Mitochondria-synthesize melatonin to improve the quality and in vitro maturation as well as the embryonic development of porcine oocytes

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Abstract

Background

The in vitro maturation efficiency of porcine oocytes is relatively low and this limits the production of in vitro porcine embryos. Since melatonin is involved in the mammalian reproductive physiology, in this study, we have explored whether the endogenously produced melatonin can help the porcine oocyte in vitro maturation.

Results

We have found for the first time, that mitochondria are the major sites for melatonin biosynthesis in porcine oocytes. This mitochondrially originated melatonin reduces ROS production increases the activity of mitochondrial respiratory electron transport chain, mitochondrial biogenesis, and mitochondrial membrane potential and ATP production. Therefore, melatonin improves the quality of oocytes and their in vitro maturation. In contrast, the reduced melatonin level caused by siRNA to knockdown *AANAT* (si*AANAT*) is associated abnormal distribution of mitochondria and decreased the ATP level porcine oocytes and inhibits their in vitro maturation. These abnormalities can be rescued by melatonin supplementation. in addition, we found that siAANAT can switches the mitochondrial oxidative phosphorylation to glycolysis, a Warberg effect. This metabolic alteration can also be corrected by melatonin supplementation. All these activities of melatonin appear to be mediated by its membrane receptors since the non-selective melatonin receptor antagonist luzindole can blunt melatonin's effects.

Conclusions

Taken together, the mitochondria of porcine oocyte can synthesize melatonin and improve the quality of oocyte maturation. The mitochondria of porcine oocyte can synthesize melatonin and improve the quality of oocyte maturation.These results provide an insight from novel aspect to study the oocyte maturation in the in vitro condition.

Background

Melatonin, also known as N-acetyl-5-methoxy tryptamine, is widely present in almost all organisms In mammals, all tissues and organs, especially the pineal gland, convert, tryptophan to,5-hydroxy tryptophan by hydroxylase, then, this intermediate is metabolized to 5-hydroxytryptamine (5HT) by decarboxylase. 5-HT is catalyzed by arylamine N-acetyltransferase (SNAT/AANAT) to N-acetylserotonin. Finally, N-acetylserotonin is converted to melatonin by the Hydroxyindole-O-methyltransferase (HIOMT/ASMT). The melatonin synthesized by pineal glans is directly released into the cerebrospinal fluid and blood [1–3] while the extrapineal synthesized melatonin is primarily for local utilization (autocrine and paracrine effects) [4]. Majority of Melatonin isy produced in in mitochondria [5] and it plays an important role in

reducing oxidative stress. Our group is the first to report that the mitochondria of mouse oocytes synthesizes melatonin [6].

Mitochondria are one of the important organelles of oocytes. They are the powerhouses of cells and also control intracellular calcium homeostasis [7, 8]. In addition mitochondria play a central role in other functions including the regulation of cell death and signaling pathways, iron metabolism, and the biosynthesis of certain organic compounds [9–11]. During folliculogenesis, the follicle undergoes substantial growth, expanding approximately 500-fold. This makes oocyte within the follicle also undergoes major structural and biochemical transitions including two meiotic divisions. To cope with such a energy-consuming process, the quantity and quality as well as distribution pattern of mitochondria in the oocyte are also required to changes [12].

The maturation of oocyte requires large amounts of ATP for continued transcription and translation, therefore, this process needs sufficient numbers of functional mitochondria for ATP production. The fact is that the immature oocytes have limited mitochondrial activity and they depend the surrounding cumulus and granulosa cells to provide additional energy to support their maturation [13]. During the process of ovulation the oocytes lose connection to the cumulus cells and this force them to activate their own mitochondria. This is the reason that the matured oocytes have a sufficient number of mitochondrial accumulation to generate ATP.. Therefore, the ATP levels are elevated during polar body expulsion, and higher ATP levels are associated with higher fertilization rates in matured oocytes[14] while the lower ATP content in oocytes is associated with poor oocyte quality and fertilization [15, 16].

As mentioned above that melatonin is synthesized in mitochondria. Thus, melatonin has been considered to have a major impact on mitochondrial functions including to increase the efficiency of the electron transport chain [17] and ATP production [18], and to reduce oxidative damage to mitochondria [19–21]. The oxidative damage caused by excess reactive oxygen species (ROS) impairs cellular function leading to enzyme inactivation, lipid peroxidation, ATP depletion, and mitochondrial disturbance. It has been found that high levels of ROS and low antioxidant activity in follicular fluid result in poor pregnancy outcomes after IVF. Increased ROS levels during in vitro oocyte maturation are associated to chromosomal errors and low developmental potential of oocytes [22, 23]. For example, the increased ROS in cultured mouse oocytes alter the chromosomal arrangement of microtubules and spindles and inhibits their maturation [5]. Melatonin as a potent antioxidant can directly scavenge toxic oxygen derivatives [24] [25],, and stimulate activities of antioxidant enzymes [26], including glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) [27]. However, whether porcine oocytes can synthesize melatonin and if so, the effects of this endogenously generated melatonin on oocyte maturation are still unclear.

In this study, we will explored the subcellular localization of melatonin synthesis in porcine oocytes, as well as the roles of locally synthesized melatonin oocyte maturation. In addition, we will knocked down the melatonin synthase AANAT gene with interfering RNA to further confirm whether melatonin is involved in porcine oocyte maturation.

Methods and material

1. Ethics Statement

All the animal studies were followed the guidelines of the Animal Care and Use Committee of China Agricultural University and approved by the Ethics Committee of the Agriculture University of China (permission number: AW01602202-1-6).

2. Chemicals

All chemicals used in this study were purchased from the Sigma- Aldrich Chemical Company (St. Louis, MO, United States) unless otherwise indicated.

3. The procedure of in vitro porcine oocyte maturation

Ovaries of sows (donated by a local slaughterhouse, Beijing Food Company, Beijing, China) were collected, packed in thermostable container (37°C) with sterilized saline, Penicillin and streptomycin, and The samples were transported to the laboratory within 2 hours, then, washed with 37°C sterilized physiological saline. Thereafter, the follicular fluid was extracted from the follicles (3-6mm in diameter) with a syringe with 20G needle. The cumulus-oocyte complex(COCS)was rinsed twice in HEPES-buffered lactate (TL-HEPES) medium and three times in hormone-free maturation medium. The COCs were then transferred into the maturation medium (50 oocytes per 0.5 ml of medium) consisting of TCM-199 with 0.57 mM cysteine, 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 10 ng/ml epidermal growth factor (EGF), 0.5 IU/ml ovine luteinizing hormone (LH), 0.5 IU/ml porcine follicle stimulating hormone (FSH), 0.1% polyvinyl alcohol (PVA), 75 mg/ml penicillin, 50 mg/ml streptomycin, 20 ng/ml LIF, 20 ng/ml IGF1, and 40 ng/ml FGF2[28] incubated at 38.5°C, 5% CO2, and 100% humidity for 42–44 hours for maturation. The maturated COCs were transferred to the culture medium containing 1 mg/mL hyaluronidase in TL-HEPES and remove cumulus cells by vertexing, and washed with TL-HEPES, then, these denuded oocytes were used for subsequent experiments.

4. Parthenogenetic Activation of Oocytes

The denuded porcine oocytes were activated in the activation medium [0.3 m mannitol, 0.05 mm CaCl2, 0.1 mm MgCl2, and 0.1% bovine serum albumin (BSA)] by an electrical pulse of DC 130 V/mm for 80 μs using a BTX Electro-Cell Manipulator 2001 (BTX, Inc., San Diego, CA, United States). The activated oocytes were then rinsed in porcine zygote medium-3 (PZM-3) and cultured in the medium containing 5 μg/ml of cytochalasin B at 38.5◦C and 5% CO2 in air with 100% humidity for 5–6 h,.

