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Letter to the Editor

Supplementation of nicotinamide mononucleotide improves the quality of postovulatory aged porcine oocytes

Dear Editor,

Postovulatory aging would occur to impair the oocyte quality in a time-dependent manner if ovulated oocytes are not fertilized within the optimal timing in the oviduct or in the dishes (Trapphoff et al., 2016), which might result in the early pregnancy failure *in vivo* and adverse outcome of assisted reproductive technology (ART) *in vitro* (Nagy et al., 1993; Wilcox et al., 1998). However, the effective approaches to preventing the postovulatory aging-induced oocyte deterioration are still underexplored. We recently reported that supplementation of nicotinamide mononucleotide (NMN), a synthetic precursor of NAD^+, which is an essential cofactor for enzymes' functioning in almost all critical cellular metabolic reactions (Imai and Guarente, 2014; Canto et al., 2015), reverses the declining quality of maternally aged mouse oocytes (Miao et al., 2020).

To ask whether NMN could also improve the quality of postovulatory aged oocytes, we first assessed the morphology of matured porcine oocytes aged for 48 h *in vitro* as described previously (Miao et al., 2018), supplemented with different concentrations of NMN. As shown in Figure 1A, the control group displayed morphologically intact oocytes surrounded by the compact cumulus cell mass. By contrast, postovulatory aging caused a significantly higher incidence of fragmented oocytes with diffuse cumulus cells (Figure 1A and B). Notably, supplementation of increasing concentrations of NMN reduced the occurrence of fragmentation in postovulatory aged oocytes to varying degrees (Figure 1B; Supplementary Figure S1). We finally used 100 μM NMN for the subsequent experiments because this concentration strikes a balance between the recovery effect and dosage of NMN.

We next tested the NMN effect on the fertilization capacity of postovulatory aged oocytes. By *in vitro* fertilization, we found that ~60% of control oocytes were fertilized and developed to 2-cell embryos, while the fertilization rate of postovulatory aged oocytes was only ~20% (Figure 1C and D). NMN supplementation considerably increased the fertilization rate of postovulatory aged oocytes to ~40% (Figure 1C and D).

In general, oocyte quality is closely related to the cytoskeleton structure. We observed that a typical barrel-shaped spindle apparatus was formed in control oocytes, coupled with the neatly arranged chromosomes at the equatorial plate (Figure 1E). However, the postovulatory aged oocytes exhibited diverse disassembled spindles with disordered chromosomes in a considerable high frequency, which was rescued by the supplementation of NMN during aging (Figure 1E–G). Actin is another important cytoskeleton that plays a critical role in the establishment of asymmetric spindle positioning, cortical polarization, and cytokinesis in oocytes (Azoury et al., 2008). The data revealed that NMN supplementation also prevented postovulatory aged oocytes from the abnormal dynamics of actin filaments by displaying the discontinuous distribution with much weaker signals (Supplementary Figure S2).

Sperm-binding ability of oocytes affects the fertilization rate and is dependent on the dynamics of cortical granules (CGs) and their component ovastacin. Confocal imaging and quantification results showed that the number of sperm binding to the oocytes was significantly decreased after postovulatory aging in comparison with the controls, but it rose in the NMN-supplemented oocytes (Figure 1H and I). Meanwhile, postovulatory aging perturbed the normal distribution of CGs and ovastacin under the subcortex by manifesting the inconsecutive or completely disappeared signals (Supplementary Figures S3 and S4). On the contrary, the abnormalities of CGs and ovastacin caused by postovulatory aging were rescued by NMN supplementation (Supplementary Figures S3 and S4).

