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# Ovarian Mitochondrial and Redox Disturbances in Prepubertal Lambs and Effects of Autophagy Modulators on Oocyte In Vitro Development

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**Abstract**

Juvenile in vitro embryo transfer (JIVET) is a promising reproductive technology that utilizes the abundant ovarian follicle pool of prepubertal lambs for additional genetic gains in small ruminants. However, the poor oocyte developmental competence in prepubertal lambs compared with adult ewes severely limits its efficiency. This study aimed to characterize the ovarian mitochondrial and redox alterations in prepubertal lambs and evaluate pharmacological modulation of autophagy-associated processes in sheep oocytes. We employed data-independent acquisition (DIA)-based quantitative proteomics to compare ovarian protein expression between gonadotropin-stimulated 6-week-old prepubertal lambs and 3-year-old adult ewes, followed by validation of abundance changes in selected proteins and determination of their ovarian localization. These analyses revealed changes in proteins associated with energy metabolism and oxidative phosphorylation, together with reduced abundance of the mitochondrial ribosomal protein MRPL28 and the autophagy-associated proteins ATG3 and MAP1LC3B. In agreement with the proteomic findings, granulosa cells from lambs exhibited a lower mitochondrial membrane potential together with higher reactive oxygen species (ROS) levels. Supplementation with autophagy activators, AICAR, corynoxine B, or ML098 during adult ewe oocyte in vitro maturation (IVM) significantly increased subsequent blastocyst development and was accompanied by enhanced antioxidant and mitochondrial activity. Notably, supplementation with these activators during IVM also improved the blastocyst rate in lambs, with a significant increase observed following AICAR supplementation. These findings suggest that disturbances in mitochondrial and redox homeostasis of prepubertal lamb ovaries may contribute to decreased oocyte developmental competence, whereas pharmacological modulation of autophagy-associated processes may improve sheep oocyte quality and subsequent embryo development, offering a potential strategy for optimizing JIVET.

**Keywords:** prepubertal lambs; ovary; oocyte developmental competence; proteomics; autophagy modulation

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## 1. Introduction

Juvenile in vitro embryo transfer (JIVET) is a powerful assisted reproductive technology with significant benefits for small ruminant breeding. JIVET can advance the reproductive age of sheep, thereby increasing female selection and utilization, while also reducing generation intervals and accelerating genetic gains [1]. Following gonadotropin stimulation, lamb ovaries can produce up to ten times more antral follicles than adult ewes, a high responsiveness attributed to their underdeveloped hypothalamic-pituitary-gonadal axis [2]. Nevertheless, the oocytes from lambs often show abnormal cytoplasmic organization, lower energy metabolism, and reduced developmental competence compared to those from adult ewes [3–6]. Although recent studies have enhanced the developmental competence of lamb oocytes, it remains lower than that of adult ewe oocytes [7,8].

Addressing this developmental defect requires closer examination of mitochondrial function and redox regulation in the follicular microenvironment. Mitochondria provide ATP for meiotic progression and cytoplasmic maturation, while their redistribution helps coordinate energy production during oocyte maturation [9,10]. Ultrastructural studies of in vitro-matured oocytes have revealed that lamb oocytes have fewer mitochondria and less pronounced mitochondrial aggregation than adult ewe oocytes, resulting in a delayed rise in ATP content and lower ATP levels in lamb oocytes [3,10,11]. Mitochondrial function is also closely linked to redox regulation. The disruption of mitochondrial function in mouse oocytes has been associated with increased reactive oxygen species (ROS) production, altered ATP levels, and delayed meiotic resumption [12,13]. Studies in sheep oocytes have similarly linked oxidative stress to altered mitochondrial distribution and function, defective cytoskeletal organization, and impaired developmental competence, and antioxidant supplementation has been shown to rescue these changes [8,14]. Within the follicle, redox homeostasis depends on a coordinated balance between the production of mitochondrial ROS, antioxidant defense, autophagy-related mitochondrial quality control, and metabolic support provided by granulosa cells and cumulus cells [15–17]. These interconnected processes provide a rationale for investigating mitochondrial and redox alterations in gonadotropin-stimulated prepubertal ovaries. Although previous studies have identified significant transcriptional and proteomic differences in in vitro-matured oocytes, granulosa cells, and follicular fluid between lambs and adult ewes [6,18,19], the ovarian proteomic profile in stimulated prepubertal ovaries remains unmapped.

In this study, we characterized ovarian protein expression in 6-week-old prepubertal lambs and 3-year-old adult ewes by data-independent acquisition (DIA)-based quantitative proteomics following gonadotropin stimulation. We hypothesized that the specific proteomic profiles of gonadotropin-stimulated prepubertal lamb ovaries might disrupt important metabolic and redox homeostasis pathways, leading to follicular microenvironment instability and subsequently inhibiting the developmental potential of oocytes. We also tested whether pharmacological modulation of autophagy-associated processes during in vitro maturation (IVM) could improve mitochondrial and redox-related characteristics and subsequent embryonic development, thereby providing new insights into the optimization of the JIVET technology.

## 2. Materials and Methods

### *2.1. Animals*

Two separate cohorts of animals were used. The first cohort comprised prepubertal lambs (5–6 weeks old, $n$ = 8) and adult ewes (3 years old, $n$ = 18) used for ovarian characterization, all of which were Merino sheep and were raised in the Aohan Banner

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Breeding Sheep Farm (Chifeng, Inner Mongolia, China). Another independent cohort of 7 lambs (5–6 weeks old) was used for the oocyte IVM experiments, all of which were Australian White sheep and were raised in the Ruimeng Animal Husbandry Co., Ltd. (Chifeng, Inner Mongolia, China). All animals experienced standardized housing conditions, with an ambient temperature of 18–22 °C and a natural photoperiod. All animal experiments were conducted in accordance with the animal care policies of China Agricultural University and were approved by the university's Animal Ethics Committee (accession number: AW20701202-3-7, date of approval: 2 April 2021).

### *2.2. Hormone Treatment*

The superovulation procedure was followed by the predefined protocol, as described previously [6,18]. Specifically, adult ewes were inserted with an intravaginal controlled internal drug release device (CIDR, containing 0.3 g progesterone; InterAg, Hamilton, New Zealand) on Day 0 (D0) for synchronization. Ten days after CIDR insertion (D10), the ewes were super-stimulated with a total dose of 270 IU follicle-stimulating hormone (FSH; Ningbo Sansheng Pharmaceutical Co., Ltd., Ningbo, China) administered in a tapering six intramuscular injections at 12-h intervals over 3 consecutive days (D10–D12). For lambs, the 270 IU FSH was divided into six injections, and the first injection coincided with administration of 400 IU of pregnant mare serum gonadotropin (PMSG; Ningbo Sansheng Pharmaceutical Co., Ltd., Ningbo, China) (Figure 1A). 12 h after the final FSH injection, the ovaries were exposed and surgically removed by midventral laparotomy under anesthesia induced by intramuscular injection of Su-Mian-Xin II (Institute of Military Veterinary, Changchun, China; 5 μL/kg body weight). Cumulus–oocyte complexes (COCs) were then aspirated from the ovaries of both animal groups. In adult ewes, the CIDR was removed at the time of oocyte collection.