5. In vitro Culture (IVC) of Embryos

The parthenogenetically activated oocytes, (approximately 20–30 oocytes per group), were placed in 100 μl droplets of PZM-3 supplemented with 0.6 mg/ml of BSA and incubated at 39◦C, 5% CO2, and 5% O2. The cleavage rate and blastocyst rate were observed and recorded at 48 and 168 h of IVC, respectively.

6. Calculation of Cumulus Cell Expansion and Polar Body Extrusion rates in Porcine Oocytes Maturation

The expanded oocytes of matured COCs at 44 hour incubation were counted under the microscope. The expanded oocytes were served as the numerator to divide the total number of oocytes in each well to calculate the expansion rate of cumulus cells. Then cumulus cells were removed by use of 0.3 mg/mL hyaluronidase. The oocytes with discharged polar bodies were selected under microscopy with 20 times eyepiece. ,The polar body discharge rate was calculated against the total number of oocytes in each well.

7. The Cortical Granule Migration Assay

The zona pellucida of MII stage oocytes was removed with 0.1% pronase and they were washed for 3 times with PBS, and incubated in a CO2 incubator to restore to normal shape. Then, they were fixed with 4% formaldehyde at room temperature for 30 minutes, the oocytes were washed 3 times with blocking solution, 5 min each time, and the blocking solution was PBS + 3mg/mLBSA + 7.5mg/mL glycine. The blocked oocytes were infiltrated with 0.5% Triton-X100-PBS-0.1%PVA for 30min, then incubated at room temperature for additional 30min under dark condition, and stained with the staining solution of 100μg/mL PBS-FITC-PNA (SigmaL-7381). The samples were washed with PBS for 3 times, placed on the glass slide and covered with the paraffin-coated cover glass.Tthe distribution of oocyte cortical granules was observed under the laser confocal microscope.

8.Mitochondria level and distribution in porcine oocytes

The oocytes were vortexed to remove the zona pellucida to obtain the naked oocytes. Mitochondria were labeled with MitoTracker Red CMXRos with 30min (PBS washing solution + 500nmol/L MitoTracker Red CMXRos), and mounted into slide analyzed under a fluorescence microscope.

9.Subcellular localization of AANAT by immunoelectron microscopy

Approximately 1000 oocytes were collected for fixation with paraformaldehyde The fixed samples were washed with PBS to remove the glutaraldehyde residuals, and dehydrated through 30%, 50%, 75%, 85%, 95%, 100% alcohol gradient in sequence and xylene in final. The samples were then soaked in epoxy resin, fixed and embedded into blocks by temperature gradient treatment in an oven. The block was trimmed with a razor blade and an obvious follicle structure on the surface of the ovary was removed. The trimmed samples were sliced with an automatic microtome in sequence. The slice thickness is 100nm. The slices containing the oocyte structure were selected and fixed on the copper grid. The AANAT antibody was diluted 1:100, and made into 30uL small droplets, the sample was submerged into the droplets, pre-incubated at room temperature for 1 hour, and then incubated at 4^{o}C for overnight, thereafter, the samples was fully washed to remove the primary antibody and, then, incubated with 30uL of gold-labeled secondary antibody (diluted 1:2000). at room temperature for 2 h. After washing away of the secondary antibody, the samples were incubated in uranyl acetate for 15 minutes. The samples were analyzed under electron microscope with photo.

10.Lipid droplet staining of porcine oocytes

After 44 hours of maturation, the COCs were harvested from the IVM medium, the cumulus cells around the oocytes were removed, and stained with 20ug/mL BODIPY 493/503 (Thermo, D3922). The stained MII oocytes were then placed in a glass petri dish and observed under a confocal microscope with image taking (Nikon A1HD25, Tokyo, Japan). The excitation wavelength was 405 nm for LipiBlue and 488 nm for BODIPY 493/503. NIS (Nikon) was used to take pictures and calculate the fluorescence intensity of lipid droplets.

11. Mitochondrial membrane potential analysis with JC-10 Staining

JC-10 is a fluorescent probe for detecting mitochondrial membrane potential △Ψm. When the mitochondrial membrane potential is high, JC-10 gathers in the mitochondrial matrix to form a polymer with red fluorescence; when the mitochondrial membrane potential is low, JC- 10 is a monomer with green fluorescence. The oocytes were incubated with the diluted (200XJC-10 to 1X) JC10 solution at 37°C for 20min, then washed with JC-10 staining buffer 3 times, the samples were placed in the covered slides and observed under a laser confocal microscope. Changes in mitochondrial membrane potential are detected by fluorescent color shifts. The relative ratio of red-green fluorescence is commonly used to measure the ratio of mitochondrial depolarization.

12.Procedure of Immunofluorescence staining

The zona pellucida of M oocyte was removed with pronase. After M oocyte restored to normal shape with incubation at a CO2 incubator, it was fixed in 4% paraformaldehyde (PFA) at room temperature for 45min and washed with PBS-0.1%PVA 3 times, 10min each time; The hole punching in the cell membrane was achieved with 0.5%Triton-X 100-PBS-0.1% PVA incubation at room temperature for 1 hour; the samples were blocked in 3% BSA-0.1 Triton-X 100-PBS-0.1% PVA solution for 1 hour at room temperature; then, MII oocytes were incubated with AANAT antibody (1:100) and ASMT(1:100) antibody (diluted in sealing solution) at 4℃ for 12 h, and washed with PBS-0.1% PVA for 3 times, 10 min each time; then, incubated with the secondary antibody (1:200) at room temperature in the dark for 1 hour, washed with DPBS-0.1%PVA 3 times, 20 minutes each time; Hoechst33342 was used to stain nuclei. The samples were mounted into slices, observed under a laser confocal microscope, and photograph was taken.

13. Melatonin asay with High Performance Liquid Chromatography-Tandem Mass Spectrometry

The 200uL of mitochondrial culture solution mixed with 800uL methanol and centrifuged 12000r/min, at 4°C for 20min. The samples were filtered with a 2um filter. The sample was injected into a HPLC -Tandem Mass Spectrometry system. The mobile phase consisted of solutions A (0.1% formic acid solution) and B (methanol). The gradient elution program was 0–1 min with 10% B-phase, 2–3.5 min with 60% B-phase, and 3.6–5 min with 10% B-phase. The column temperature was set at 40 ◦C. The mobile phase was delivered at a flow rate of 0.4 mL/min. 2 μL of the prepared sample is injected into the LC-MS/MS system. The MS/MS system consisted of a triple quadrupole mass spectrometer equipped with

electrospray ionization (ESI). MS/MS data are acquired in the positive mode. MT and MT-d4 were identified by multiple reaction monitoring (MRM). The Gas Temp and Gas Flow were maintained at 350 ℃ and 6 L/min, respectively. The nebulizer was 50 psi. The capillary voltage was set to 3500 V. The sheathgasheater was 300 ℃. The sheathgasflow was 10 mL/min. Precursor ion (m/z) of MT was 233.1 and production ions (m/z) were 174.2 and 159.1. The Collision Energy of 233.1 > 174.1 was 10 V. The Collision Energy of 233.1 > 159.1 was 25 V. Precursor ion (m/z) of MT-d4 was 237.1 and production ions (m/z) were 178.2 and 163.1. The Collision Energy of 237.1 > 178.2 and 237.1 > 163.1 was 25 V. All above Fragmentor were 75 V.

14.Assay of ATP

The zona pellucida of the oocytes were removed with pronase, and the cleaned oocytes were washed 3 times with TL solution; 12 oocytes was transferred into 50 μL lysate solution, vortexed until their fully lysed and the samples were kept at 4 ℃ or on the top of the ice for a short moment; A 96-well light-proof enzyme labeling plate was used for the study. The 50 μL of ATP detection solution were added to the standard and sample wells of this plate respectively and let them at room temperature for 5 minutes to consume background ATP; then the lysed samples were added to the preprepared wells of the plate and mixed well. The InfiniteF200 microplate reader was used to detect the ATP content. The ATP content of each sample well was calculated based on the ATP standard curve, and the value was divided by the number of oocytes to obtain the ATP content of each oocyte.

