Functional role of increased acetylated tubulin in porcine oocyte microtubule structure, meiotic maturation and embryogenesis

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Abstract

Acetylated microtubule improves porcine oocyte microtubule structure, meiotic maturation and subsequent embryonic development. HDAC6 can specifically deacetylate α-tubulin in assembled microtubules, increased acetylated microtubule treatment with tubacin, a HDAC6-selective inhibitor, is beneficial for porcine oocytes maturation and early embryogenesis. Here it is shown that α-tubulin acetylation gradually decreased from MI to IVF pronuclear stage. The increased acetylation of α-tubulin significantly reduced the abnormal rate of microtubules, furthermore, the proportion of mitochondria in the vicinity of IVF nucleus was significantly enhanced in MI and MII stages. The expression levels of microtubule assembly genes (TUBA1A , α TAT1 and MAP2) significantly up-regulated in MI and MII stages. In addition, the oocytes with high acetylation level of α-tubulin significantly improved maturation, syngamy and IVF blastocyst formation compared with the control oocytes. In present study, these indicate functional role of increased acetylated α-tubulin advances normal spindle formation and mitochondrial concentration, moreover, improves porcine maturation, syngamy and preimplantation embryo development.

Background

Microtubules (MTs) are required for the cascade of events including the spindle assembly, mitochondria distribution, meiotic maturation, syngamy and early embryo development(1–4). The inhibition of microtubule assembly has adverse effects on these important events in different species(1, 5–7). Abnormal microtubule is caused by aging(8), in vitro maturation(9), chemical exposures(10) and mutations(11). Some drugs could hinder microtubule assembly (colchicine(12) and nocodazole(13)) or prevent depolymerization (taxol(14)) during oocytes meiotic maturation. These data suggest that the disruption of dynamic balance may result in abnormalities of microtubules.

Acetylated microtubule is a reversible post-translational modifications (PTMs) which catalyzes the acetylation of Lys-40 sites on the inner surface of microtubules(15). Although it is not clear whether the stability of microtubules is related to the acetylation of microtubules (16, 17), acetylated tubulin appears more stable treated by colchicine(12) and nocodazole(13, 18). With TSA and Tubacin treatment or catalytically inactive HDAC6, increased acetylated tubulin enhances the chemotactic motility of these cells(19, 20). The acetylated α-tubulin of mouse is predominantly at the spindle of MII and first fertilization cleavage(21). HDAC6 knockout in mice increased microtubules acetylation without affecting the growth and development(22). It is still unclear how the functional roles of increased acetylated alters maturation, fertilization, and early embryonic development.

Histone deacetylase 6 (HDAC6) removed the α-K40 acetylation (19), and localized to the cytoplasm and operated on the free tubulin dimer. Tubacin, a selective and reversible HDAC6 inhibitor, could increase α-tubulin acetylation to make microtubules dynamic and stable(20, 23). αTAT1 catalyzes the α-K40 acetylation (24) and is homology to histone acetyltransferases(25). The recruitment of microtubule-associated proteins (MAPs) into acetylated tubulin can lead the change in dynamic balance (26–28).

However, it has not been addressed whether increased α-tubulin acetylation causes the changes of αTAT1 and MAPs in oocyte maturation and fertilization.

Our understanding of increased α-tubulin acetylation should keep stable after microtubules assembly, however, not disrupt dynamic balance of microtubules structure to advance the maturation, fertilization and development. We explored the functional roles of increased α-tubulin acetylation in spindle formation, mitochondrial distribution, PN syngamy, embryo development, and the transcription of α-tubulin acetyltransferase and microtubule-associated proteins in porcine oocyte maturation and fertilization.

Results

Effect of drug treatment on the α-tubulin acetylation

As indicated by mean pixel intensity after immunofluorescence staining (Fig. 1), the dynamic pattern of α-tubulin acetylation gradually decreased from MI to MII to PN syngamy. The intensity of α-tubulin acetylation treatment with tubacin were stronger than control in MI and MII (P<0.05), but they were not different in PN syngamy (P>0.05).

Effect of increased α-tubulin acetylation on spindle morphology

GV oocytes displayed a large number of α-tubulin in the cortex (Fig. 2A). The normal spindle was barrel shaped or slightly pointed (Fig. 2B,D,E,H), and the abnormal spindle was disorganized, asymmetrical, round or elongated (Fig. 2C,F,G,I). With increased α-tubulin acetylation, the normal spindle was higher than the control in MI and syngamy (87.73 % vs. 70.93 % and 22.22 % vs. 11.94 %, P<0.05; Table 2), and the abnormal spindles were lower than the control in MI and MII (4.94 % vs. 20.25 % and 17.26 % vs. 35.65 %, P<0.05; Table 2). These suggest that the increased α-tubulin acetylation have a critical effect to improve microtubules after meiotic apparatus assembly.

