The Age-Associated Increase In Ovarian Stiffness Impairs Follicle Development And Oocyte Quality Through Early Modulation Of Follicles' Transcriptome

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Oocyte quantity and quality are severely impacted by multiple extrinsic factors, with female reproductive aging being among the most prevalent in our society. We and others reported that with aging the mouse ovary assumes a pro-fibrotic milieu, which is associated with ovarian stiffness. However, the role of stiffness on ovarian function and oocyte quality is unknown. The objective of this work is to use an alginate-encapsulated *in vitro* follicle culture system that mimics age-associated changes in the ovarian microenvironment to identify whether stiffness is a novel mechanism mediating the progressive decline in oocyte quality observed with advanced maternal age. To evaluate how stiffness impacts follicle development and oocyte quality, we synthesized hydrogel that replicated the soft (young: 0.5%, 1.79±0.08 kPa) and stiff (old: 2%, 4.56±2.03 kPa) environments. Secondary follicles from CD1 (D12-D13, N=3 replicates) were cultured in 0.5% or 2% alginate for up to 12 days. Follicles cultured in stiff environments showed significant reduction in follicle size compared to follicles in soft environments (0.5% 226.9±17.4um, 2% 160.8±9.9um,$p < 0.0001$). These differences were triggered by granulosa layer since no changes were detected in oocyte size (oocytes: 0.5% 66.19±5.7um, 2% 60.6±4.5um,$p=0.401$; granulosa cells: 0.5% 160.04±13.4um, 2% 103.37±16.5um,$p < 0.0001$) suggesting that granulosa cells are not proliferating in a stiff environment. To explore this, we assessed estradiol synthesis and granulosa cell death. Estradiol levels were reduced in 2% (0.5% 11.3±15.9ng/ml, 2% 0.3±0.5ng/ml,$p=0.296$) while CC3 (apoptosis-marker) intensity was increased (CC3intensity/area, 0.5% 20.4±5.8a.u., 2% 27.1±4.3a.u.,$p < 0.05$), confirming that stiff environments impact granulosa cells viability. We then evaluated whether stiff environment impact gamete quality. Oocytes from follicles at D12 were isolated and inspected morphologically. Oocyte quality significantly declined in 2%, with 68.9±16.8% of oocytes degenerated, compared to 23.6±9.2% in 0.5%. We repeated all the analyses using the CB6F1 strain yielding comparable results. Since the effects of stiffness on follicle growth were already evident at day 4, we investigated early-changes in follicles' transcriptome that might mediate the observed phenotypes. 32 secondary follicles were cultured in 0.5% or 2%, and analyzed by RNAseq at 3h, 6h, 12h and 24h. We analyzed follicles' gene expression (*DESeq2*,R/Bioconductor) at each timepoint comparing 0.5% and 2% (significance at padj< 0.05;

log2FoldChange±2). Surprisingly, we found few differentially expressed genes (DEG) at 3h, a peak of DEG at 6h, followed by a dramatic decrease which reached a plateau at 12h and 24h. DEGs at 6h were involved in response to external stimuli and ERK1/2 cascade, pathways of proliferation and differentiation, suggesting that at 6h follicles' transcriptome is more sensitive to changes in ovarian biomechanics. To further evaluate how stiffness impacts ovarian transcriptome, we analyzed DEGs in a time-dependent manner. We identified 1033 DEGs in follicles cultured in 2% at 24h compared to 3h. Most of the upregulated genes were associated with apoptosis and matrix remodeling. In the 0.5% condition, 1295 DEGs were identified, with upregulation of genes related to metabolism and cell cycle at 24h. Overall, we demonstrated that the age-associated increase in ovarian stiffness impacts follicle development and oocyte quality by impairing granulosa cell viability. In addition, our transcriptome analysis reveals that follicles quickly respond to stiffness triggering the expression of well-known genes associated with advanced maternal age when exposed to a stiff environment. These results show that stiffness might be a novel regulator of folliculogenesis and oocyte quality.

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