15. RNA interference assay

The porcine AANAT gene was used as a template to design interfering RNA by Suzhou Gemma Gene Co., Ltd, and the designed sequence was compared with BLAST to exclude homology with other genes. The final designed porcine siRNA sequence is as follows:

siAANAT: sense(5’-3’): GGGACUGAAAUAAAGAGAUTT;

antisense(5’-3’): AUCUCUUUAUUUCAGUCCCTT.

The cumulus oocytes were removed from the cumulus granulosa cells, and 10 plsiRNA (20 μM) was injected into the cytoplasm of each oocyte using a micromanipulator[29].

16.Real-time fluorescent quantitative PCR

The oocytes extracted from COCs were washed three times with PBS, and stored at 80°C until RNA extraction. Total RNA was extracted using TRIzol(Invitrogen Inc., Carlsbad, CA, United States), quantified by measuring absorbance at 260 nm, and stored at -80°C until assayed. The mRNA levels of relevant genes were assessed in LightCycler(Roche Applied Science, Mannheim, Germany) by quantitative RT-PCR using the OneStep SYBR PrimeScript RT-PCR kit (Takara Bio. Inc., Tokyo, Japan). After melting curve analysis, the accumulated level of fluorescence was analyzed by the second derivative method, and then

the expression level of the target gene in each sample was normalized to that of GAPDH. Primer pairs for mRNAs are as follows.

17.Statistical Analysis

Unless otherwise specified, the data are expressed as mean ± SME. Analysis of variance (ANOVA) was used to analyze the normality among the groups followed by, Dunnett's test. All tests were performed by SPSS26.0 statistical software $P < 0.05$ was set up as significant difference.

Results

1. Melatonin synthesis in mitochondria during porcine oocyte maturation in the in vitro condition

By use of immunofluorescence staining and confocal microscopy, both melatonin synthetic enzymes AANAT and ASMT were found to express in oocytes and co-localized with mitochondria (Fig. 1A). The immunoelectron microscopy results confirmed that major portion of AANAT was distributed in mitochondria but some were also present in cytoplasm (Fig. 1B). In order to explore whether melatonin is synthesized during the process of maturation in porcine oocytes, 5HT, a precursor of melatonin, was added to the maturation medium. Then, the medium was collected melatonin assay by ultra-high performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) The results showed that during the process of maturition, the level of melatonin gradually increased, and the increased sharply from MI to MII stage compared to the control group ($P < 0.01$) (Fig. 1E). accordingly,after adding 5-HT, the protein level of AANAT was also significantly increased compared to the control group ($P < 0.01$) (Figure C,D). To further explore the subcellular sites of melatonin synthesis, the mitochondria of oocytes and cumulus cells were extracted and incubated with 5-HT, The culture fluid was collected at 0, 1, and 2 hours respectively, for melatonin detection. The results showed that melatonin was detected in the the mitochondrial culture medium. extracted from oocytes supplemented 5-HT supplementation significantly increased the mitochondrial melatonin production compared to the controls after 1 h incubation (Fig. 1F).These results indicate that the mitochondria of porcine oocytes can synthesize melatonin during in vitro maturation.

2.5-HT supplementation improves the quality of porcine oocytes

Since 5-HT could increase melatonin production in oocytes, 5-HT was added to the maturation medium. The results showed that 5-HT significantly increased the expansion rate of porcine cumulus cells ($P < 0.05$) (Fig. 2A,B), the porcine oocyte polar body excretion rate ($P > 0.05$) (Fig. 2C) and the normal migration of cortical granules ($P < 0.05$) (Fig. 2D-E) compared to the control group. While addition of Luzindole, a melatonin receptor inhibitor, significantly reduced the normal mobility of cortical granules ($P < 0.001$), and the cumulus expansion rate ($P < 0.05$) (Fig. 2B) compared to the control group. Luzindole also reduced the polar body excretion rate,, but this decrease was not significant difference compared to the control group($P > 0.05$) (Fig. 2C). All the results indicated the promotive effects of melatonin on both

nuclear and cytoplasmic maturation of porcine oocytes, and these activities might be mediated by melatonin receptors .

of oocytes after 44 h of maturation, scale: 200μm; (B) The statistical analyses of cumulus cells expansion, Control (n = 789) 5-HT(n = 741) 5-HT + Lu(n = 686); (C) The statistical analyses of polar body expulsion, Control(n = 570) 5-HT(n = 596) 5-HT + Lu(n = 391); (D) The statistical analyses of ortical granules migration, Control(n = 22) 5-HT(n = 25) 5-HT + Lu(n = 29); (E) Fluorescence staining of cortical granules, green arrows indicate normal migration, red arrows indicate abnormal migration. Scale bar:100 μm.

3. 5-HT supplementation improves the distribution and function of mitochondria in oocyte

To explore the endogenously produced melatonin on mitochondrial properties in oocytes, 5-HT was supplied to the cell culture medium to improve melatonin production of oocytes. The result showed that this treatment significantly increased the mitochondrial density ($P < 0.05$) (Fig. 3A, B), and their normal distributions in oocytes ($P < 0.05$). Accordantly, mitochondrial biogenesis-related gene *SIRT1* were significantly upregulated compared to controls (Fig. 3C). Again, the melatonin receptor inhibitor, Luzindole significantly blunted all these beneficial alterations of mitochondria, ($P < 0.05$) (Fig. 3B and D indicating that the beneficial effects of melatonin is mediated by its receptor It is worth noting that the 5-HT supplementation also increased the number of lipid droplets in porcine oocytes ($P < 0.05$), while the Luzindole reduced this increase ($P < 0.05$) (Fig. 3A, E).

Mechanistically, To explore whether endogenous MT promotes the energy metabolism of oocytes, oocytes were stained with was calculated by confocal (FigureS 1A-C). The results showed that 5-HT supplementation significantly increased the mitochondrial membrane potential ($P < 0.0001$) indicated by increased JC-10 fluorescence intensity (Fig. 3F, S1A-C), and the ATP content of oocytes ($P < 0.05$) (Fig. 3G). Luzindole significantly blunted mitochondrial membrane potential ($P < 0.0001$), and the ATP content ($P < 0.05$) that was increased by 5-HT. At the molecular level, 5-HT treatment increased activities of mitochondrial complex (*ND1*), complex (*COX3*), complex (*CytB*), but the differences did not reach statistically significant; while complex (*ATPase6*) had little change compared to the control (FigureS 1E-H). 5-HT treatment up-regulated the gene expressions of *SIRT3* and *SOD1* (Figure S 1C, D) and significantly decreased the ROS level in oocytes ($P < 0.05$), but this decrease was blunted by Luzindole ($P < 0.001$) hindering the antioxidant effect of endogenous melatonin ($P < 0.0001$) (Fig. 3H, I).

4.Effects of siAANAT on the maturation quality of porcine oocytes

siAANAT was designed to suppress the melatonin production in oocytes. siAANAT was microinjected into GV stage oocytes and cultured vitro for 44 hours, then the M stage oocytes were selected for immunofluorescence staining.. The results showed that the siAANAT oocytes had significantly lower AANAT protein expression that in siNC oocytes ($P < 0.01$), indicating that siRNA successfully knocked down the expression of AANAT in oocytes. The polar body excretion rate of oocytes in the siAANAT group was significantly lower than that in the control group ($P < 0.01$) (Fig. 4D) and after parthenogenetic

activation the cleavage rate ($P < 0.05$) and blastocyst rate ($P < 0.01$) of the embryos in the siAANAT group were also significantly lower than those in the siNC group, and melatonin supplementation could improve the pig oocyte production rate. Maturity quality and its embryo developmental potential ($P < 0.05$) (Fig. 4E-F). it was significantly increased after supplementing with 10^{-7}M melatonin The decrease in polar body excretion rate due to knockdown of AANAT was observed ($P < 0.05$). However, melatonin supplementation rescued all these abnormalities caused by siAANAT ($P < 0.05$) (Fig. 4E-F).