Effect of increased α-tubulin acetylation on mitochondrial distribution and fertilization process

The mitochondria aggregation in the vicinity of treated nucleus was more obvious than the control (47.02 % vs. 25.59 %, P<0.05; Table 3). After *in vitro* fertilization, The mitochondria aggregation was associated with the pronucleus (Fig. 3B) or syngamy chromosome (Fig. 3C). With increased α-tubulin acetylation, the syngamy proportion was higher than the control (16.04 vs. 6.15, P<0.05; Table 4). These indicated that increased α-tubulin acetylation can promote mitochondria concentration and pronucleus formation.

Effect of increased α-tubulin acetylation on meiotic maturation and IVF embryos development

The rates of maturation and blastocyst on the treated groups were higher than the control (72.90 ± 5.13 % vs. 64.06 ± 2.87 %; 13.28 ± 1.79 % vs. 9.80 ± 1.93 %; $P<0.05$; Table 5). However, there were not different in 2-cells and total number of blastocyst cells between two groups (P>0.05). These showed that the functional effects of increased α-tubulin acetylation can improve *in vitro* maturation and fertilization development.

Effect of increased α-tubulin acetylation on the transcription of related genes

From MI to MII, the expression of *αTAT1*, *MAP2*, *HSP90* and *Katanin* was significantly up-regulated ($P<0.05$), but the expression of *TUBA1A* was significantly down-regulated ($P<0.05$) (Fig. 5). At the MI stage, the *TUBA1A* transcription in the treatment had significantly higher levels than the control ($P<0.05$) (Fig. 5A). The *αTAT1* and *MAP2* patterns of the MII oocytes in the treatment had significantly higher expression than the control ($P<0.05$) (Fig.5B,5C). The results support a molecular mechanism of increased α-tubulin acetylation for recruiting MAPs to improve the configuration of microtubule dynamics. The above results indicated that increased α-tubulin acetylation promoted the binding of tubulin to MAPs and motor proteins, which improved microtubule configuration, maturation and fertilization.

Discussion

Tubulin acetylation could affect microtubule associated proteins recruitment to microtubules and alter microtubule assemble(29). The study investigated the dynamic pattern of α-tubulin acetylation gradually decreased from MI to MII to PN syngamy. The intensity of α-tubulin acetylation treatment with tubacin was significantly higher in MI and MII (Fig. 1), but they were not different in PN syngamy. Abnormal microtubule acetylation of GV oocytes influenced spindle structure, which lead to abnormal maturation and failure of chromosome division(30). Thus, tubacin increased porcine α-tubulin acetylation from MI to MII stages. With increased α-tubulin acetylation, the normal rates of spindle formation were significantly higher than the control in MI and syngamy, and the abnormalities of MI and MII spindles were significantly lower (Table 2). The oocyte development required microtubule assembly and chromosome association. Post-translational modifications promoted microtubule associated proteins binding to the microtubule, which affected microtubule assemble and dynamic stability to perform different physiological functions. Some drugs could hinder microtubule assembly (colchicine(12) and nocodazole(13, 18)) or prevent depolymerization (taxol(14)) during oocytes meiotic maturation, which significantly inhibit spindle morphology in vitro maturation and fertilization. These data supported increased porcine α-tubulin acetylation is critical to microtubules dynamic stabilization in meiotic maturation and fertilization.

This study showed the proportion of mitochondria aggregation in the vicinity of treated nucleus was significantly higher (Table 3). The mitochondrion of fertilized egg was close to the pronucleus (Fig. 3B), or associated with syngamy chromosome (Fig. 3C). Mitochondria represented the areas of ATP

concentration that contributed to embryonic development. Microtubules were thought to promote mitochondrial transport and concentration in neuron cell (31). Microtubules of porcine oocytes could regulate active mitochondrial distribution to improve the embryo development (32). The abnormal mitochondrion would greatly affect early embryo development, reduce the post-implantation development of the fetus and even lead to diseases in adulthood (33). Thus, it was conducted orcein stain to analyze changes in fertilization process. Increased α-tubulin acetylation embryos showed higher proportion of the syngamy than the control embryos (Table 4). Microtubule is the key factor affecting the fusion of male and female pronucleus in mouse fertilized eggs(1). The functional effects of increased α-tubulin acetylation can promote mitochondria concentration distribution and pronucleus formation. It was required to test maturation efficiency and embryo development. The rates of maturation and blastocyst were significantly enhanced (Table 5). The cumulative data suggest that increased α-tubulin acetylation have a critical effect in oocytes maturation and fertilization.

