

# **Impact of Long In Vitro Culture on Oocytes from Sheep Early Antral Follicles: A Comparison of Mitochondrial Activity and ROS Levels between Single and Group Culture**

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It has been demonstrated that long in vitro culture (LIVC) of cumulus-oocyte complexes (COCs) from early antral follicles (EAFs) is a promising approach for increasing the number of available oocytes for assisted reproductive technologies. However, suitable culture conditions must be clarified and further optimized. In this study, we compared the efficacy of single versus group cultures of COCs using LIVC. Additionally, we analyzed the mitochondrial function and reactive oxygen species (ROS) levels in cultured oocytes to evaluate the system's efficiency. For this purpose, sheep ovaries were collected from the slaughterhouse, and COCs were retrieved from EAFs (350-450  $\mu\text{m}$ ). The COCs were then cultured either individually (n=327) or in groups (n=312) in TCM199 supplemented with 0.15  $\mu\text{g}/\text{mL}$  zinc sulfate,  $10^{-4}$  IU/mL FSH, 10 ng/mL estradiol, 50 ng/mL testosterone, 50 ng/mL progesterone, and 5  $\mu\text{M}$  Cilostamide. Chromatin configuration, mitochondrial activity, mitochondrial distribution, and oocyte ROS levels were analyzed using confocal microscopy and fluorescent probes before and after 5 days of LIVC. Additionally, meiotic competence was assessed after in vitro maturation (IVM).

Our findings indicated no statistically significant differences in chromatin configuration, intracellular ROS levels, mitochondrial activity, and distribution pattern after LIVC ( $P < 0.05$ ) between treatments. However, following IVM, the group culture showed a significant increase in oocyte maturation capability (34.78 vs. 16.67,  $P < 0.01$ ) and a tendency toward lower levels of ROS compared to the single culture ( $P > 0.086$ ). Additionally, mitochondrial distribution patterns showed a noticeable shift from a 'fine' to a 'granular' pattern in the group culture ( $P < 0.01$ ), which may benefit oocyte competence. In conclusion, the study demonstrated that both single and group culture conditions effectively supported chromatin configuration changes and mitochondrial activity while decreasing ROS levels in COCs collected from sheep EAFs. However, COCs cultured in groups showed higher rates of meiosis resumption, lower levels of ROS, and a more granular mitochondrial pattern compared to the single cultured ones. These findings suggest insights for optimizing the culture conditions of COCs from EAFs, thus widening the availability of competent gametes for assisted reproductive technologies applied to animal breeding and endangered species conservation.