1. (A) siRNA Flowchart; (B) Immunofluorescence staining of AANAT protein in pig oocytes. Scale bar: 100 μm; (C) Statistic analysis of Fluorescence intensity of AANAT protein. siNC (n = 39) siAANAT (n = 23) and siAANAT + MT (n = 19) ; (D) Polar body extration rate. siNC(n = 355), siAANA T(n = 473), and siAANA T + MT(n = 254) ; (E) Cleavage rate. siNC (n = 125) siAANA T(n = 94), and siAANA T + MT(n = 93) groups; (F) Blastocyst rate. siNC (n = 125) siAANA T(n = 94), and siAANA T + MT(n = 93) groups.

1. 5.Effects of siAANAT on mitochondrial distribution and ATP production in porcine oocytes

Since lipid is an important substrate of mitochondrial metabolism the lipid levels in oocytes were measured. The results showed that the number of lipid droplets in siAANAT oocytes were no significant difference compared to other groups (FigureS 2A, B). The number of mitochondria in the siAANAT oocytes had no significant difference with siNC group, but had higher abnormal distribution rate than that in the siNC oocytes ($P < 0.001$) (Fig. 5E). Interestingly, melatonin treatment rescued the mitochondrial abnormal distribution caused by siAANAT ($P < 0.01$), and also significantly increased the number of mitochondria ($P < 0.01$) (Fig. 5A,D). In addition, the expression of *SIRT1* in the melatonin treatment group was significantly upregulated compared to other groups ($P < 0.05$) (Fig. 5G). At the same time, the ROS level the siAANAT group was significantly increased ($P < 0.0001$), and reduced by melatonin supplementation ($P < 0.0001$) (Fig. 5B,C). The mitochondrial membrane potential in the siAANAT oocytes was lower than that in the control group and the melatonin treatment group, but the difference was not significant ($P > 0.05$) (FigureS 2C,D). However, the ATP level of the siAANAT oocytes was significantly lower than that in the control and melatonin treated oocytes ($P < 0.05$), (Fig. 5F).

6. Effects of siAANAT on the metabolic pattern of porcine oocytes

In the study, we also found that melatonin slightly increased expression of mitochondrial complex III (*COX3*) in siAANAT oocytes but significantly upregulated the expressions of complex IV (CytB) and complex V compared to the other groups ($P < 0.05$) (Fig. 5H-J). So we speculated whether knocking down AANAT made oocytes mainly produce energy through glycolysis. To further understand the effects of siAANAT on metabolic pattern of porcine oocytes, the key gene expression in the glycolytic pathway were measured.The results showed that the expressions of *HIF1A* and *GLUT1* in siAANAT oocytes were slightly up-regulated (Fig. 5K, L), however, At the same time, the expressions of phospho-6-gluconate dehydrogenase (*PGD*) and lactate dehydrogenase (*LDHA*) were significantly upregulated compared to the

control oocytes ($P < 0.05$) (Fig. 5N, O). Melatonin supplementation significantly down-regulated the expression levels of PGD ($P < 0.05$) and serine-threonine protein kinase 1 (*AKT1*) ($P < 0.05$) (Fig. 5M,N).

Discussion

The maturation of mammalian oocyte requires substantial energy, and studies have shown that porcine oocytes consume glucose to support their final maturation[30].Recently, evidence has emerged that lipids are a key nutrient and even a major energy source for porcine oocytes [31]. Numerous studies have shown that in the early embryo development, mitochondria are responsible to provide sufficient ATP for most cellular processes through oxidative phosphorylation. The number of mitochondria increases substantially during embryonic development to provide the energy required for blastocyst formation, therefore, mitochondrial dysfunction leads to developmental arrest in early embryos [32].

Evidence has been shown that mitochondrial functions can be influenced by several factors and melatonin is one of them. Melatonin can regulate mitochondrial functions by scavenging free radicals, activating uncoupling proteins, maintaining optimal mitochondrial membrane potential and promoting mitochondrial biogenesis [25, 33–37]. The activities of melatonin on mitochondria may relate to its effects to promote oocyte maturation, fertilization, and early embryonic development in mammals [38]. AANAT is the enzyme that converts serotonin to melatonin [39]. Our previous study found that melatonin was synthesized in mitochondria of mouse oocyte during its maturation [6]. In current study, we confirmed that AANAT was co-localized with mitochondria in porcine oocytes and 5-HT supplementation during the in vitro porcine oocyte maturation significantly increased the melatonin production compared to the control group ($P < 0.01$). In addition, the in vitro cultured mitochondria isolated from cumulus cells or oocytes both can release melatonin to the culture medium and with 5-HT supplementation, the mitochondria produced significantly higher melatonin than that of the control group. The results showed that the mitochondria of porcine oocytes synthesized melatonin during oocyte maturation.

During maturation, the oocytes demand substantial amounts of ATP for the continuous transcription and translation activities. Mitochondria is the primary source of ATP production and the sufficient numbers of functional mitochondria are critical are required for the maturation. Therefore, the quality of oocytes is positively related to mitochondrial DNA copy number and ATP content. Under physiological condition, the copy number of mitochondrial DNA as well as mitochondrial distribution were significantly improved during oocytes maturation.. However, the in vitro maturation (IVM) may result in altered mitochondrial morphology and expression of genes related to mitochondrial function [40]. In this study, we found that 5-HT supplementation significantly increased melatonin production and this increased melatonin production increased the number of mitochondria, and promoted the uniform distribution of mitochondria and, the ATP content in oocytes. These activities of melatonin were probably mediated by its receptor since melatonin receptor inhibitor Luzindole blunted all these activities. To further identify the effects of melatonin on the mitochondrial function and oocyte maturation the *AANAT* was silenced by siAANAT which can significantly reduce melatonin production. The siAAMAT caused abnormal mitochondrial distribution and mitochondrial dysfunction while melatonin supplementation rescued these

abnormalities. The results showed that melatonin is necessary for mitochondrial function and oocyte maturation and embryonic development.

To further prove the endogenously generated melatonin is involved in oocyte quality and its maturation the 5-HT was used to increase endogenous melatonin production. The results showed that 5-HT supplementation had the similar effects as melatonin supplementation and diminished the siAANAT induced abnormalities in oocytes. Melatonin is a mitochondrial targeted antioxidant and it, up regulated the expression of *SIRT3* and *SOD1*[41]. Melatonin also protects against mitochondrial depletion and energy deficiency caused by environmental toxin exposure by activating the *SIRT1/PGC-1α* pathway, thereby promoting mitochondrial biogenesis, suggesting that melatonin can be used to early embryonic development to rescue the state of mitochondrial deficiency [42]. In this study, we found that 5-HT significantly up-regulated the expression level of SIRT1 after SIAANAT. He et al found that melatonin can reduce the mitochondrial membrane potential, resulting in the quiescence of mitochondrial respiration and maintaining a state of low metabolism[43]. However, our results found that melatonin increased the mitochondrial membrane potential, which may be due to a compensatory mechanism caused by the in vitro culture environment in which it had an energy supply shortage.

It has been reported that 5-HT and its receptors were present in mouse and human oocytes and cumulus cells [44–46], indicating their involvements in mammalian reproductive activity including normal embryonic development. Other studies have reported that 5-HT administration caused blastocyst cell apoptosis, decrease in blastocyst cell number and blastocyst rate [45, 47]. 5-HT at the concentration of 10^{-4}M inhibited the maturation of porcine oocytes by inhibiting the synthesis of estradiol in granulosa cells while its antagonists also inhibited mouse embryonic development even caused embryonic development to block at high concentrations in the in vitro condition [48]. In current study, we speculated that some activities of 5-HT on oocytes and embryonic development were mediated by its metabolite, melatonin. The evidence obtained from the study strongly supported our speculation. Our results showed that 5-HT supplementation not only upregulated the expression of *AANAT* but also increased the melatonin production in oocytes. The increased melatonin level was positively associated with oocyte quality and the embryonic development.