### *2.3. Proteome of Ovaries*

For the DIA experimental design, three ovarian tissue samples were prepared for each lamb group or adult group. Ovaries were rinsed with saline and stored at −80 °C. Ovarian proteins were extracted using RIPA protein extraction buffer (Beyotime, Shanghai, China, Cat. No. P0013B) for DIA proteomics analysis and western blotting. Total protein concentration was checked with the Pierce BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

The proteins (100 μg per sample) were processed by filter-aided sample preparation (FASP) method using 10-kDa ultrafiltration devices [20]. Equal peptide aliquots from the six samples were pooled, and 100 μg of the pooled peptides were fractionated by high-pH reversed-phase chromatography for data-dependent acquisition (DDA) and spectral library generation. For DIA quantification, peptides from each sample were analyzed separately using an UltiMate 3000 UHPLC system coupled via a nanoelectrospray ionization source to an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific) [21]. The DDA data were searched against the sheep UniProt fasta database (TaxID: 9940) based on Sequest HT in Proteome Discoverer 2.1.0182. Identifications were filtered at a peptide or protein FDR < 1%. The resulting spectral library was imported into Skyline (v21.1) for DIA signal extraction and quantification.

The differentially expressed proteins (DEPs) were identified using mapDIA (v3.1.0). The criteria for identifying DEPs were $\log_2$ fold change ≥0.58 or ≤–0.58 and a $p < 0.05$. Functional enrichment of DEPs in Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways was assessed with clusterProfiler in R software (v4.2.1). Terms meeting $p < 0.05$ were identified as significant.

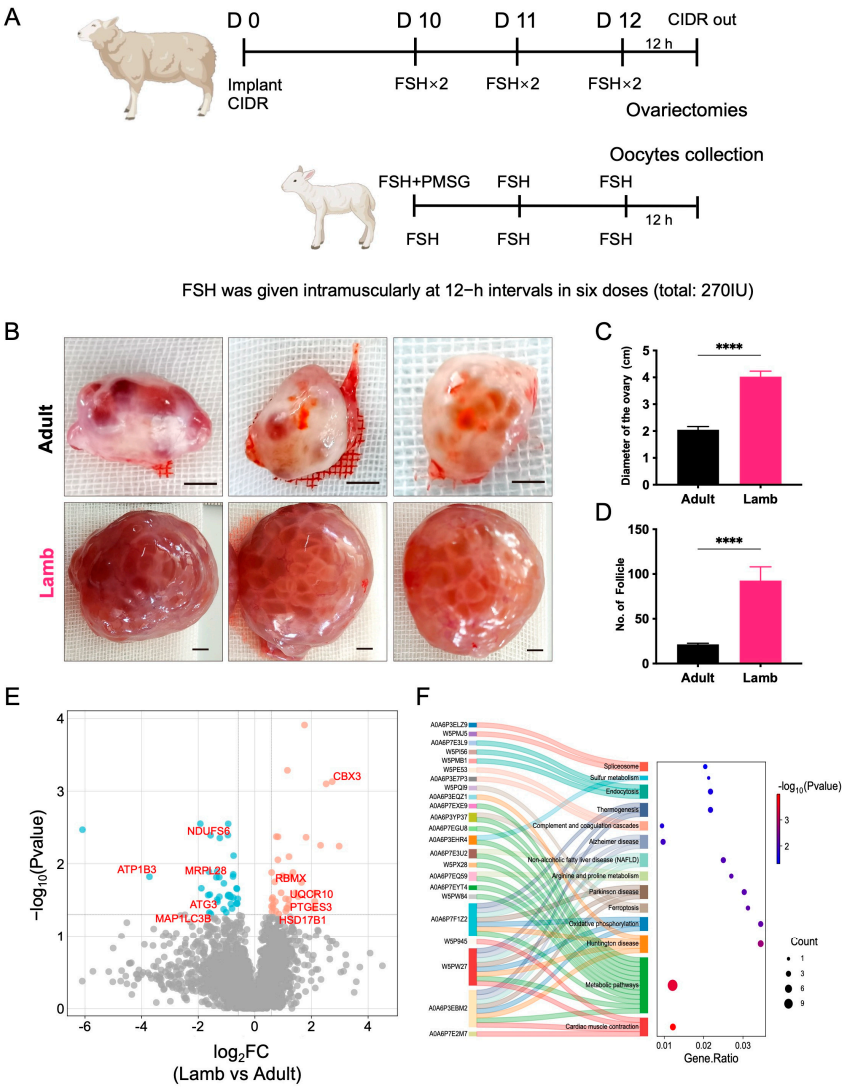



**Figure 1.** Comparison of ovarian morphological characterization and differentially expressed proteins (DEPs) between adult ewes and lambs following gonadotropin stimulation. (**A**) Gonadotropin stimulation treatment protocols of adult ewes and lambs. (**B**) The representative gross morphology of whole ovaries from adult ewes and lambs after hormonal stimulation. Scale bar: 0.5 cm. (**C**) The ovarian diameter and (**D**) number of visible surface follicles in the two groups. **** $p < 0.0001$. (**E**) Volcano plot showing DEPs between adult ewe and lamb ovaries. Red and blue dots indicate DEPs meeting both thresholds ($p < 0.05$ and $|\log_2FC| \geq 0.58$), and gray dots indicate proteins that did not meet both thresholds. The *x*-axis shows $\log_2FC$, with positive and negative values indicating higher and lower protein abundance in lamb ovaries, respectively. The *y*-axis shows $-\log_{10}(p)$. (**F**) Enrichment of DEPs in Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Bubble color denotes the *p* value; the number of DEPs assigned to a pathway determines its bubble size.

### *2.4. In Vitro Oocyte Maturation, Fertilization, and Embryo Culture*

The COCs were aspirated from slaughterhouse-derived adult ewe ovaries or gonadotropin-stimulated prepubertal lamb ovaries. The IVM medium was comprised of TCM 199 supplemented with 10% (*v*/*v*) fetal bovine serum (FBS), 10 μg/mL FSH (Folltropin-V; Bioniche Inc., Belleville, ON., Canada), 10 μg/mL LH (Bioniche Inc.), 1 μg/mL 17β-estradiol, 100 IU/mL penicillin, and 100 μg/mL streptomycin. To evaluate the

effects of autophagy activators, COCs were collected from slaughterhouse-derived adult ewe ovaries and were cultured in IVM medium containing AICAR (0, 50, 100, or 200 μM; MedChemExpress, Monmouth Junction, NJ, USA), corynoxine B (Cory B; 0, 5, 20, or 50 μM; MedChemExpress), or ML098 (0, 0.1, 1, or 5 μM; MedChemExpress). For lamb oocyte experiments, COCs were cultured in IVM medium or in medium containing 50 μM AICAR, 50 μM Cory B, or 5 μM ML098 for 24 h at 38.5 °C in a humidified atmosphere of 5% $CO_2$.