Post-translational modifications of the microtubules were in the C-terminal tails or on the outer surface of microtubules. The modifications of α-tubulin were closely linked to microtubule-associated proteins, and specifc modifications would change the interaction between α-tubulin and microtubule-associated proteins. It was determined to measure the relative level of *αTAT1* and *MAP2* in oocytes maturation. From MI to MII, the expression of *αTAT1* and *MAP2* significantly rose (Fig. 5). The *αTAT1* and *MAP2* patterns of the treated MII oocytes were higher than the control (Fig.5B, C). αTAT1 is a member of GCN 5 superfamily of acetyltransferase, and is still not clear to catalyze acetylation. αTAT1 may be the only enzyme to catalyze acetylation in K40 site, and tubulin acetylation plays a key role in cytoplasmic structure (24). MAP2 can bind microtubules to promote dynamic stability and extension. Phosphorylation and dephosphorylation can reversibly regulate the binding of MAP2 and microtubule. MAP2 dephosphorylation can promote the dynamic stability of microtubules (34, 35). Few studies have focused on the relationship between the MAP2 and maturation. The study shows that increased levels of α-tubulin acetylation can promote the expression of *MAP2*, which is conducive to microtubule stability. These results support a molecular mechanism of increased α-tubulin acetylation for recruiting *αTAT1* and *MAP2* to improve the configuration of microtubule dynamics.

Conclusions

The hyperacetylation of α-tubulin significantly reduced the abnormal rate of microtubules, furthermore, the proportion of mitochondria in the vicinity of IVF nucleus was significantly enhanced in MI and MII stages. The expression levels of microtubule assembly genes (*TUBA1A*, *αTAT1* and *MAP2*) significantly up-regulated in MI and MII stages. In addition, the oocytes with high acetylation level of α-tubulin significantly improved maturation, syngamy and IVF blastocyst formation compared with the control oocytes. These suggest that functional role of increased acetylated α-tubulin promotes normal spindle formation and mitochondrial concentration, moreover, improves porcine maturation, syngamy and preimplantation embryo development. The present work provides evidence supporting a functional role of acetylated microtubule in porcine oocyte microtubule structure and that specific stimulatory actions of meiotic maturation and early embryogenesis are observed.

Materials And Methods

Our chemicals and reagents were from the Sigma-Aldrich. All experiments were performed according to the guidelines of Guangxi University.

In vitro maturation

Oocyte maturation process and formula follow our previous experiments(36). Cumulus oocyte complexes (COCs) were cultured in the maturation medium containing 15 IU/mL eCG and 10 IU/mL hCG for 20-22h, and transferred to free hormone maturation medium for the remaining 20-22h. Maturation medium was supplemented with tubacin (20mM stock in DMSO, 2μM final; selleck Chemicals) during MI (28h) or MII (44h). Oocytes treated with tubacin were use for the experiments of *in vitro* fertilization or fluorescence labeling.

Production of IVF embryos

Fertilization process and formula follow our previous experiments(36). The matured cumulus was gently blown and digested. Oocytes with polar body and uniform cytoplasm were coincubated with semen of appropriate concentration for 4h. The presumptive zygotes were selected for subsequent experiments or cultured in PZM-3. The rates of cleavage and blastocyst were recorded at 24h and 168h. The total number of blastocysts was recorded after staining.

Confocal microscopy

The Immunofluorescence procedures were performed according to our previous study(36). Samples were incubated with α-tubulin antibody (1:100; Santa cruz) and α-tubulin acetylation antibody (1:100; Proteintech) primary antibodies, and was labeled with fluorescein isothiocyanate co-njugated (1:200; Sigma) secondary antibody. Live oocytes or embryos was incubated in PZM-3 containing Mito-tracker Green (Molecular Probes). They were detected by a confocal microscope (Cofocal microscope, Leica, Inc.). The brightest area of every sample was automatically quantified and recorded. The fluorescence intensity represents the acetylation level used for statistical analysis.

Evaluation of *in vitro* fertilization

After IVF 22-24 h (starting from culture), After IVF 22-24 h (starting from culture), presumptive zygotes were transferred into PBS on the slide. The four corners of the droplet was dotted with Vaseline. The

cover glass was gently pressed down to keep oocytes intact on vaseline. About 100 μL of fixing liquid (3:1 ethanol:acetic acid) was carefully pushed into the space between the cover glass and the slide. According to the above method, 1% (w/v) orcein was gently pushed to cover fixed oocyte. The changes of fertilization pronucleus were observed and recorded under inverted microscope at 200×.

Reverse transcription and QRT-PCR

Reverse transcription and QRT-PCR procedures were performed according to our previous study (36). These primers (table 1) were synthesized from Sangon Biotech (Shanghai, China). Five embryos was collected into the lysis solution, and messenger RNA was reverse transcribed. The expression of different genes was automatically acquired and recorded. Each gene in different sample was repeated three times, and detected compared with the expression of the endogenous control 18s. The expression levels of the genes were calculated by $2^{-\triangle\triangle CT}$ calculation method.

Statistics

Results are represented as mean SEM. Statistical analysis was proceeded by one-way analysis of variance and tested by Duncan's multiple comparison. Only $p<0.05$ represented significant difference.