Traditionally, It was believed that the energy metabolism in the COCs was collaborated among the cells. The cumulus cells are responsible to metabolize glucose to form pyruvate and lactate, and then both of them were transferred to the oocyte through gap junctions, and finally through the TCA cycle and oxidation Phosphorylated to produce ATP in oocytes [49–51]. However, the porcine COCs may prefer to use fat acids as the energy source [52]. Fatty acids undergo beta oxidation to produce ATP[53]. Reduced abundance of *CPT1* impairs transport of fatty acids into mitochondria, leading to reduced β-oxidation and the compensated elevation of glucose metabolism in porcine embryos, suggesting that fatty acid oxidation is prevalence to the alternative energy pathway of glucose metabolism in this cell [54] Further evidence to show that AANAT knockdown significantly up-regulated the expression of the key genes of *PGD* and *LDHA* related to the Warburg effect to increase the glycolytic activity but with decreased ATP production in *siAANAT* oocytes compared to the controls. Melatonin supplementation reduced these

upregulated genes. The results indicate that melatonin may induce porcine oocytes to preferentially use lipids for fatty acid β-oxidation to provide energy for porcine oocyte maturation.

Conclusions

In summary, in this study, for the first time we have identified that the mitochondria of porcine oocytes are the major sites for melatonin biosynthesis. This mitochondrially originated melatonin reduces ROS, upregulates the expression of *SIRT1*, increases the number of mitochondria and their uniform distribution as well as their oxidative phosphorylation, therefore, improves the maturation efficiency of porcine oocytes. The reduced melatonin level by si*AANAT* up-regulates the expression of *PGD* and *LDHA* switches the mitochondrial oxidative phosphorylation to glycolysis and reduces the maturation efficiency of porcine oocytes. These abnormalities are rescued with melatonin supplementation. All these effects of melatonin at lease are partially mediated by its receptors since non-selective melatonin receptor blocker, luzindole blunts these activities. The research results provide an experimental basis for further revealing the metabolic mode of melatonin on porcine oocyte maturation. If this observation is confirmed by others or in different mammalian species It will provide new insights to treat human infertility and conservation of germplasm resources in animal husbandry.

Abbreviations

ROS
reactive oxygen species
ATP
Adenosine triphosphate
AANAT
Arylalkylamine-N-Acetyltransferase
5HT
5-hydroxytryptamine
ASMT
Hydroxyindole-O-methyltransferase
IVF
In Vitro Fertilization
COCS
Cumulus-oocyte complexs
IVC
In vitro Culture
LC-MS/MS
Liquid Chromatography Mass Spectrometry Mass Spectrometry
MI
Metaphase I

MII
Metaphase II
GV
Germinal vesicle
GVBD
Germinal vesicle breakdown
Mito
Mitochondria
Lu
Luzindole
MT
Melatonin
IBMX
Isobutylmethylxanthine
NC
Negtive Control
IVM
in vitro maturation.

Declarations

Author Contributions: Guoshi Liu proposed the research and revised the manuscript. Tianqi Zhu and Laiqing Yan conceived the study, performed experiments and writing the manuscript.Wenkui Ma, Fan Xia, Likai Wang, Xiao Ma, Guangdong Li, Zixia Shen, Yiwei Wang, Yao Fu, Bingyuan Wang and Lu Zhang and Pengyun Ji performed experiments and collected data.All authors provided helpful discussion and helped shape the research, analysis, and manuscript.

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Acknowledgments

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate:

The animal study protocol was approved by the AnimalCare and Use Committee of China Agricultural University (permission No. AW01602202-1-6).

Consent for publication: Not applicable

Competing interests: The authors declare no conflict of interest.

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Figures

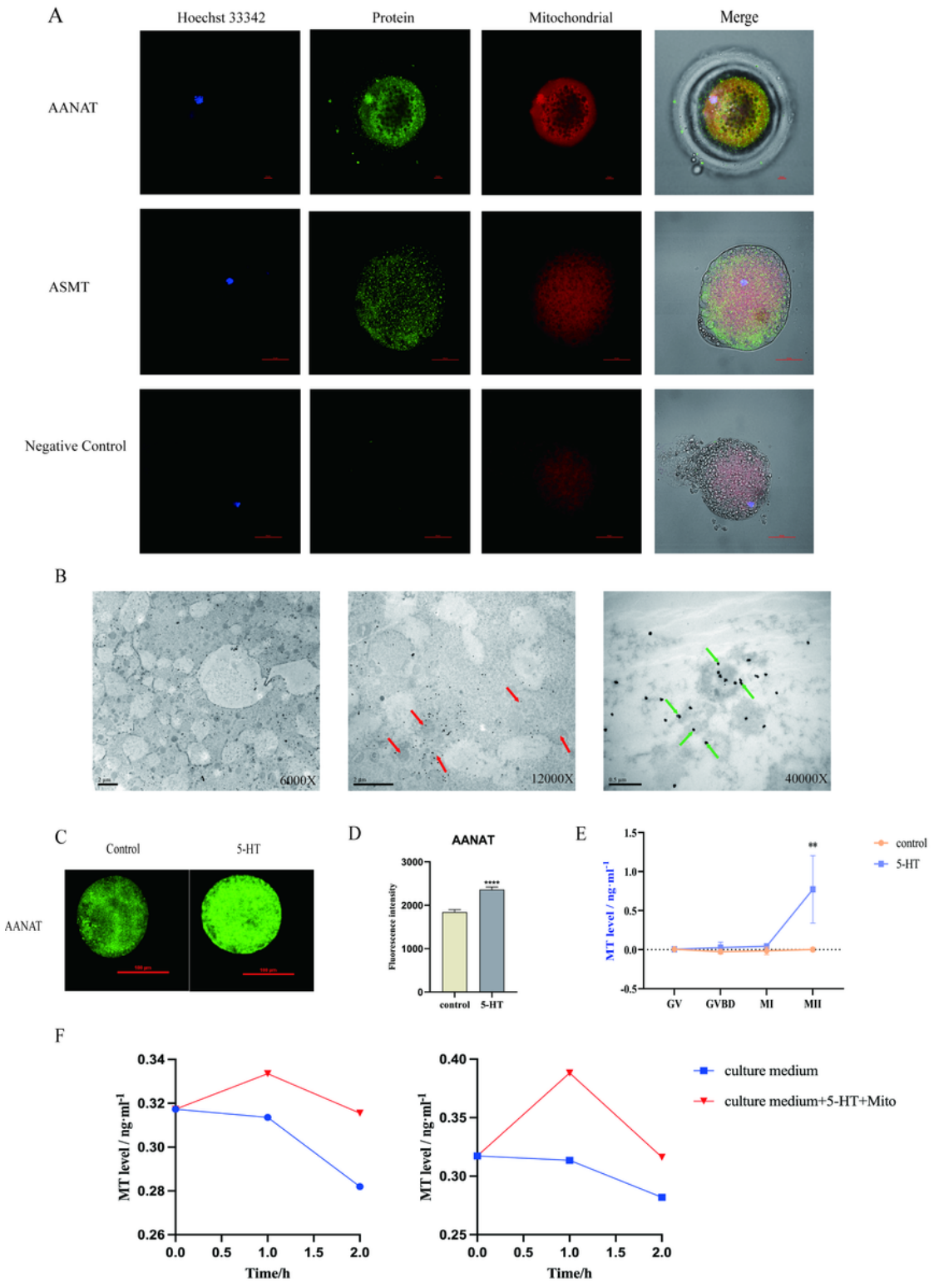

Figure 1

Melatonin synthesis in mitochondria during the process of in vitro maturation of porcine oocytes (A) Immunofluorescent staining of AANAT and ASMT in porcine oocytes. Scale bar: 50μm; (B) AANAT subcellular distribution in porcine oocytes, red arrows point to mitochondria, green arrows point to

AANAT synthetase (C) Immunofluorescent staining of AANAT in porcine oocytes with 5HT treatment, scale bar: 100μm; (D) Statistical analysis of AANAT level of oocytes (n=25); (E) Melatonin levels in culture medium of oocytes at each mature stage, n=8; (F) Melatonin levels in mitochondrial culture medium of granulosa cells (left) and oocytes (right), n=3.