After IVM, the expanded cumulus cells were removed from oocytes by gentle pipetting, and matured oocytes were transferred to an IVF medium containing 2% estrus sheep serum in synthetic oviduct fluid (SOF) [22]. The oocytes were co-incubated with 2 × $10^6$ spermatozoa/mL for 16 h at 38.5 °C in an atmosphere comprising 5% $CO_2$. The zygotes were subsequently transferred to embryo culture medium, the SOF containing 8 mg/mL bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA), 2% (*v*/*v*) essential amino acids (Sigma-Aldrich), and 1% (*v*/*v*) non-essential amino acids (Sigma-Aldrich), and cultured at 38.5 °C in 88% $N_2$, 5% $CO_2$, and 7% $O_2$. Cleavage and blastocyst formation were recorded at 48 h and 6–8 days after IVF, respectively.

### *2.5. Immunohistochemistry (IHC) Staining*

Ovaries were fixed in 4% paraformaldehyde for 24 h at room temperature, processed for paraffin embedding, and cut into approximately 5-μm sections. The sections were mounted on glass slides, deparaffinized in xylene, and rehydrated through graded ethanol. The ovarian sections were then retrieved by microwave heating with a buffer of Citrate Antigen Retrieval Solution (Beyotime, P0081, pH 6.0) for 20 min. Endogenous peroxidase activity was quenched by incubation with 3% $H_2O_2$ for 10 min, and the sections were blocked with 5% goat serum for 30 min at room temperature. Sections were incubated overnight at 4 °C with rabbit primary antibodies against MRPL28 (BBI, Shanghai, China, Cat. No. D122700; 1:100), UQCR10 (BBI, Cat. No. D263661; 1:100), ATG3 (BBI, Cat. No. D221649; 1:100), or MAP1LC3B (BBI, Cat. No. D263557; 1:50). After incubation, the sections were washed three times in phosphate-buffered saline (PBS) for 10 min each. Then the sections were incubated with horseradish peroxidase-labeled secondary antibody at room temperature for 1 h. Negative control sections were treated with secondary antibody without primary antibody. The 3,3′-diaminobenzidine (DAB) solution was applied to the sections for 5 min at room temperature in the dark. Sections were rinsed with tap water, counterstained with hematoxylin for 1 min, briefly differentiated in acid alcohol, and rinsed again with water. Sections were observed, and images were acquired using an Olympus BX51 microscope (Tokyo, Japan).

### *2.6. Western Blotting*

Equal quantities of protein were separated by SDS–PAGE and wet-electroblotted onto PVDF membranes. The membranes were blocked with blocking solution (Beyotime, P0252) at room temperature for 1 h. Then, the membranes were incubated at 4 °C overnight with the following primary antibodies: ATG3 (mouse; Santa Cruz, Dallas, TX, USA, Cat. No. sc-393660; 1:1000), MRPL28 (rabbit; BBI, Cat. No. D122700; 1:2000), NDUFS6 (rabbit; BBI, Cat. No. D222750; 1:500), and MAP1LC3B (rabbit; BBI, Cat. No. D263557; 1:200). The β-ACTIN (rabbit; Abcam, Cambridge, UK, Cat. No. ab8227; 1:5000) was selected as the loading reference. After washing three times, the membranes were incubated with the appropriate HRP-conjugated secondary antibodies (ZSGB-bio, Beijing, China; Goat Anti-Mouse IgG, Cat. No. ZB-2305, or Goat Anti-Rabbit IgG, Cat. No. ZB-2301; 1:8000) at room temperature for 2 h. The membrane was treated with Super ECL Plus (Beyotime, P0018S), and the Western blot signal was detected using a Tanon 5200 chemiluminescence imaging system (Beijing, China). ImageJ (National Institutes of

Health, Bethesda, Maryland, USA, v2.1.0) software was used to delineate relevant bands on the picture, thereby quantifying the intensity of the blot signal. Relative abundance was calculated by dividing the background-subtracted density of each target band by that of its corresponding β-ACTIN band.

### *2.7. Quantitative Reverse Transcription PCR (qRT-PCR)*

Ovarian samples were thoroughly ground and processed with TRNzol Universal (Tiangen, Beijing, China, Cat. No. DP424) following the protocol. The RNA was reverse-transcribed with the TIANScript II RT Kit (Tiangen, Cat. No. GKR107). For oocyte analysis, 10–15 IVM oocytes were pooled per replicate and subjected to reverse transcription and target-specific preamplification using the Single Cell Sequence Specific Amplification Kit (Vazyme, Nanjing, China, Cat. No. P621-01) following the manufacturer's instructions. The qPCR was performed using SuperReal PreMix Plus (Tiangen) on an ABI 7500 System (Applied Biosystems, Foster City, CA, USA). Cycling consisted of 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. Primer sequences are listed in Supplementary Table S1. The relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, with *YWHAZ* serving as the internal reference gene.

### *2.8. Fluorescence Staining*

Granulosa cells (GCs) were isolated by centrifugation from aspirated follicular content after removal of COCs and cultured in DMEM/F12 (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum at 37 °C. When the confluence reached approximately 80%, the cells were stained with DCFH-DA (1 mM; Beyotime, Cat. No. S0034S) or JC-1 (10 μM; Beyotime, Cat. No. C2003S) for 30 min at 37 °C to assess ROS-related fluorescence and mitochondrial membrane potential (MMP), respectively.

For oocyte staining, immediately after IVM, cumulus cells were removed by hyaluronidase treatment for 1 min at 37 °C followed by gentle pipetting. The denuded metaphase II oocytes were washed three times in PBS containing 0.2% bovine serum albumin. Separate sets of oocytes were stained for 20 min at 38.5 °C with DCFH-DA or 5-chloromethylfluorescein diacetate (CMFDA; 10 μM; Beyotime, Cat. No. C2231) to assess ROS and GSH levels, respectively. MitoTracker Red CMXRos (200 nM; Beyotime, Cat. No. C1035) and JC-1 were used for oocyte mitochondrial staining and assessment of MMP, respectively. Monodansylcadaverine (MDC; Beyotime, Cat. No. C3018S) was diluted 1:1000 in 1× assay buffer and used to assess autophagy-associated vesicular fluorescence. The fluorescence was measured under an epifluorescence microscope equipped with a DP72 digital camera (Olympus, Tokyo, Japan). Within each assay, the same acquisition settings were used across groups. Background-corrected fluorescence intensity was quantified using ImageJ, and JC-1 results were expressed as the red-to-green fluorescence intensity ratio.

### *2.9. Statistical Analyses*

Statistical analyses used SPSS version 26.0 (IBM Corp., Chicago, IL, USA). Student's t-test was used to assess lamb–adult differences, whereas one-way analysis of variance (ANOVA) with Fisher's least significant difference (LSD) post hoc test was adopted to analyze the differences among multiple treatment groups. Results are reported as means with their standard errors (SEM). Statistical significance was defined by $p < 0.05$, and figure captions specify the significance levels.