Declarations

Our experiment was approved by the animal ethics committee of Guangxi University. Each of our authors agreed to publish. Thank all the authors for their hard work. Thanks to Professor Deshun Shi, Professor Qingyou Liu, Professor Ben Huang and Professor Kuiqing Cui for guiding the experiment. Thanks to Dr. Zhipeng Li and Dr. Bangjun Gao for qRT-PCR experiment, immunofluorescence experiment and data collection. Thanks to Ms. Jianrong Jiang for her interest in the experiment. We have no conflict of interest. This study was supported by grants from the China National High Technology Research and Development Program (863) Project (2011AA100607), National Natural Science Foundation Project (31260552 and 31401267), Guangxi Natural Science Foundation (Grant No. 2014GXNSFCB118003) and Guangxi Medical University Youth Science Foundation Project (GXMUYSF201829).

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Tables

Table 1. Primer Sequences, and Expected Product Sizes for QRT-PCR Analysis

Target Genes	Primers Sequences (5'to 3')	GenBank Accession Number	Product Size (bp)
18s	F: GATGGGCGGCGGAAAATTG R: TCCTCAACACCACATGAGCA	NR_046261	107
TUBA1A	F: CTCCCTGCTGATGGAACGTCT R: TACCATGAAGGCACAATCAGAG	NM_001315710	163
αTAT1	F: CGCCTTCTGCTCGCTACTGACC R: GTTTCCTGCTCCCCCTGCTTCA	XM_005665740	170
MAP2	F: ACAGAAGACAGAACCAAGCC R: CAACTAAACCCCACTCGTCC	XM_013984450	131
HSP90	F: CAAACACAACGATGACGAGCAG R: AACCTTTGTTCCACGACCCAT	NM_213973	103
Katanin	F: CGGAACGATTGCTGAAACCT R: TTTGACTAACTGCTTGGCTG	XM_013991829	155

Table 2. Effects of increased α-tubulin acetylation on the microtubules configuration

s	Normal MI (%)	Abnormal MI (%)	Others (%)	Normal MII (%)	Abnormal MII (%)	Others (%)	IVF tubulin (%)
l	37(70.93)^{b}	12(20.25)^{a}	5(8.82)	28(62.26)	16(35.65)^{a}	1(2.22)	5(11.94)^{b}
	36(87.73)^{a}	2(4.94)^{b}	3(7.14)	32(70.24)	8(17.26)^{b}	6(12.80)	8(22.22)^{a}

[a, b] Values with different superscripts are significantly different (P<0.05).

Table 3. Effects of increased α-tubulin acetylation on the mitochondrial distribution

Groups	Oocytes	PN syngamy focus(%)	PN syngamy diffusion (%)	Others(%)
Control	126	28(25.59)[b]	83(65.68)[a]	15(8.73)
Treat	123	51(47.02)[a]	58(47.01)[b]	14(5.97)

[a, b] Values with different superscripts are significantly different (P<0.05).

Table 4. Effects of increased α-tubulin acetylation on the IVF process

Groups	Oocytes	Syngamy(%)	Unfertilized(%)	Polysperm(%)	Others(%)
Control	227	15(6.15)[b]	55(25.34)	120(48.95)	37(19.56)
Treat	314	52(16.04)[a]	67(21.32)	157(48.73)	38(13.91)

[a, b] Values with different superscripts are significantly different (P<0.05).

Table 5. Effects of increased α-tubulin acetylation on the maturation and IVF embryos

Groups	Oocytes	MII (%)	2-cell (%)	Blastocyst (%)	Blastocysts cells
Control	770	503(64.06±2.87)[b]	262(59.96±4.54)	43(9.80±1.93)[b]	48.65±16.56
Treat	771	563(72.90±5.13)[a]	284(59.75±3.40)	63(13.28±1.79)[a]	49.65±18.66

[a, b] Values with different superscripts are significantly different (P<0.05).