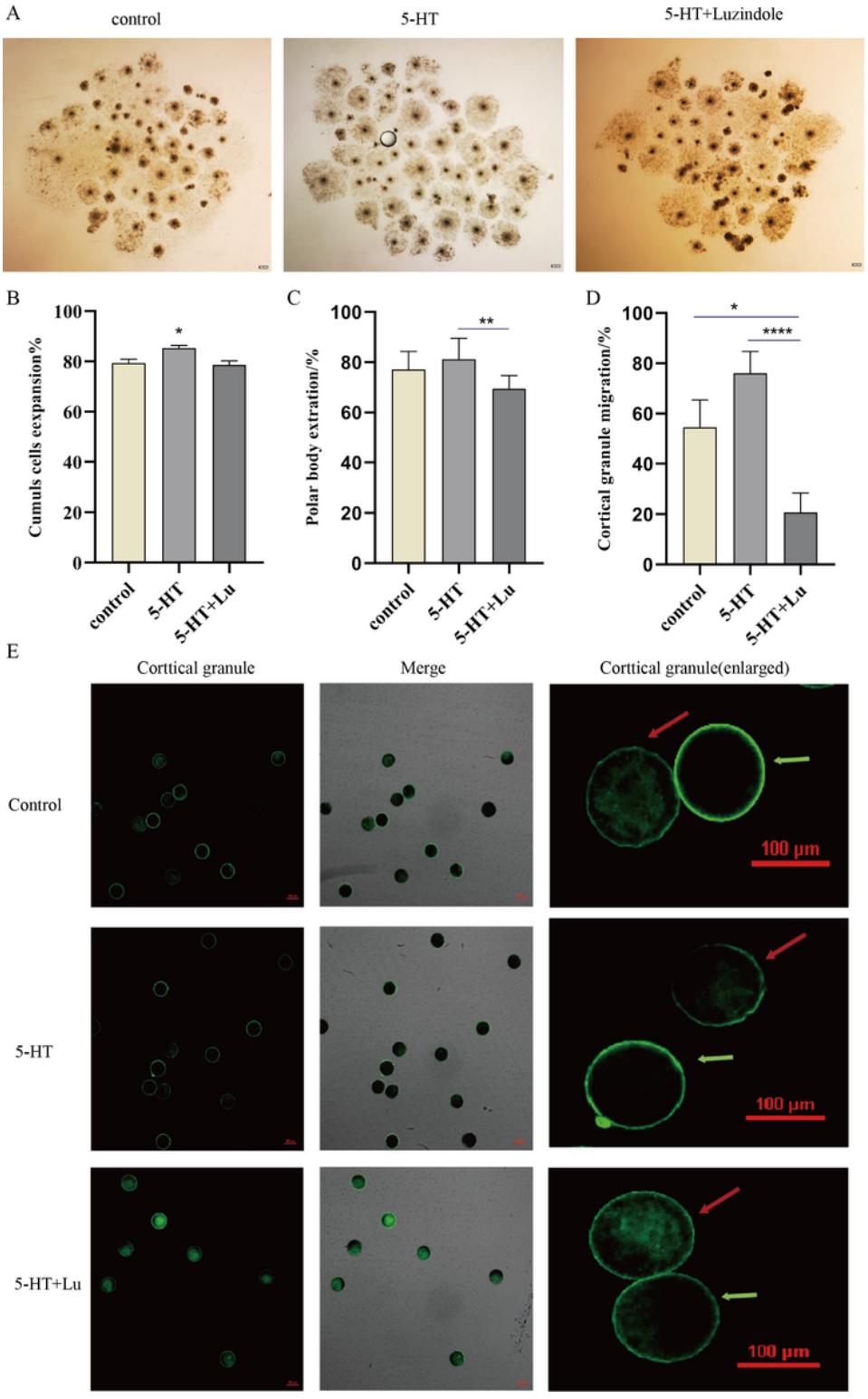

Figure 2

The effects of 5-HT and Luzindole on the maturation of porcine oocytes. (A) Cumulus cells expansion of oocytes after 44 h of maturation, scale: 200μm; (B) The statistical analyses of cumulus cells expansion, Control (n=789) 5-HT(n=741) 5-HT+Lu(n=686); (C) The statistical analyses of polar body expulsion, Control(n=570) 5-HT(n=596) 5-HT+Lu(n=391); (D) The statistical analyses of ortical granules migration, Control(n=22) 5-HT(n=25) 5-HT+Lu(n=29); (E) Fluorescence staining of cortical granules, green arrows indicate normal migration, red arrows indicate abnormal migration. Scale bar:100 μm.