## 3. Results

### *3.1. Comparison of Ovarian Morphology and Proteomics*

Gross morphological observation of the ovaries after hormonal stimulation revealed that ovaries from lambs were substantially larger and displayed a more even distribution of surface follicles than those from adults (Figure 1B). The average diameter of lamb ovaries was significantly greater than that of adults (average 4.03 ± 0.21 vs. 2.05 ± 0.12 cm, $p < 0.05$, Figure 1C). An average of 21.39 ± 1.36 surface follicles (≥2 mm) was counted and recorded in the adult ewe group, whereas the lamb ovaries had an average of 92.63 ± 15.38 surface follicles per animal ($p < 0.05$, Figure 1D).

Proteomic analysis identified 75 DEPs between adult and lamb ovaries, with 38 DEPs upregulated and 37 downregulated in lambs (Figure 1E and supplementary Table S2). KEGG pathway enrichment linked many of the DEPs to metabolic processes including oxidative phosphorylation, ferroptosis, and arginine/proline metabolism (Figure 1F).

### *3.2. Validation of DEPs*

Due to the limitation of available specific antibodies to confirm the expression of DEPs, qRT-PCR was performed to verify the data and investigate the transcriptional expression patterns of the ovaries of lambs and adults after hormonal stimulation (Figure 2A). Compared with the adult group, the mRNA levels of *MRPL28*, *ATP1B3*, *ATG3*, and *MAP1LC3B* were significantly lower, but the expression of *CBX3*, *PTGES3*, *HSD17B1*, and *RBMX* was markedly higher in lambs. Overall, the transcriptional profiles of these randomly selected genes were largely consistent with the DIA-based proteomic data (Table S2).

In addition, the western blot analysis was performed to validate the expression of selected proteins. The mitochondrial proteins MRPL28 ($p = 0.0048$) and NDUFS6 ($p = 0.0254$), together with the autophagy-associated protein MAP1LC3B ($p = 0.0177$), were less abundant in lamb ovaries than in adult ewe ovaries. ATG3 showed the same directional trend, although the difference was not statistically significant ($p = 0.0881$; Figure 2B,C). Consistent with these observations, the IHC staining showed that MRPL28, UQCR10, ATG3, and MAP1LC3B were predominantly localized in ovarian GCs. UQCR10 exhibited a stronger staining signal in lamb ovaries, while MRPL28, ATG3, and MAP1LC3B showed stronger signals in adult ovaries (Figure 2D), suggesting that ovarian GCs may represent an important cellular component contributing to the differences in ovarian function between adult ewes and lambs.

### *3.3. Assays in Cultured GCs*

To further clarify the differences in ovarian GC status between adult ewes and lambs, we isolated and cultured GCs in vitro and assessed the mitochondrial activity and intracellular ROS levels. The results showed that the ratio of high mitochondrial potential to low potential in lamb GCs was lower than that in adults (Figure 3A,B), while the ROS level was significantly higher in lambs than that in adults (Figure 3C,D). These findings indicated that cultured GCs from prepubertal lambs experienced higher oxidative stress relative to adult ewes.

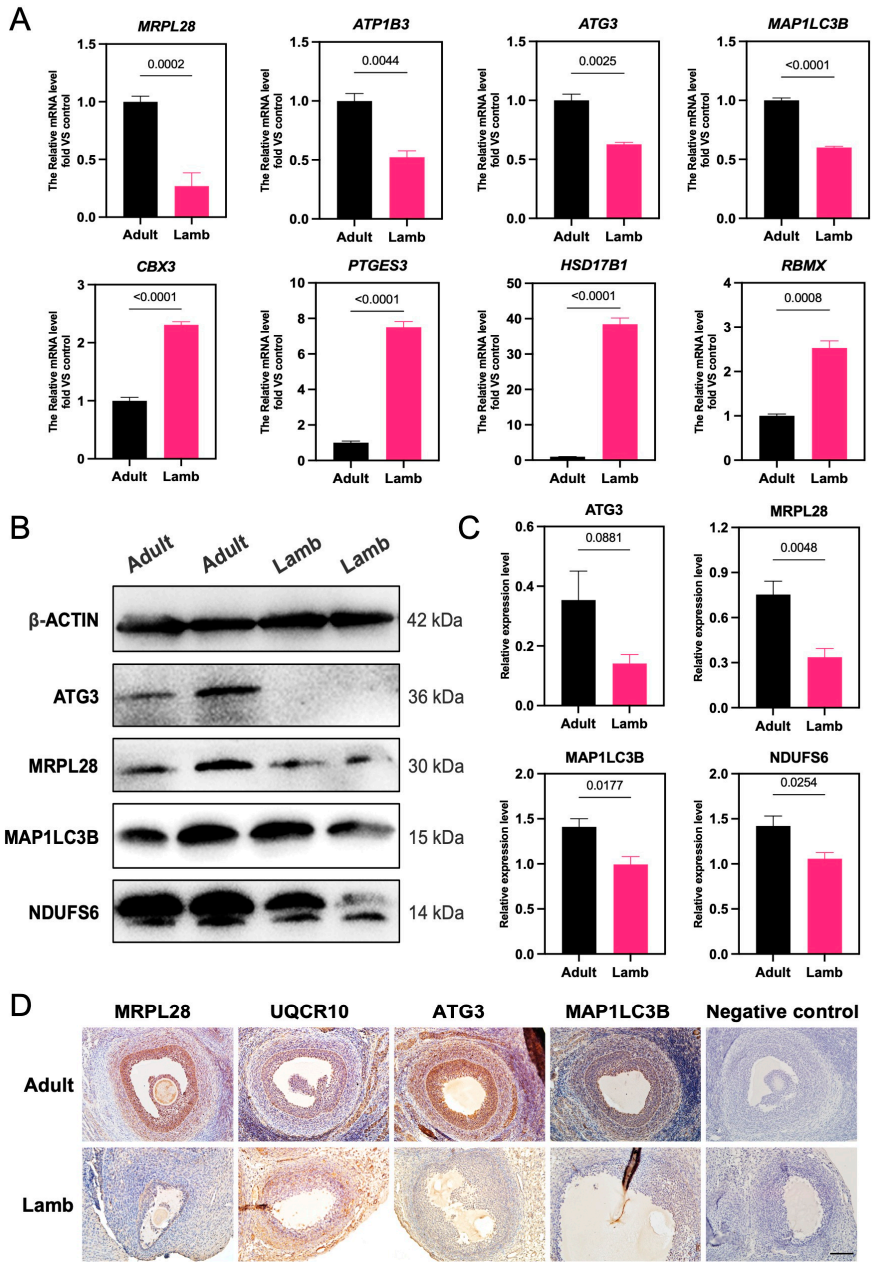



**Figure 2.** Validation and ovarian localization of selected DEPs. (**A**) qRT-PCR measurements of mRNA corresponding to 8 DEPs. The $2^{-\Delta\Delta Ct}$ calculation used *YWHAZ* as the reference gene and adult ewe samples as the normalization calibrator. Values represent mean ± SEM ($n = 3$). (**B**) Western blots validating the expression of 4 representative DEPs. Each target protein was examined in at least three independent western blot experiments. (**C**) Relative protein expression derived from band densitometry, with each target band's intensity divided by its corresponding β-ACTIN signal. (**D**) The expression of 4 representative DEPs in adult and lamb ovaries detected by immunohistochemistry imaged at 20× magnification. Signal specificity was checked with a negative control lacking primary antibody. Scale bar, 100 μm.