Figures

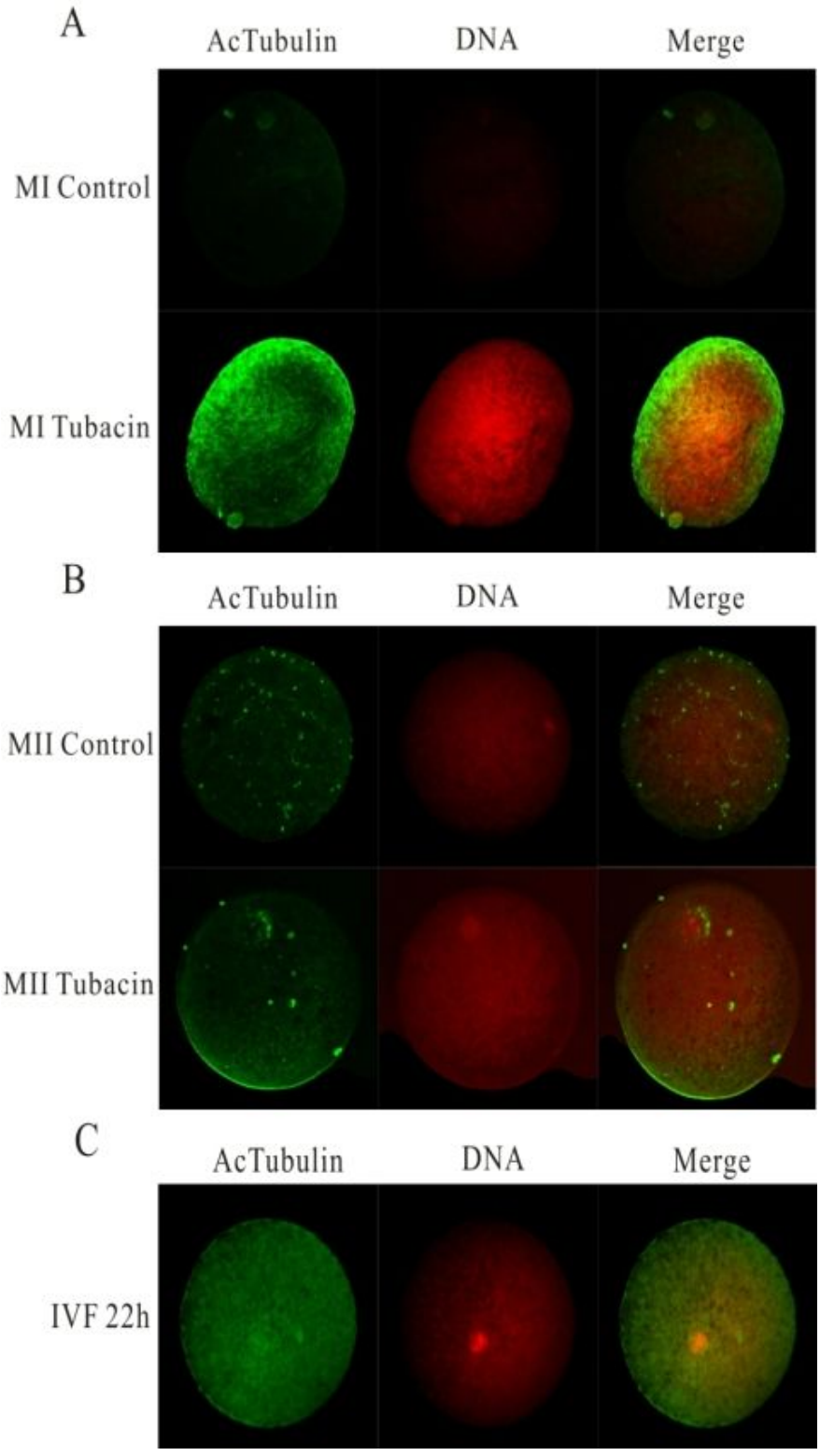

Figure 1

Comparison of ac-α-Tubulin status of porcine oocytes and embryos during IVM and IVF. Original magnification × 200. Note: A: MI stage; B: MII stage; C: Syngamy; Green: Ac-α-Tubulin; Red: DNA