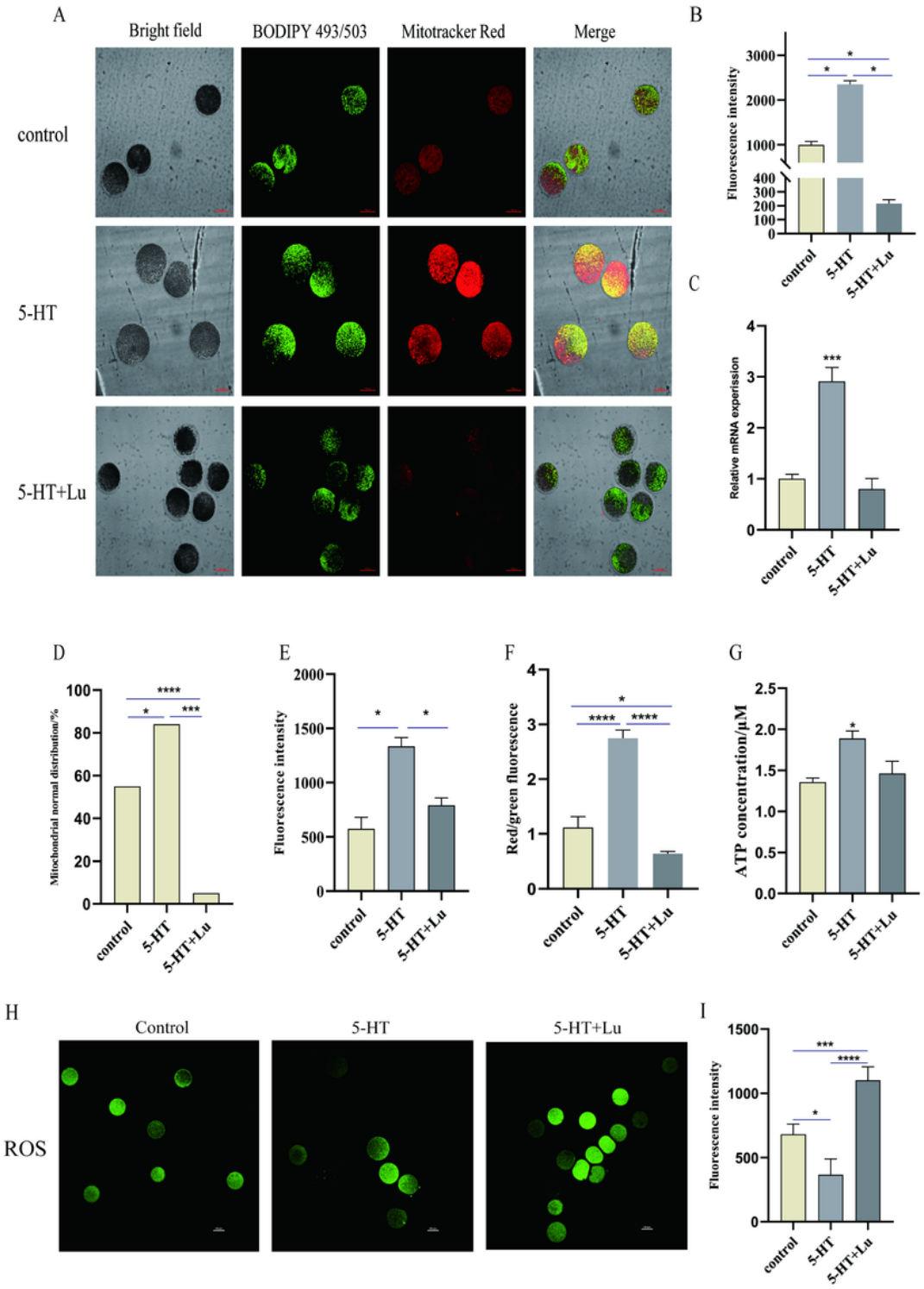

Figure 3

Effects of 5HT on mitochondrial function in porcine oocytes. (A) Lipid droplet and mitochondrial staining images of oocytes; (B) Statistical analysis of Mitotracker Red fluorescence staining intensity, Control (n=20) 5-HT(n=18) 5-HT+Lu(n=18); (C) Relative *SIRT1* mRNA level, n=4; (D) Mitochondrial distribution, Control (n=20) 5-HT(n=19) 5-HT+Lu(n=17); (E) BODIPY493/503 fluorescence staining intensity, Control(n=19) 5-HT(n=18) 5-HT+Lu(n=19). Scale bar: 100 μm; (F) Mitochondrial membrane potential; (G)

ATP level of porcine oocytes, n=36; (H) ROS staining images of porcine oocytes; (I) ROS levels in oocytes. Control (n=22) 5-HT(n=31) 5-HT+Lu(n=34). Scale bar: 100 μm.

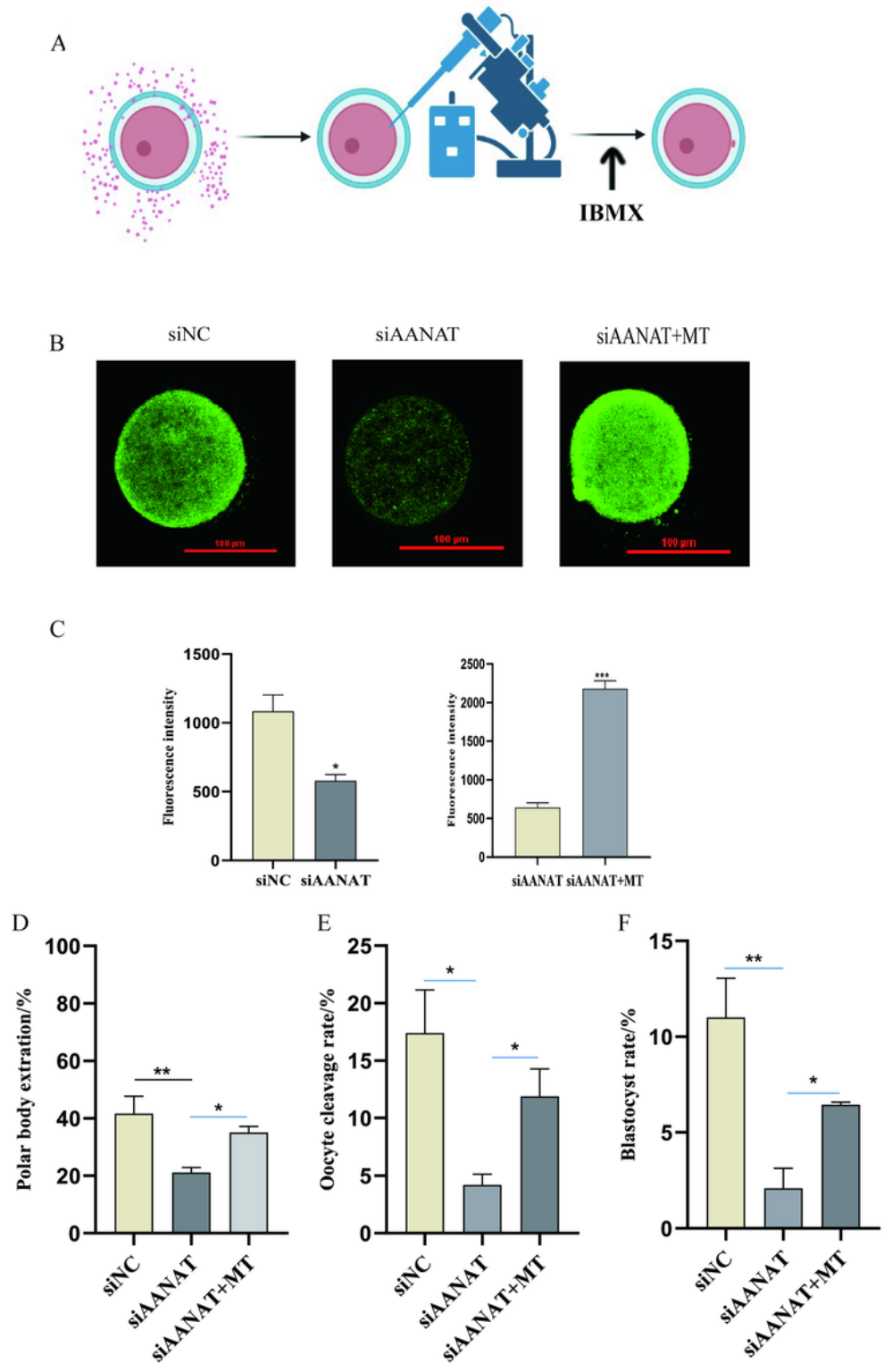

Figure 4

Effects of siAANAT on the maturation quality of porcine oocytes

(A) siRNA Flowchart; (B) Immunofluorescence staining of AANAT protein in pig oocytes. Scale bar: 100 μm; (C) Statistic analysis of Fluorescence intensity of AANAT protein. siNC (n=39) siAANAT (n=23) and siAANAT+MT (n=19) ; (D) Polar body extration rate. siNC(n=355), siAANA T(n=473), and siAANA T+MT(n=254) ; (E) Cleavage rate. siNC (n=125) siAANA T(n=94), and siAANA T+MT(n=93) groups; (F) Blastocyst rate. siNC (n=125) siAANA T(n=94), and siAANA T+MT(n=93) groups.