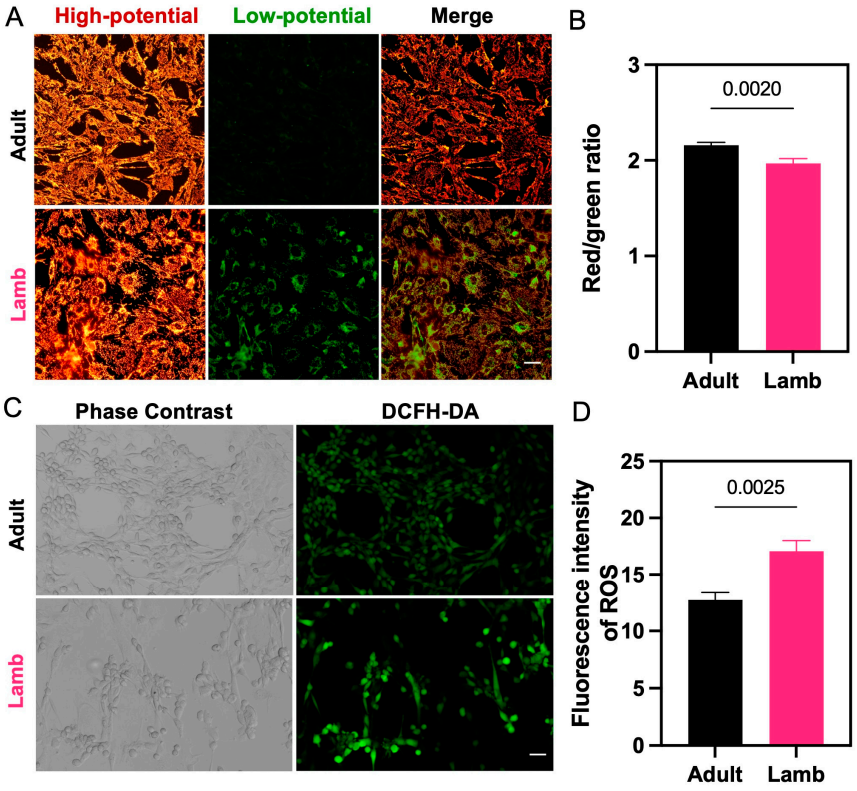



**Figure 3.** Mitochondrial activity and ROS levels of ovarian GCs from adult ewes and lambs. (**A**) JC-1 staining of GCs from adult ewes and lambs. Red fluorescence indicates high MMP, and green fluorescence indicates low MMP. Scale bar: 50 μm. (**B**) MMP assessed by the JC-1 red-to-green fluorescence intensity ratio. (**C**) DCFH-DA staining showing intracellular ROS levels in granulosa cells from adult ewes and lambs. Scale bar, 50 μm. (**D**) Quantification of ROS levels, expressed as relative fluorescence intensity. Data are presented as mean ± SEM. Each experiment was performed in at least three replicates.

### *3.4. Effects of Autophagy Activators on Adult Ewe Oocytes*

Given that gonadotropin-stimulated lamb ovaries exhibited altered expression of mitochondrial- and autophagy-associated proteins, accompanied by increased oxidative stress markers, we investigated whether pharmacological modulation of autophagy could improve sheep oocyte developmental competence during IVM. Three autophagy-related substances, AICAR, Cory B, and ML098, which are reported to activate different stages of the autophagic process, were supplemented during IVM to evaluate their effects on oocyte developmental potential.

For AICAR treatment, the 50 μM AICAR group showed the highest blastocyst rate among all tested concentrations, increasing blastocyst rate by 26.8% compared with the control group (49.36 ± 2.60% vs. 22.56 ± 1.96%, Table 1). The supplementation of Cory B also significantly enhanced oocyte developmental potential, and treatment with 50 μM Cory B resulted in the greatest improvement in blastocyst formation in this assay (Table 2). Similarly, all three concentrations of ML098 supplementation significantly improved blastocyst development when compared to the control, and the 5 μM ML098 yielded the highest blastocyst rate (Table 3). These results demonstrated the beneficial roles of autophagy activators on oocyte in vitro development.

**Table 1.** Effect of supplementation with AICAR on the developmental competence of adult oocytes during IVM.

| Groups | No. of Oocytes | No. of Cleaved (Mean ± SEM, %) | No. of Blastocysts (Mean ± SEM, %) |
|---|---|---|---|
| control | 158 | 131 (82.67 ± 2.46) | 29 (22.56 ± 1.96) [d] |
| 50 μM AICAR | 180 | 149 (82.66 ± 1.55) | 73 (49.36 ± 2.60) [a] |
| 100 μM AICAR | 168 | 140 (83.35 ± 1.62) | 59 (42.03 ± 0.64) [b] |
| 200 μM AICAR | 164 | 138 (84.45 ± 2.20) | 47 (33.95 ± 2.64) [c] |

Groups: The control medium consisted of the basal IVM medium without AICAR supplementation. For the treatment groups, the control medium was supplemented with different concentrations of AICAR (0, 50, 100, or 200 μM). Percentage of cleaved embryos: No. of cleaved embryos/No. of oocytes. Percentage of blastocysts: No. of blastocysts/No. of cleaved embryos. Within a column, different lowercase letter labels (a, b, c, d) denote a significant difference between values ($p < 0.05$).

**Table 2.** Effect of supplementation with corynoxine B on the developmental competence of adult oocytes during IVM.

| Groups | No. of Oocytes | No. of Cleaved (Mean ± SEM, %) | No. of Blastocysts (Mean ± SEM, %) |
|---|---|---|---|
| control | 162 | 136 (83.46 ± 2.39) [ab] | 32 (23.54 ± 2.47) [b] |
| 5 μM Cory B | 175 | 142 (81.27 ± 1.74) [b] | 53 (37.12 ± 2.23) [a] |
| 20 μM Cory B | 178 | 153 (86.01 ± 2.71) [ab] | 60 (38.76 ± 4.23) [a] |
| 50 μM Cory B | 182 | 156 (85.74 ± 2.10) [a] | 66 (41.62 ± 3.87) [a] |

Groups: The control medium consisted of the basal IVM medium without corynoxine B (Cory B) supplementation. For the treatment groups, the control medium was supplemented with different concentrations of Cory B (0, 5, 20, or 50 μM). Percentage of cleaved embryos: No. of cleaved embryos/No. of oocytes. Percentage of blastocysts: No. of blastocysts/No. of cleaved embryos. Within a column, different lowercase letter labels (a, b, c, d) denote a significant difference between values ($p < 0.05$).