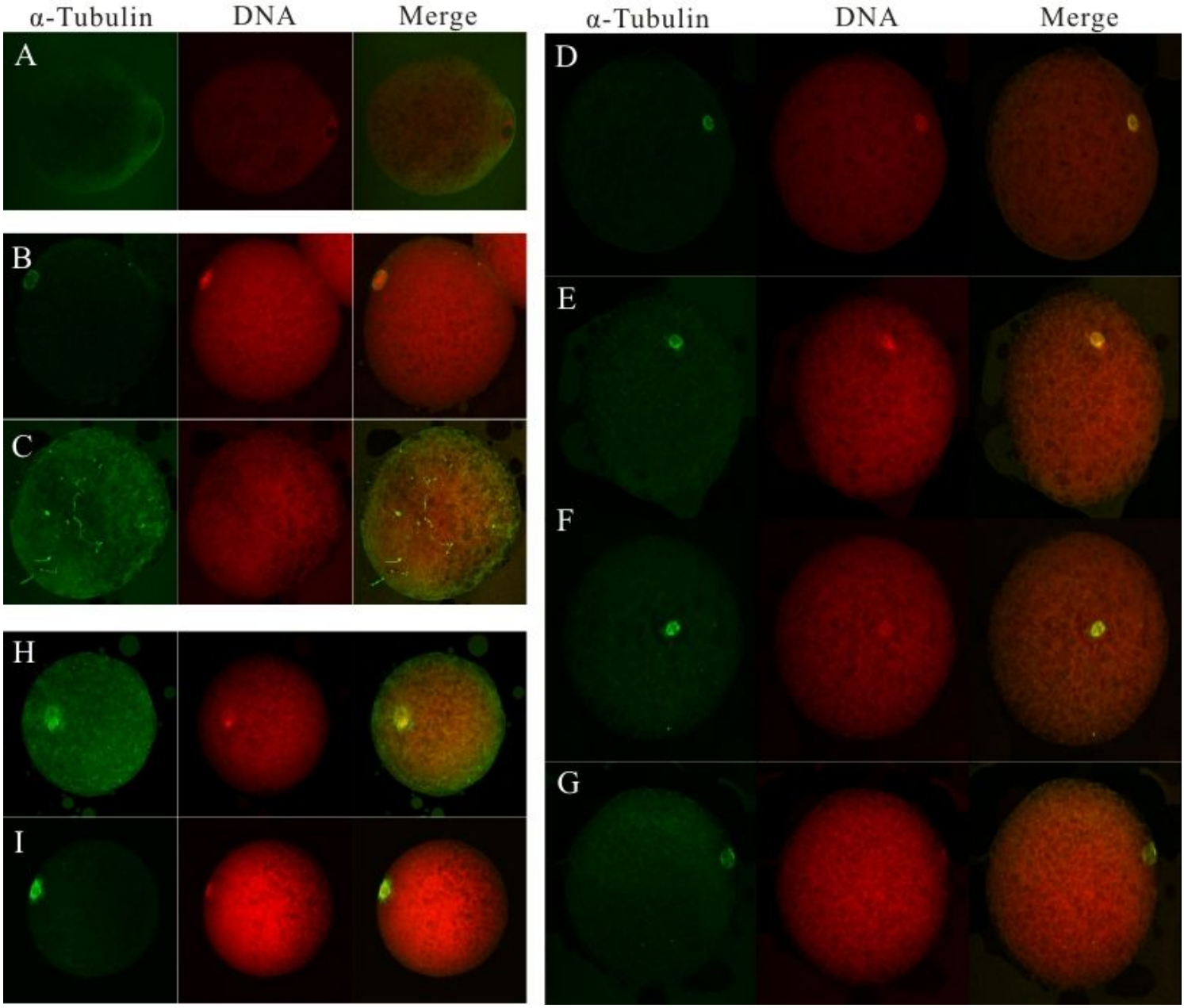

Figure 2

Comparison of α-tubulin status of porcine oocytes and embryos during IVM and IVF. Original magnification × 200. Note: A: Dispersive distribution of GV oocytes; B: Normal status of MI oocytes; C: Abnormal status of MI oocytes; D, E: Normal status of MII oocytes; F, G: Abnormal status of MII oocytes; H: Syngamy; I: Unfertilized MII embryo; Green: α-tubulin; Red: DNA.