To further gain insights into the underlying mechanism of the NMN effects on the quality of postovulatory aged oocytes, we performed single-cell RNA sequencing. The heatmap and volcano plot data indicated that postovulatory aging dramatically changed the gene expression pattern of matured oocytes, which could be partially restored by NMN supplementation (Supplementary Figure S5). Particularly, Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) analyses displayed that differentially expressed genes (DEGs) were enriched in the oxidative phosphorylation and cellular senescence pathways (Figure 1J), as well as mitochondrial organization processes (Figure 1K), suggesting that the

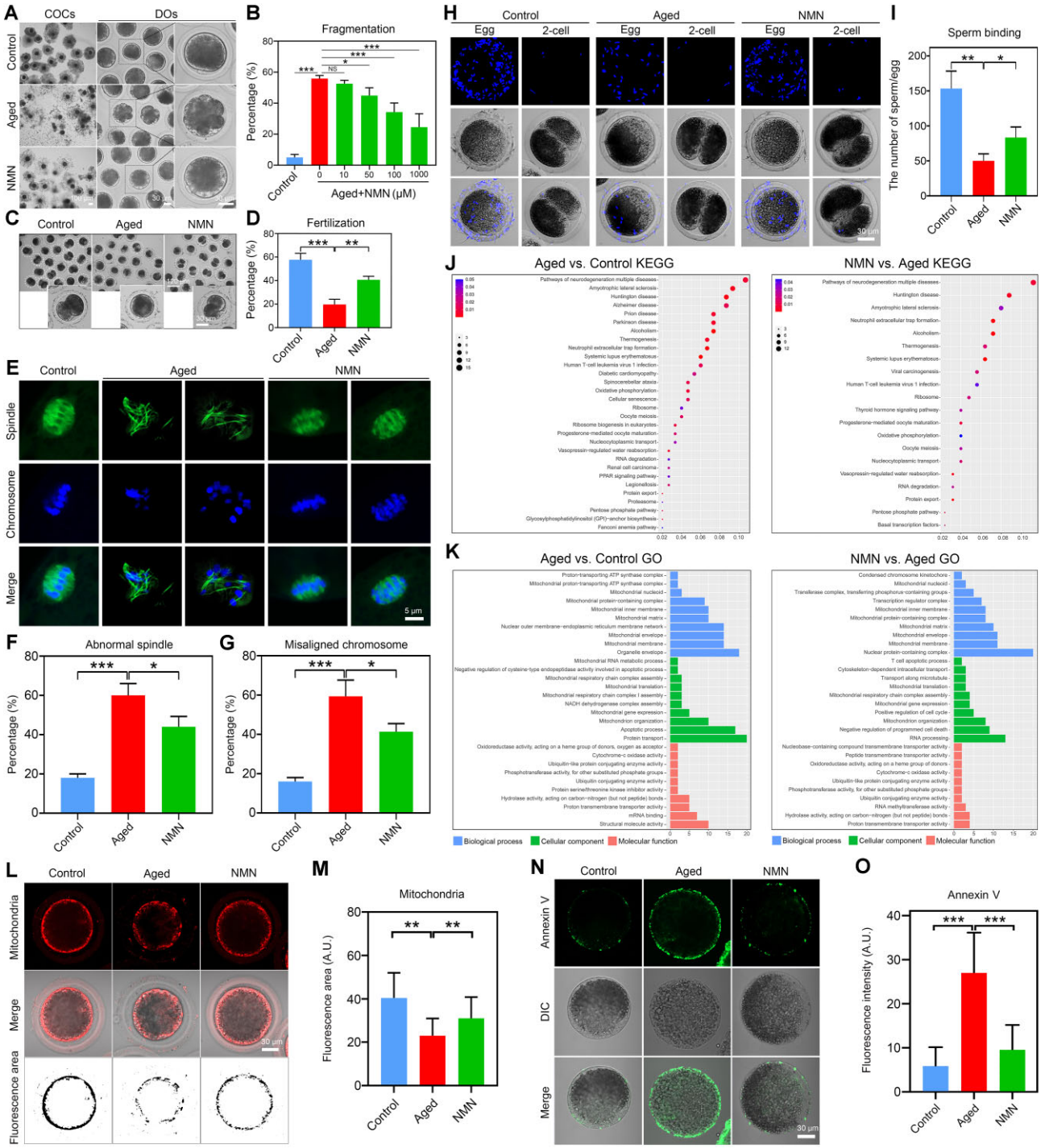

Figure 1 Effects of NMN supplementation on the quality of postovulatory aged oocytes. (**A**) Representative images of oocyte morphology and cumulus cell expansion in control, aged, and NMN-supplemented (100 μM) groups. COCs, cumulus–oocyte complexes; DOs, denuded oocytes. (**B**) The rate of fragmentation in control ($n = 112$), aged ($n = 98$), and NMN-supplemented (10 μM: $n = 104$; 50 μM: $n = 112$; 100 μM: $n = 100$; 1000 μM: $n = 104$) oocytes. (**C**) Representative images of 2-cell embryos fertilized from the control, aged, and NMN-supplemented oocytes. (**D**) The percentage of *in vitro* fertilization in control ($n = 52$), aged ($n = 46$), and NMN-supplemented ($n = 50$) oocytes. (**E**) Representative images of spindle morphologies and chromosome alignment in control, aged, and NMN-supplemented groups. (**F**) The rate of aberrant spindles in control ($n = 98$), aged ($n = 104$), and NMN-supplemented ($n = 100$) oocytes. (**G**) The rate of misaligned chromosomes in control ($n = 96$), aged ($n = 102$), and NMN-supplemented ($n = 90$) oocytes. (**H**) Representative images of sperm binding to control, aged, and NMN-supplemented oocytes. (**I**) The number of sperm binding to oocytes in control ($n = 110$), aged ($n = 114$), and NMN-supplemented ($n = 114$) groups. (**J**) KEGG enrichment analysis of DEGs in postovulatory aged oocytes compared to control oocytes and

restoration of the quality of postovulatory aged oocytes by NMN might be mediated through the mitochondria.