**Table 3.** Effect of supplementation with ML098 on the developmental competence of adult oocytes during IVM.

| Groups | No. of Oocytes | No. of Cleaved (Mean ± SEM, %) | No. of Blastocysts (Mean ± SEM, %) |
|---|---|---|---|
| control | 177 | 152 (85.79 ± 1.02) [a] | 32 (20.65 ± 1.86) [b] |
| 0.1 μM ML098 | 185 | 153 (82.91 ± 1.03) [b] | 64 (41.42 ± 3.09) [a] |
| 1 μM ML098 | 187 | 158 (84.63 ± 1.46) [ab] | 64 (40.52 ± 2.04) [a] |
| 5 μM ML098 | 179 | 149 (83.29 ± 1.19) [ab] | 65 (44.20 ± 2.52) [a] |

Groups: The control medium consisted of the basal IVM medium without ML098 supplementation. For the treatment groups, the control medium was supplemented with different concentrations of ML098 (0, 0.1, 1, or 5 μM). Percentage of cleaved embryos: No. of cleaved embryos/No. of oocytes. Percentage of blastocysts: No. of blastocysts/No. of cleaved embryos. Within a column, different lowercase letter labels (a, b, c, d) denote a significant difference between values ($p < 0.05$).

### *3.5. Effects of Autophagy Activators on Mitochondrial Redox Homeostasis of Adult Ewe Oocytes*

The potential mechanisms underlying the beneficial effects of autophagy modulation were investigated in oocytes treated with AICAR (50 μM), Cory B (50 μM), or ML098 (5 μM). All three treatments significantly increased the autophagy-related signals, elevated intracellular GSH levels, and reduced ROS accumulation in sheep oocytes (Figure 4A). Among the three treatments, ML098 exhibited the strongest autophagy-associated vesicular fluorescence signals (Figure 4B), whereas AICAR showed the most pronounced

improvement in antioxidant capacity, as characterized by increased GSH levels and reduced ROS accumulation (Figure 4C,D). In addition, all three treatments increased active mitochondrial fluorescence intensity and improved MMP in sheep oocytes (Figure 4E–H).

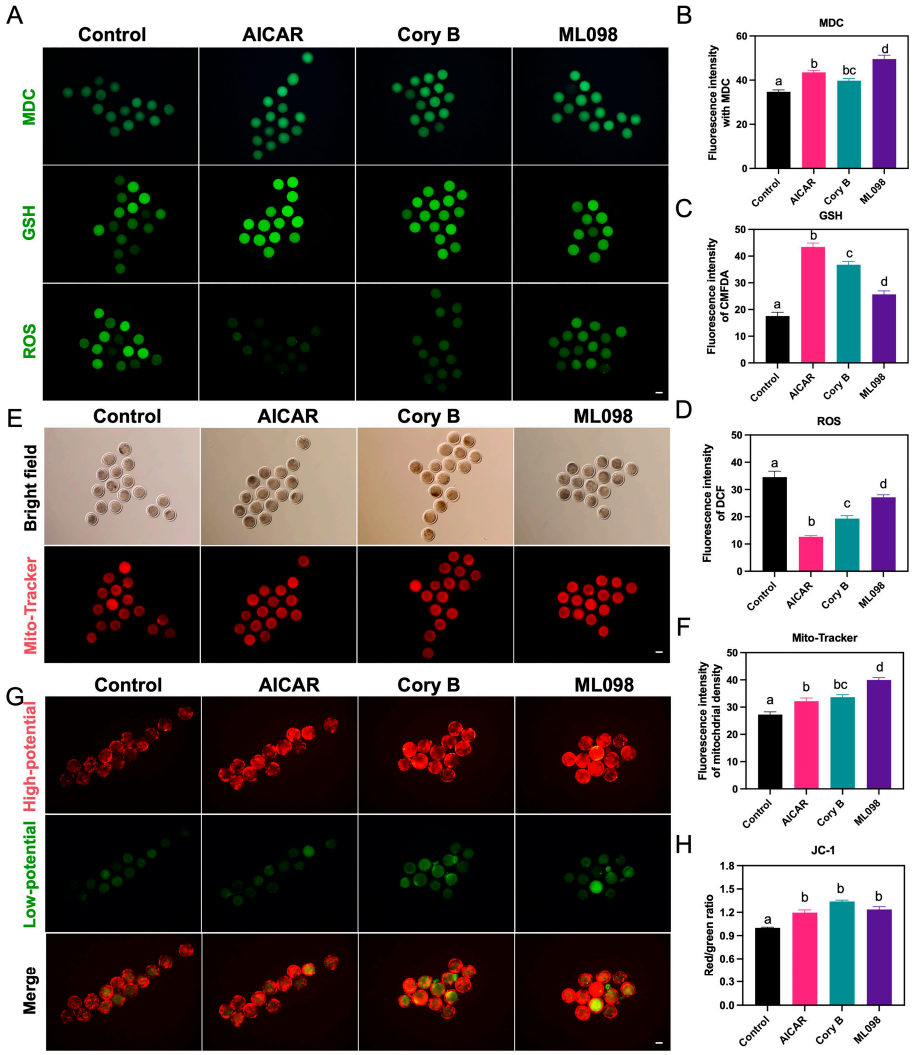



**Figure 4.** Effects of AICAR, corynoxine B, and ML098 supplementation during in vitro maturation on autophagy-associated vesicles, redox status, and mitochondrial function in adult ewe oocytes. (**A**) Representative fluorescence images of denuded oocytes stained with MDC, CMFDA, or DCFH-DA following in vitro maturation under control conditions or with AICAR, corynoxine B (Cory B), or ML098 supplementation. (**B–D**) Quantification of MDC (**B**), CMFDA (**C**), and DCFH-DA (**D**) fluorescence intensity. (**E**) Representative bright-field and MitoTracker Red CMXRos fluorescence images. (**F**) Quantification of MitoTracker Red CMXRos fluorescence intensity. (**G**) Representative JC-1 images. (**H**) Quantification of MMP. MMP assessed by the JC-1 red-to-green fluorescence intensity ratio. Data are presented as mean ± SEM from at least three independent biological replicates, with at least 11 oocytes analyzed per group in each experiment for each assay. Groups that do not share the letter differ significantly ($p < 0.05$). Scale bars, 100 μm.

