Polo-like kinase-1 regulates first cleavage of one-cell embryos in culture during assisted reproduction

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ABSTRACT

الأهداف: التحقق من دور إنزيم بولو كيناز (PLK1) في الانقسام الأول للخلية الملقحة في المزرعة.

الطريقة: أجريت التجربة في مركز الإنجاب الطبي – مستشفى تونجي – وهان – الصين خلال الفترة من 1 يونيو 2009م حتى 20 نوفمبر 2009م. في البداية، اكتشفنا ظهور إنزيم بولو كيناز خلال الانقسام الأول للخلية الملقحة باستخدام لطخة ويسترن، و قمنا بالتقليل من ظهور إنزيم بولو كيناز، و التقليل من ظهور إنزيم بولو كيناز خلال انقسام الخلية الملقحة عن طريق إضافة الحمض النووي الريبوزي (RNA). (شاملة المجموعات الأربع إنزيم بولو كيناز، و إضافة قليلة للحمض الريبوزي، و السيطرة على siRNA، و الالتهاب الكاذب، وإزالة الغشاء الشفاف (ZP). وفي النهاية، قمنا بتقييم و مقارنة معدلات الانقسام الأول للمجموعات الأربع.

النتائج: بلغت ذروة ظهور إنزيم بولو كيناز في الطور الأول لانقسام الخلية الملقحة (3 عينات/ لكل مجموعة، 100 خلية ملقحة/ لكل عينة). قلت الكمية النسبية لإنزيم البولو كيناز في الخلية الملقحة للفأر بعد دخول إنزيم بولو كيناز. كان معدل الإنقسام الأول لمجموعة إنزيم بولو كيناز أقل من المجموعات الأخرى (التحكم في siRNA، و الالتهاب الكاذب، و إزالة الغشاء الشفاف ZP p=0.000.

خاتمة: يلعب دور إنزيم بولو كيناز دور مهم خلال الانقسام الأول للخلية، كما أن الخلية الملقحة لن تتمكن من الانقسام بنجاح بدون دور إنزيم بولو كيناز الوظيفي.

Objectives: To investigate the role of polo-like kinase -1 (PLK1) in the first cleavage of a zygote in culture.

Methods: This experiment took place in the Reproductive Medicine Center of Tongji Hospital, Wuhan, China, from 1st June 2009 to 20th November 2009. First, we detected the expression of PLK1 during the first zygotic division by using Western blotting, and then we reduced the expression of PLK1 during the first zygotic division by ribonucleic acid (RNA) interference (including 4 groups: PLK1 small interfering RNA (siRNA), siRNA control, mock transfection, and only zona pellucida (ZP) removal), finally we evaluated and compared the first cleavage rates of the 4 groups.

Results: The expression of PLK1 peaked in the first M phase of zygotic cleavage (3 samples/group, 100 zygotes/sample). The relative amount of PLK1 of the mouse zygotes was reduced significantly after siRNA transfection. The first cleavage rate of the PLK1 siRNA group was significantly less than that of other groups (siRNA control, mock transfection, and only ZP removal, p=0.000).

Conclusion: The PLK1 plays a crucial role during the first cleavage of one-cell embryos, and the zygotes are unable to divide successfully without functional PLK1.

Saudi Med J 2010; Vol. 31 (3): 247-252

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Received 23rd November 2009. Accepted 16th February 2010.

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During the procedure of *in-vitro* fertilization/ intracytoplasmic sperm injection (IVF/ICSI), some zygotes are unable to achieve successful cleavage. In fact in some patients, the majority of their zygotes are unable to complete their first cell cleavage. The causes and underlying mechanisms are largely unknown. Therefore, it is of utmost interest and importance to determine the major regulatory factors affecting the first cell cleavage of the zygotes. The male and female pronuclei of the one-