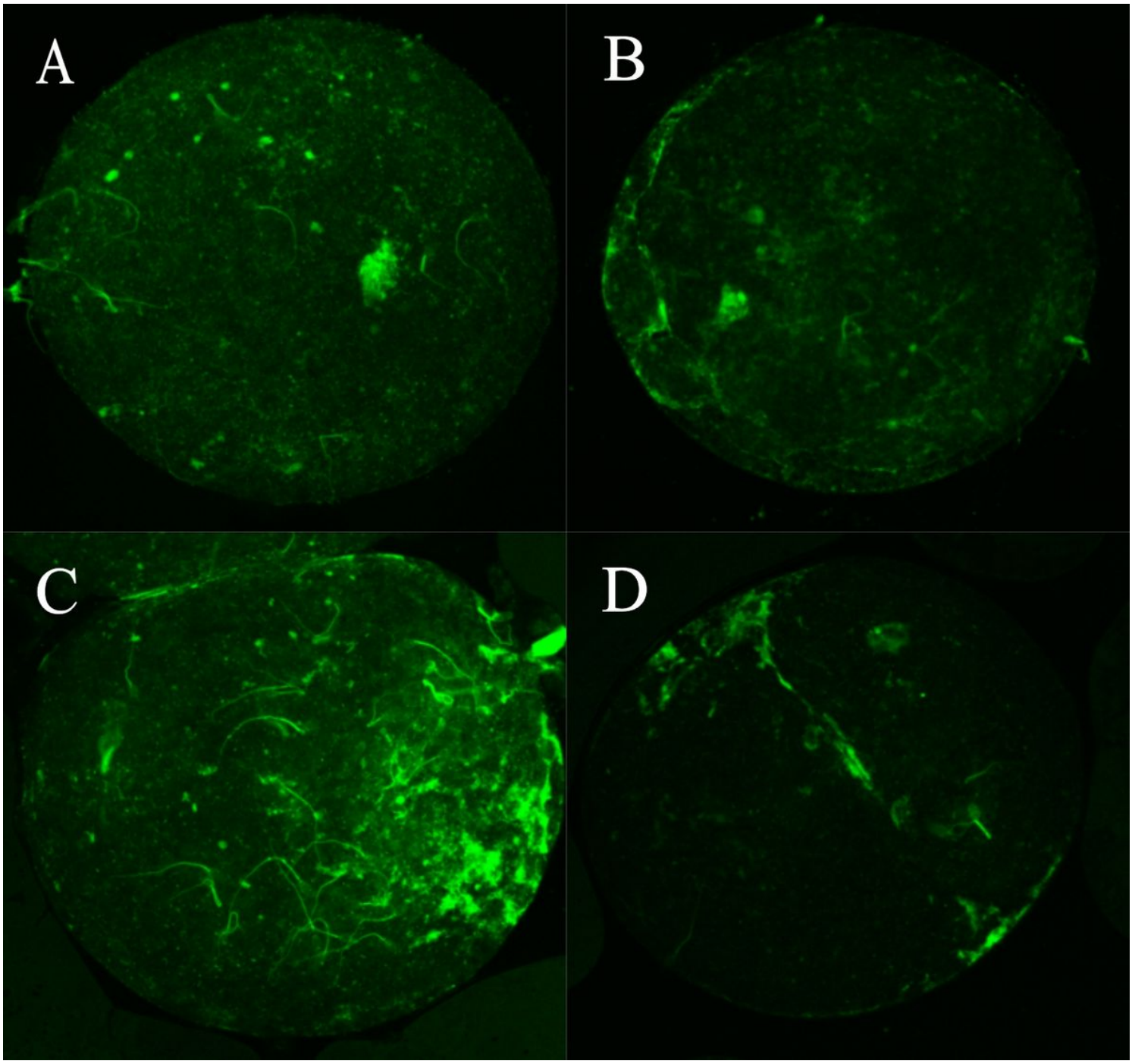

Figure 3

The mitochondrial distribution in porcine oocytes and embryos. Original magnification × 200. Note: A: Unfertilized MII oocytes; B: Pronuclear stage; C: Syngamy; D: Two-cells stages; Green: mitochondria.