Consistent with these observations, qRT-PCR analysis demonstrated that autophagy- and oxidative stress-related genes were significantly upregulated following treatment with AICAR, Cory B, or ML098. AICAR treatment significantly increased the expression of genes involved in autophagy initiation and elongation, including *BECN1* and

*MAP1LC3B*. Cory B treatment was associated with elevated expression of genes related to autophagosome formation and maturation, including *VPS34*, *BECN1*, *MAP1LC3B*, and *RAB7*. ML098 preferentially increased the expression of genes involved in autophagosome maturation and lysosomal fusion, including *MAP1LC3B*, *RAB7*, and *LAMP1* (Figure 5A–C). Furthermore, all three treatments enhanced the expression of mitophagy-related genes (*PINK1* and *PRKN*) and antioxidant defense genes (*SOD2* and *CAT*) (Figure 5D–F).

Collectively, these findings indicate that pharmacological modulation of autophagy-associated processes was associated with improved mitochondrial homeostasis and antioxidant capacity during sheep oocyte maturation.

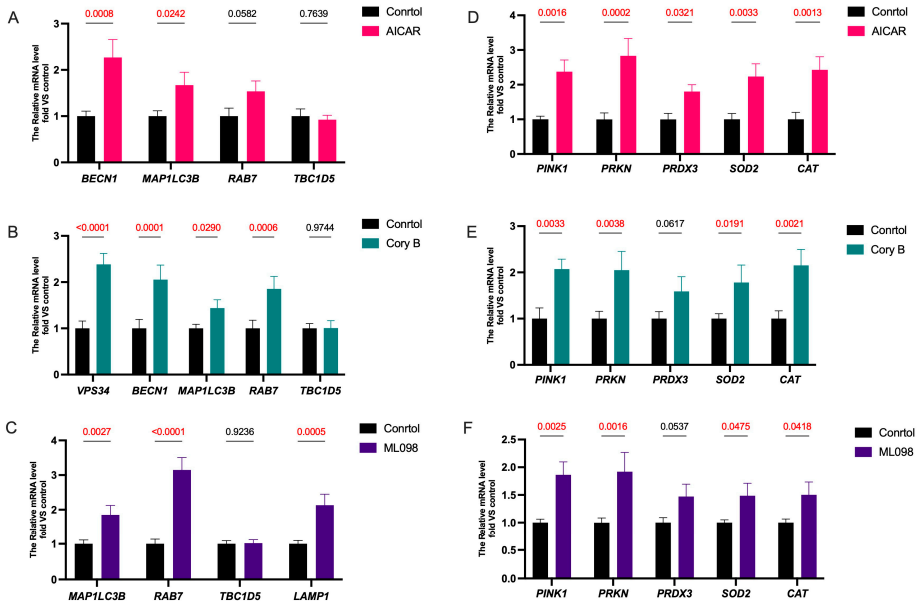



**Figure 5.** Effects of AICAR, Cory B, and ML098 supplementation during in vitro maturation on autophagy-, mitophagy-, and antioxidant-related gene expression in adult ewe oocytes. (**A**–**C**) Expression of genes associated with autophagy regulation following AICAR (**A**), Cory B (**B**), or ML098 (**C**) treatment. The genes examined were *BECN1*, *MAP1LC3B*, *RAB7*, and *TBC1D5* in (**A**); *VPS34*, *BECN1*, *MAP1LC3B*, *RAB7*, and *TBC1D5* in (**B**); and *MAP1LC3B*, *RAB7*, *TBC1D5*, and *LAMP1* in (**C**). (**D**–**F**) Expression of the mitophagy-related genes *PINK1* and *PRKN* and the antioxidant-related genes *PRDX3*, *SOD2*, and *CAT* following AICAR (**D**), Cory B (**E**), or ML098 (**F**) treatment. Data are presented as mean ± SEM from three independent biological replicates.

### *3.6. Effect of Autophagy Activators on Lamb Oocytes*

To test whether modulation of autophagy-related processes could improve the developmental competence of prepubertal lamb oocytes, we supplemented the IVM medium with AICAR (50 μM), Cory B (50 μM), or ML098 (5 μM). AICAR supplementation resulted in a significant improvement in blastocyst development when compared to the control (49.52 ± 6.04% vs. 29.89 ± 4.46%; Table 4). Although treatments with Cory B (37.00 ± 7.00%) and ML098 (42.97 ± 4.87%) also showed a trend toward increased blastocyst rates, these improvements did not reach statistical significance. These findings support pharmacological modulation of autophagy-related processes as a potential approach to improve lamb oocyte developmental competence, with a significant benefit observed for AICAR under the conditions tested.

**Table 4.** Effect of supplementation with different supplements on the developmental competence of lamb oocytes during IVM.

| Groups | No. of Oocytes | No. of Cleaved (Mean ± SEM, %) | No. of Blastocysts (Mean ± SEM, %) |
|---|---|---|---|
| control | 112 | 54 (48.30 ± 2.37) | 16 (29.89 ± 4.46) [b] |
| 50 μM AICAR | 88 | 50 (56.82 ± 4.55) | 25 (49.52 ± 6.04) [a] |
| 50 μM Cory B | 85 | 45 (52.88 ± 5.26) | 17 (37.00 ± 7.00) [ab] |
| 5 μM ML098 | 84 | 44 (52.36 ± 1.14) | 19 (42.97 ± 4.87) [ab] |

Groups: The control medium consisted of the basal IVM medium without any supplementation. For the treatment groups, the control medium was supplemented with 50 μM AICAR, 50 μM Cory B, or 5 μM ML098. COCs from 7 individual prepubertal lambs were separately allocated among the control, AICAR, Cory B, and ML098 groups. Percentage of cleaved embryos: No. of cleaved embryos/No. of oocytes. Percentage of blastocysts: No. of blastocysts/No. of cleaved embryos. Within a column, different lowercase letter labels (a, b, c, d) denote a significant difference between values ($p < 0.05$).

## 4. Discussion

The JIVET technology offers the advantage of exploiting the large ovarian follicle pool in prepubertal lambs. However, the developmental competence of lamb oocytes is significantly lower than that of adult ewe oocytes. This deficiency is mainly attributed to impaired maturation of oocyte cytoplasm, as well as a suboptimal follicular microenvironment within the lamb ovary [11,23]. In the present study, comparative ovarian protein profiles in prepubertal lambs and adult ewes, together with GC and oocyte analyses, identified defects in mitochondrial, autophagy-related, and antioxidant processes following gonadotropin stimulation. Pharmacological modulation of autophagy-associated processes improved mitochondrial-related characteristics, antioxidant capacity, and developmental outcomes. These findings indicate that mitochondrial-redox homeostasis may be a potential target for improving oocyte maturation and subsequent development in sheep.

