_2 condition promoted blastocyst development, similar to 5-5% culture conditions. In conclusion, the initial exposure of OCGCs to the low O_2 environment during the IVG culture using the GP culture device maintained an increasing E_2 production pattern compared to the constant 20% O_2 environment in previous reports. In addition, a low O_2 environment promoted blastocyst rates; however, the modulation of the O_2 environment from low to high with Ax supplementation also promoted the nuclear maturation and development competence of day-8 IVG oocytes.

Finally, this study has improved IVG culture systems for incompetent bovine oocytes derived from early antral follicles by shortening the duration of culture from 12 to 8 days and increasing the O_2 availability with Ax supplementation. A constant low O_2 environment or O_2 modulation with Ax supplementation are ideal culture conditions to improve blastocyst rates of oocytes derived from these IVG culture systems. The improved IVG culture system described in this study can provide an alternative source of oocytes that can be submitted for conventional IVP. In addition, while this IVG culture system requires further optimization, it can be used as an *in vitro* model to study oogenesis and folliculogenesis in other large mammalian species such as human beings.