Revolutionizing Female Fertility: Pioneering *In-Vitro* Culture of Multiple Ovarian Follicles on a Reproductive Tissue Engineering Scaffold for the Advancement of Transplantable Artificial Ovaries

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Implementing tissue engineering for safe *in-vivo* transplantation of an artificial ovary poses challenges in reproductive medicine. Taking advantage of the recent developed of ovarian biomimetic scaffolds, the present research has been designed to demonstrate the role in supporting ovine folliculogenesis as a key proof-of-concept to translate its use in clinic herald new strategy for fertility restoration.

The Reproductive Tissue Engineering (REPROTEN) approach has been used to demonstrate the bio-functionality of poly(epsilon-caprolactone)(PCL) electrospun patterned scaffolds in orchestrating sequential *in-vitro* folliculogenesis/*iv*F from preantral follicles/PA up to oocyte maturation. This study proposes an innovative multiple-follicles long-term culture (18-days) to reproduce a fragment of ovary (Artificial Ovary/AO). The goal is to replicate the natural microenvironment guiding follicles transition from PA to early-antral/EA stages, completing the phase of oocyte-growth and acquiring meiotic competence. Upon reaching the antral-stage, the follicle-enclosed-oocyte is exposed to human Chorionic Gonadotropin/hCG surge to trigger meiotic maturation.

PA (250±4), mechanically isolated from slaughterhoused lamb ovaries, were cultured on scaffold individually (Control/Ctrl) or in groups (10 PA/well; AO) on PCL-Patterned scaffolds. To preserve the ratio scaffold-surface and volume/follicle, Ctrl and AO were placed on 96 and 48-well plates, respectively. The cultural media were serum-free, supplemented with 4IU/mL equine Chorionic Gonadotropin (growth-phase) or 25 IU/ml hCG (maturation-phase). Follicle development was assessed by considering follicle-growth, antrum differentiation and CYP19A1-expression. The culture influence on germinal compartment is recorded by considering oocyte increase, chromatin configuration, Metaphase-II and parthenogenetic activation-rate.

AO synchronously supported follicle and oocyte growth. The AO displayed slower follicular growth with a delay in antrum differentiation on Day 12 (44.8%) *vs.* Ctrl (84.6%;$p<0.001$). Therefore, the long-term *iv*F was better faced by AO with a consistent lower rate of degeneration (5% *vs.* 28% Ctrl;$p<0.001$). Furthermore, almost the totality of follicles in AO differentiated the antrum cavity (95% *vs.* 72% Ctrl). Additionally, the transition from PA to EA in AO group was accompanied from a great upregulation of CYP19A1 (2.5-fold change PA *vs.* EA;$p<0.0001$), significantly higher than that recorded in Ctrl-EA (1.5-fold;$p<0.01$).

The synchronous *in-vitro* development of somatic and germinal compartments is confirmed by the meiotic competence acquisition (2.6-fold change PA *vs*. EA in AO;$p<0.001$). The long-term culture in AO improved also the quality of the collected oocyte in term of chromatin configuration (53.3% Surrounding-Nucleolus; 26.7% Surrounding-Nuclear-Envelope *vs*. 100% Non-Surrounding-Nucleolus in Ctrl;$p<0.001$), Metaphase-II (62% *vs.* 48% Ctrl;$p<0.05$) and parthenogenetic activation rate (66.7% *vs.* 15.4% in Ctrl;$p<0.001$).
The ability to bypass the pronuclear block in AO-system may be attributed to a more mature chromatin organization in AO-derived oocytes, with a nuclear remodeling distribution rate exceeding 50% in Surrounding-Nucleolus and Surrounding-Nuclear-Envelope (compared to 100% Non-Surrounding-Nucleolus in Ctrl;$p<0.0001$).
The study delves into the prospective application of the REPROTEN solution to face female infertility. PCL-scaffolds showcase the ability to replicate functionally the native ovarian matrix by supporting multiple and coordinate follicle development. This opens two potential applications: optimizing current *iv*F protocols with

biomimetic scaffolds, potentially mimicking gonadotropin-insensitive/low-sensitive phases; using PCL to construct ovarian fragments from frozen ovaries, onto which early-stage developing follicles can be transferred for transplantation, reducing cancerous cell transfer risk. The study emphasizes the crucial role of assisted-reproductive-techniques in enhancing female fertility by recovering early-stage follicles, highlighting potential future applications in ovarian transplantation.

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