The ovarian proteomics analysis showed that the NDUFS6 and MRPL28 abundance were reduced, whereas those of UQCR10 and COX5B were increased in lamb ovaries. NDUFS6 supports complex I assembly and function, and its deficiency can impair NADH oxidation [24,25], potentially impairing the activity of $NAD^+$-dependent enzymes and promoting the excessive production of ROS [26,27]. The reproductive relevance of $NAD^+$ metabolism is supported by studies showing that $NAD^+$ precursor supplementation improves ovarian or oocyte function in pigs and mice [28,29]. UQCR10 is a subunit of respiratory chain complex III, which can contribute to mitochondrial ROS production [30]. The COX5B protein complexes in oxidative phosphorylation complex IV, promoting the terminal transfer of electrons during oxidative phosphorylation [31]. Reduced levels of the mitochondrial ribosomal protein MRPL28, which is a component of the mitochondrial large ribosomal subunit [32], further suggest potential alterations of mitochondrial translation in lamb ovaries. This is consistent with previous findings where the MRPL28 protein expression level was downregulated in lamb ovarian GCs [33]. Together, these findings point to differences in proteins involved in both mitochondrial respiration and translation which may have implications for mitochondrial function and redox regulation [12,34]. However, the ovarian proteomic profiles revealed differences at the entire tissue level between prepubertal lambs and adult ewes, which may reflect variation in the relative proportions of follicles, stroma, and luteal tissue, as well as changes in protein abundance within individual cell types.

The IHC confirmed the location of proteins associated with autophagy and oxidative phosphorylation in follicular GCs. In separate assays, cultured GCs from lambs exhibited lower MMP and elevated ROS levels than those from adult ewes, indicating redox differences in GCs. These findings complement the previous proteomic investigations, which reported that lamb follicular fluid exhibited a distinct protein profile from adults, particularly characterized by a downregulation of proteins associated with oxidoreductase activity and antioxidant defense, including GCLC and SOD [19]. Additionally, it has been reported that oocytes from prepubertal animals possess a weaker capacity for antioxidants than those from adult animals [13,35]. Together, these findings suggest the follicular microenvironment of prepubertal lambs may provide less antioxidant protection to developing oocytes.

The reduced protein abundance of ATG3 and MAP1LC3B in lamb ovaries further suggests a possible disturbance in autophagy-associated homeostasis; however, this does not establish reduced autophagic flux, which was not directly measured in this study. It has been reported that inhibition of autophagy impaired meiotic maturation and subsequent embryonic development in mice, pigs, and bovines [36–38], whereas moderate enhancement of autophagy could improve the developmental potential of oocytes affected by maternal aging, oxidative stress, or in vitro conditions [38,39]. These studies demonstrate that alterations in autophagy have a significant impact on the developmental competence of oocytes. We further explored the question of whether modifying the oocyte IVM environment could improve subsequent embryo development. In the present study, supplementation with pharmacological modulators of autophagy-associated processes during IVM, including AICAR, Cory B, or ML098, could improve subsequent sheep oocyte development and was accompanied by enhanced antioxidant and mitochondrial activity. These developmental responses are consistent with reports that supplementation with AICAR [40] and Cory B [41] during IVM improved blastocyst formation in bovine or aged mice. The short-term exposure to ML098 improved meiotic progression in mouse oocytes with experimentally induced mitochondrial damage [39]. However, excessively high concentrations of AICAR can also inhibit meiotic resumption and nuclear maturation in bovine oocytes [42]. These findings suggest an important role for regulation of autophagy-associated processes in oocyte maturation and developmental competence.

Although the three substances participate in the autophagy biological process, they appear to act through distinct regulatory mechanisms. AICAR is widely used as an AMPK activator and can promote autophagy initiation through AMPK–ULK1 signaling and the coordinated suppression of mTORC1 activity [43]. Cory B has been shown to enhance BECN1–VPS34 complex activity, thereby facilitating autophagosome formation [41], whereas ML098 increases RAB7 activity and can promote late endosomal and autophagic trafficking, including autophagosome–lysosome maturation [39]. Notably, recent evidence demonstrates that VPS34 activation by Cory B improves oocyte quality in aged mice, while RAB7 activity is required for the coordination of mitophagy during oocyte meiosis and reproductive aging [39,41]. Across the three treatment experiments, improved oocyte developmental competence was accompanied by increased expression of the autophagy-related gene (*MAP1LC3B*) and the antioxidant genes (*SOD2* and *CAT*). In addition, increased *PINK1* and *PRKN* expression was accompanied by improved mitochondrial activity and a more favorable intracellular redox state. Previous studies have shown that properly regulated *PINK1*/*PRKN*-mediated mitophagy supports mitochondrial quality control, reduces oxidative stress, restores ATP production capacity, and enhances the developmental capacity of aging and deficient oocytes [44,45]. The coordinated developmental, transcriptional, and functional responses in these treatments suggest that autophagy regulators may improve oocyte developmental capacity by modulating mitochondrial quality control.

Notably, the present study further showed that AICAR-treated lamb oocytes yielded a higher blastocyst rate than controls, providing evidence supporting the development-promoting effect of this autophagy activator. However, ML098 or Cory B treatment could not achieve significant improvement of lamb oocyte development, suggesting that deficiencies in the upstream, but not downstream, autophagy-related pathway might contribute to the lamb oocyte development. Future studies are required to further clarify the influence of autophagy-related processes on oocyte development.

## 5. Conclusions

In conclusion, the ovarian DIA proteomics analysis and GC experiment showed mitochondrial and redox disturbances in lambs. Pharmacological modulation of autophagy-associated processes during IVM could improve redox-related indicators and subsequent developmental competence in sheep oocytes. In particular, the promoting effect of AICAR on lamb oocytes provides a candidate strategy to improve the efficiency of JIVET technology.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antiox15111522/s1, Table S1: Primer sequences used for qRT-PCR in this study; Table S2. Differentially expressed proteins between lamb and adult ovaries.

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**Data Availability Statement:** The datasets supporting the conclusions of this article have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository and are accessible under the dataset identifier PXD078894.

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## Abbreviations

The following abbreviations are used in this manuscript:

| | |
|---|---|
| JIVET | Juvenile in vitro embryo transfer |
| DIA | Data-independent acquisition |
| CIDR | Controlled internal drug release device |
| FSH | Follicle-stimulating hormone |
| PMSG | Pregnant mare serum gonadotropin |
| FASP | Filter-aided sample preparation |
| DEPs | Differentially expressed proteins |
| KEGG | Kyoto Encyclopedia of Genes and Genomes |

| | |
|---|---|
| IHC | Immunohistochemistry staining |
| qRT-PCR | Quantitative reverse transcription PCR |
| MMP | Mitochondrial membrane potential |
| ROS | Reactive oxygen species |
| GCs | Granulosa cells |
| PTGES3 | Prostaglandin E synthase 3 |
| HSD17B1 | Hydroxysteroid 17-β-dehydrogenase 1 |
| GCLC | Glutamate–cysteine ligase catalytic subunit |
| RBMX | RRM domain-containing protein |
| MRPL28 | Mitochondrial ribosomal protein L28 |
| MAP1LC3B | Microtubule associated protein 1 light chain 3 beta |
| ATG3 | Autophagy related 3 |
| UQCR10 | Ubiquinol-cytochrome C reductase complex III Subunit X |
| NDUFS6 | Ubiquinone oxidoreductase subunit S6 |
| Cory B | Corynoxine B |

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