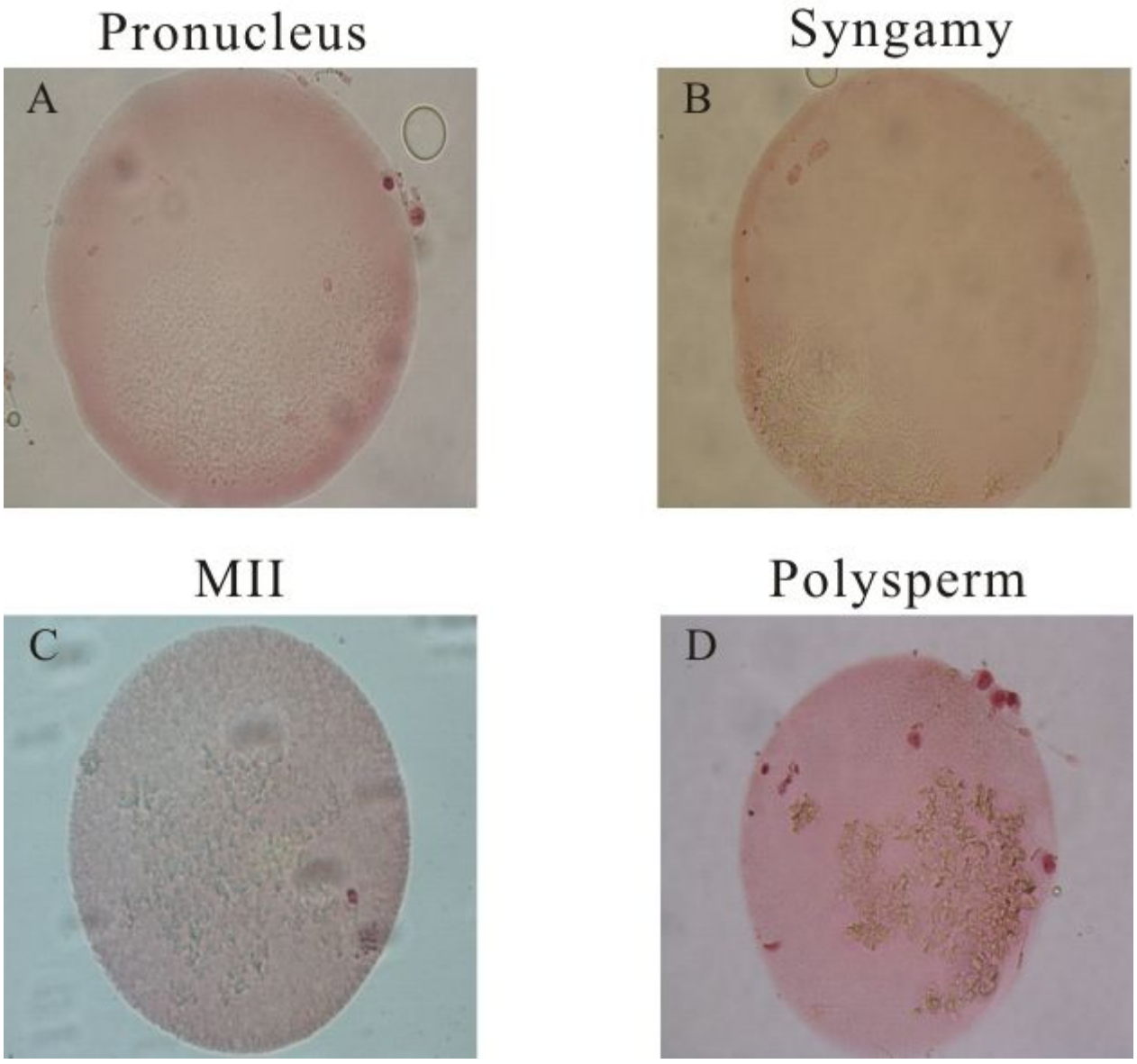

Figure 4

Different nuclear stages of porcine embryos after in vitro fertilization. Original magnification × 200. Note: A: Pronucleus; B: Syngamy; C: Unfertilized; D: Polysperm

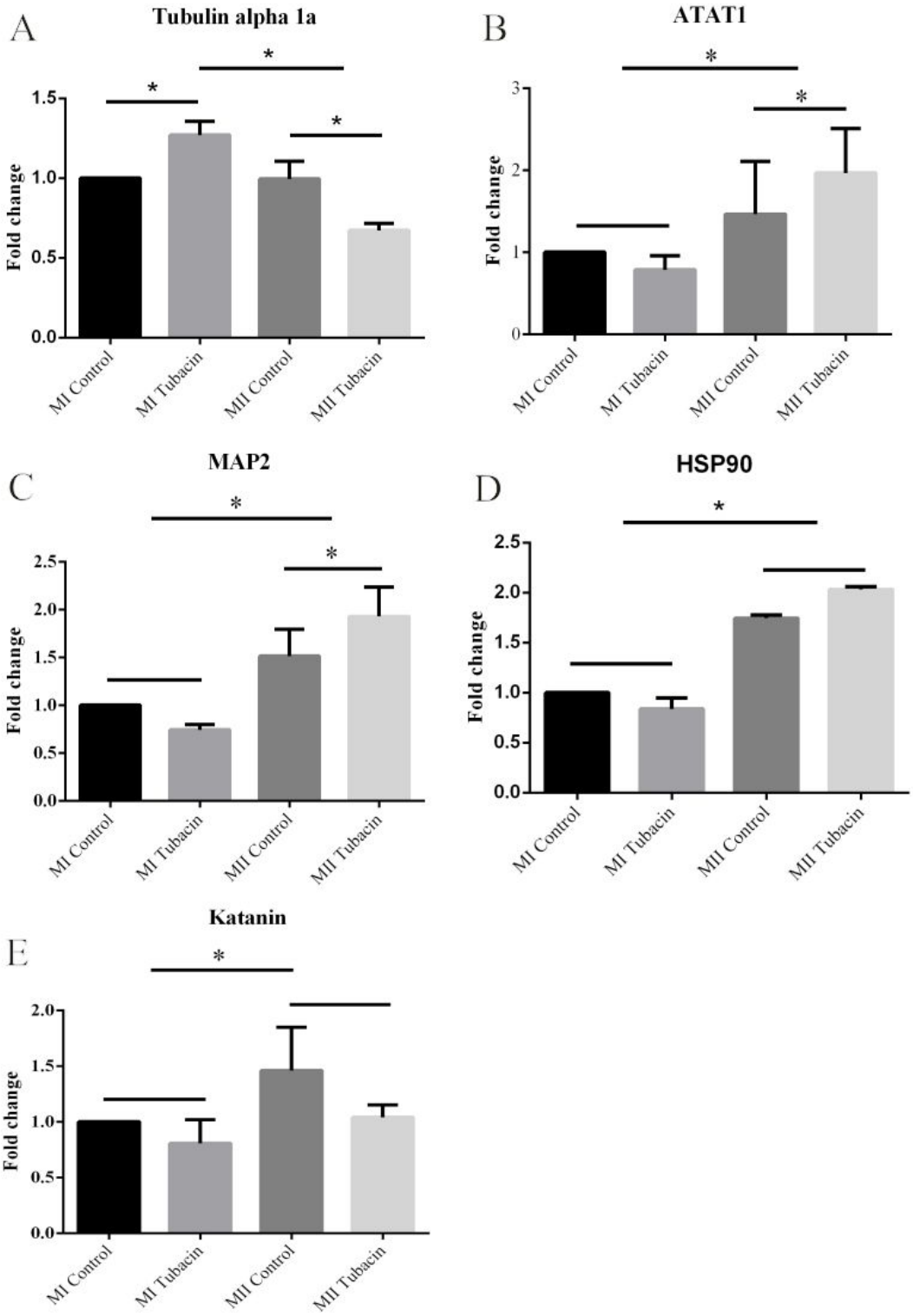

Figure 5

Comparison of relative expression levels of microtubule assembly genes. Note: * Asterisk was significantly different (P<0.05).