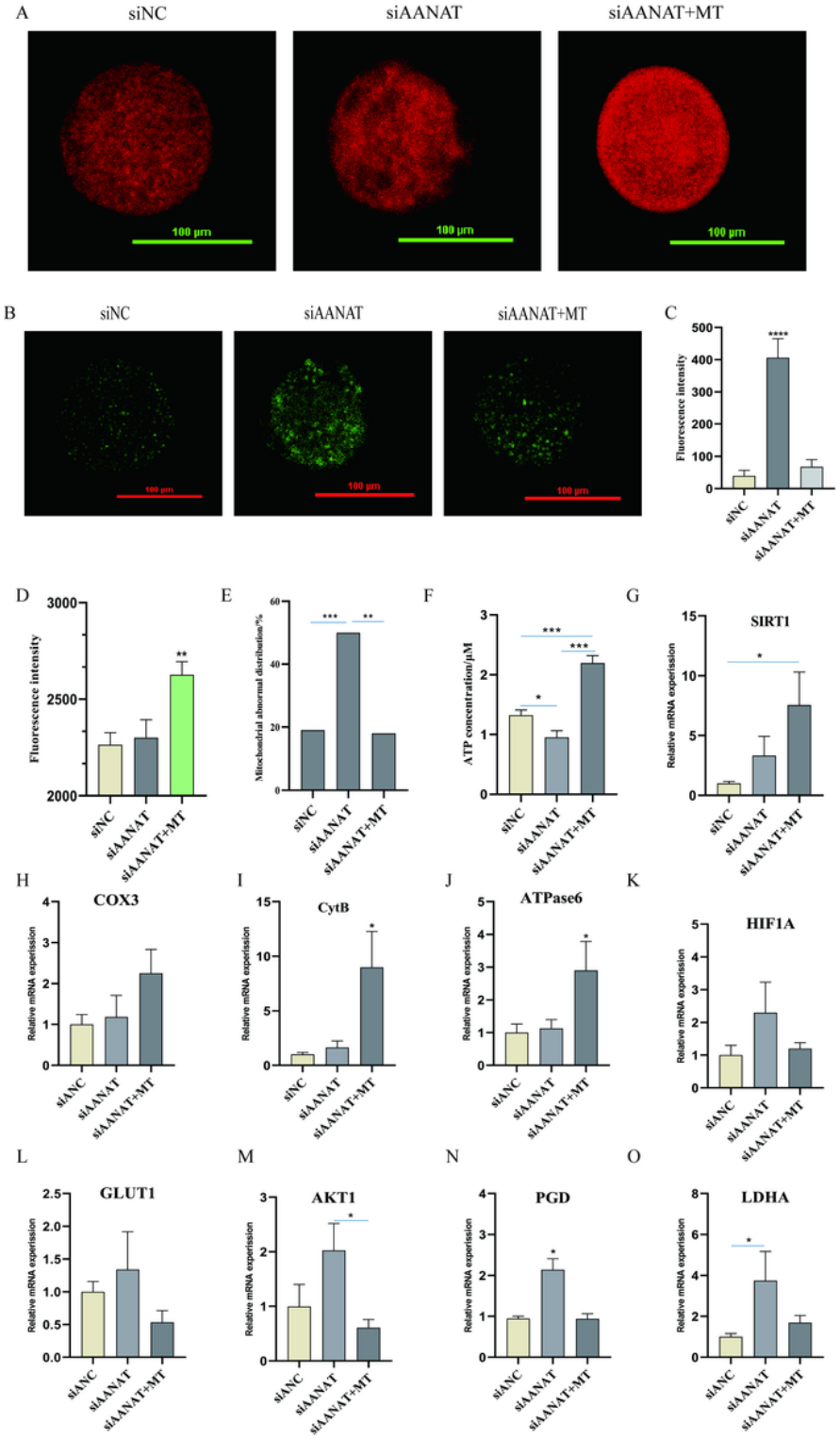

Figure 5

Effects of siAANAT on the metabolic patterns in porcine oocytes. (A) Fluorescence staining images of mitochondria in porcine oocytes. Scale: 100μm; (B) ROS fluorescent staining in oocytes; (C) Statistic analysis of ROS fluorescence staining intensity in oocytes. siNC(n=6), siAANAT(n=5) and siAANAT+MT(n=8) ; (D) Fluorescence intensity of mitochondrial fluorescent staining. siNC(n=32), siAANA T (n=17) and siAANAT+MT(n=19) ; (E) Abnormal distribution ratio of mitochondria. siNC(n=71), siAANA T (n=34) and siAANAT+MT (n=22) group; (F) ATP content. siNC(n=48), siAANA T(n=48) and siAANA T+MT(n=48)group; (G) Relative *SIRT1* mRNA experission level, n=3; (H) Relative *COX3* mRNA experission level, n=3; (I) Relative *CytB* mRNA experission level, n=3; (J) Relative *ATPase6* mRNA experission level, n=3; (K) Relative *HIF1A* mRNA experission level, n=3; (L) Relative *GLUT1* mRNA experission level, n=3; (M) Relative *AKT1* mRNA experission level, n=3; (N) Relative *PGD* mRNA experission level, n=3; (N) Relative *LDHA* mRNA experission level, n=3.

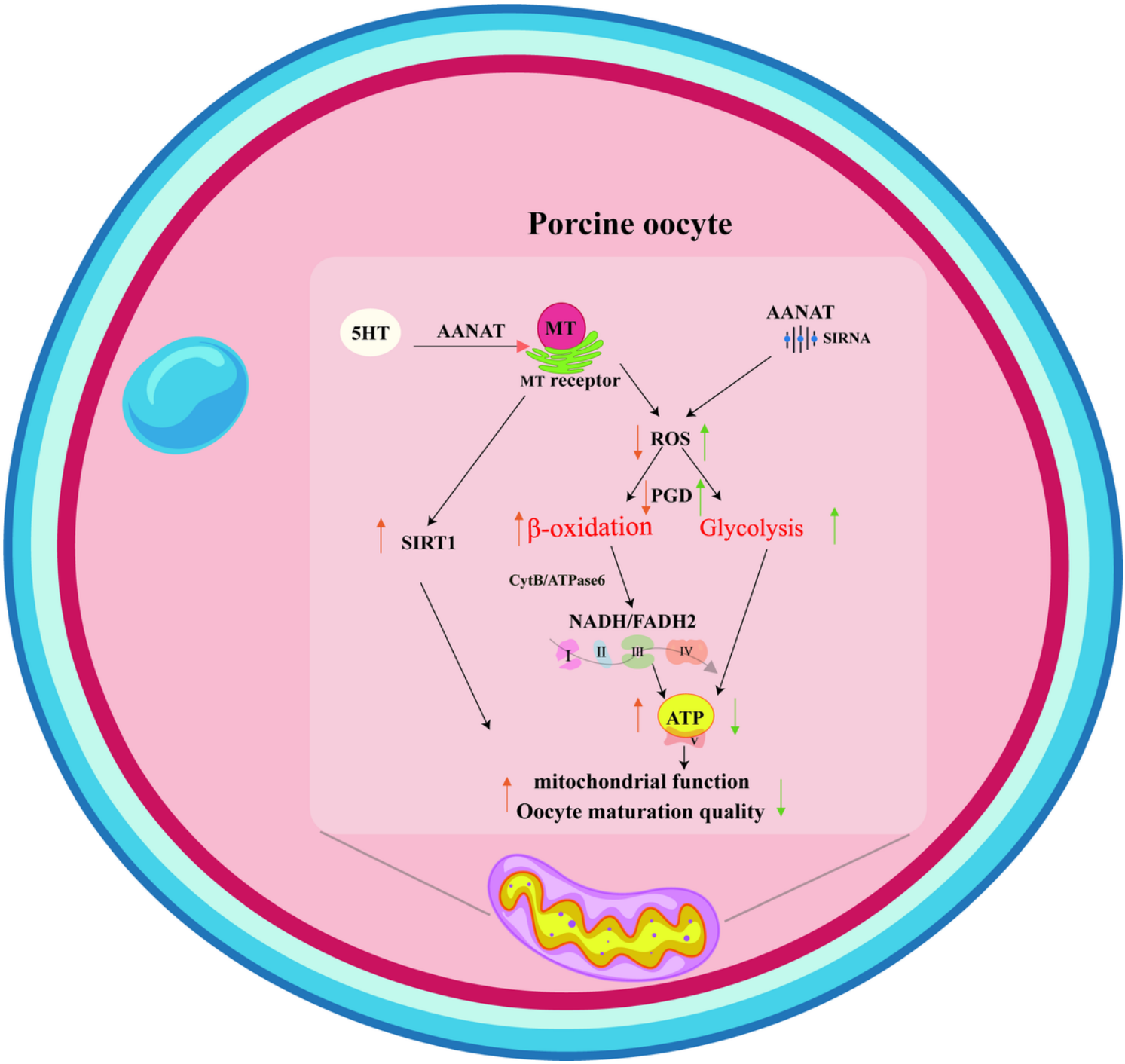

Figure 6

Pig oocyte mitochondria synthesize melatonin and enhance its function by promoting electron transport chain to improve oocyte quality

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