To test this possibility, we evaluated the mitochondrial localization by MitoTracker staining and the mitochondrial membrane potential ($\Delta\Psi m$) by JC-1 staining. The fluorescence imaging and quantitative analysis results revealed that, in control oocytes, most of the mitochondria accumulated around the lipid droplets under the subcortical region with robust signals, showing high $\Delta\Psi m$ (Figure 1L and M; Supplementary Figure S6). However, in postovulatory aged oocytes, a large proportion of mitochondria partially or completely lost this normal distribution with discrete and weak signals, showing low $\Delta\Psi m$ (Figure 1L and M; Supplementary Figure S6). Meanwhile, these abnormalities could be alleviated by NMN supplementation (Figure 1L and M; Supplementary Figure S6), suggesting that NMN can mitigate the mitochondrial dysfunction induced by postovulatory aging.

Because mitochondrial dysfunction is always associated with the induction of oxidative stress, we therefore measured the reactive oxygen species (ROS) levels in oocytes by 2′,7′-dichlorofluorescin diacetate staining. The imaging and quantification data displayed that ROS signals were hardly detected in the cytoplasm of control oocytes but prominently present in postovulatory aged oocytes (Supplementary Figure S7). NMN supplementation effectively eliminated the ROS caused by aging (Supplementary Figure S7), and thus suppressed the occurrence of apoptosis in postovulatory aged oocytes as assessed by annexin V staining (Figure 1N and O).

In summary, we provide several lines of evidence validating that NMN supplementation is an effective approach to improving the quality of postovulatory aged oocytes possibly by strengthening the mitochondrial function and suppressing the oxidative stress-induced apoptosis, which potentially contributes to enhance the efficiency of ART in human clinics.

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Figure 1 *(Continued)* in NMN-supplemented oocytes compared to postovulatory aged oocytes. (**K**) GO analysis of DEGs in postovulatory aged oocytes compared to control oocytes and in NMN-supplemented oocytes compared to postovulatory aged oocytes. (**L**) Representative images of mitochondrial distribution in control, aged, and NMN-supplemented oocytes. Mitochondria were stained with MitoTracker Red CMXRos. (**M**) The fluorescence area of mitochondrial signals in control ($n = 110$), aged ($n = 98$), and NMN-supplemented ($n = 102$) oocytes. (**N**) Representative images of apoptotic oocytes in control, aged, and NMN-supplemented groups. DIC, differential interference contrast. (**O**) The fluorescence intensity of annexin V-FITC signals in control ($n = 108$), aged ($n = 110$), and NMN-supplemented ($n = 100$) oocytes. Data in **B**, **D**, **F**, **G**, **I**, **M**, and **O** were presented as mean percentage or value (mean ± SEM) of at least three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.