cell zygote fuse during syngamy, duplicate their DNA, and then undergo cytokinesis, with many regulatory factors controlling these processes. Polo-like kinases (PLKs) are a family of distinct serine/threonine protein kinases that play important roles in the multiple steps of mitotic and meiotic progression, such as entry into, and exit from the mitotic (M) phase, spindle organization, and cytokinesis.[1,2] Humans and other mammals possess 4 distinct, but closely related PLKs, which share small conserved domains named polo-box that are required for protein localization. Of these isoforms, only PLK1, essential for mitosis, is studied most extensively, on the other hand, PLK2, PLK3, and PLK4 are more likely to be involved only in interphase.[2] The PLK1 play multiple roles during mitosis; it participates in the G2/M transition, if inhibited, this protein will delay the cell's entry into mitosis. Among the PLK1 substrates involved in the G2/M transition are CDC25, Myt1, and cyclin B1.[3-5] The PLK1 is also involved in the feedback loop that controls the activation of Cdk1/cyclin B.[6,7] During centrosome maturation, PLK1 activity is required to recruit proteins necessary to nucleate the microtubules that will participate in a bipolar spindle assembly. It also interacts with and phosphorylates many proteins involved in microtubule dynamics.[8,9] In addition to being localized and active at the centrosome level, PLK1 also localizes to the chromosome kinetochores,[10] where its activity results in part, in the localization of spindle checkpoint proteins. The PLK1 is also required to activate the E3 ubiquitin ligase anaphase-promoting complex/cyclosome (APC/C) needed to trigger mitotic protein degradation.[11] Although PLK1 directly phosphorylates APC/C subunits, the effect of this phosphorylation on APC/C activity is minor,[12] as compared to the phosphorylation by Cdk1/cyclin B1 on such activity.[13] However, PLK1 contributes indirectly to APC/C activation by phosphorylating the APC/C-cdc20 inhibitor early mitotic inhibitor 1 (Emi1) in somatic cells. The phosphorylation of Emi1 by PLK1 triggers Emi1 degradation and APC/C-cdc20 activation.[14,15] This contributes to the metaphase-anaphase transition, which is controlled by APC/C-cdc20, and the M/G1 transition, which is controlled by APC/C-cdh1. Fertilization is a multi-step process involving penetration of the oocyte by sperm, activation of the oocyte, reorganization of the male chromatin, formation of the pronuclei, and establishment of the one-cell embryo ready to start mitotic divisions. The first mitotic cell cycle of one-cell embryos differs from somatic cells in several ways. In the mouse embryos, the duration of the first mitosis (120 minutes) is almost twice as long as the second (70 minutes). This increase seems to be due to a transient metaphase arrest.[16] The duration of the early mitotic divisions tends to shorten gradually to a certain extent as development proceeds. The zygotic genome activation is initiated at the end of the first division, and takes place during the G2 phase of the second division in the embryo.[17] Thus, the first zygotic cleavage may be controlled only by maternal messenger ribonucleic acids (mRNAs), and proteins accumulated during oogenesis. Despite the numerous studies on PLK1 in somatic cells, few reports concerning its functions in mammalian one-cell embryo cleavage are available. Considering PLK1's multiple roles in the eukaryotic cell cycle regulation and the unique characteristics of zygotic cleavage, we hypothesized that PLK1 has a key role in the first cleavage of one-cell embryos. Thus, in the present study, we investigated the expression of PLK1 during the first zygotic division, the correlation between PLK1 expression and the process of the first zygotic division, and the effects of PLK1 on the one-cell embryo cleavage during culture *in-vitro*. Mouse one-cell embryos were used in this study. It is hoped that the results would provide important information for a better understanding of the process of zygotic cleavage, and for improving the treatment of patients whose major zygotes are unable to complete their first cell cleavage.

Methods. This study was conducted in the Reproductive Medicine Center of Tongji Hospital, Wuhan, China, from 1st June 2009 to 20th November 2009. Ethical approval for this study was obtained from the Ethics Committee of Tongji Hospital. All of our experiments meet the ethical requirements.

Animals and treatment. Adult (Kunming white) mice weighing 20-25 g were purchased from Hubei Laboratory Animal Research Center in Wuhan, China. They were housed in a controlled environment with a 14-h light/10-h dark cycle, and were bred in the laboratory animal care facility of Tongji Hospital. All animal care, use, and procedures were approved by the laboratory animal care facility of Tongji Hospital and conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Mouse embryo collection. The mice were superovulated by intraperitoneal injection of 10 IU of pregnant mare serum gonadotropin (PMSG [Hangzhou Animal Medicine Factory, Hangzhou, China]), and 48 hours later, with 10 IU of human choriogonadotropin (hCG [Livson Pharmaceutical Group Inc., Zhuhai, China]). On the same day of hCG injection, the female mice were mated with fertile males of the same strain to induce pregnancy. Pregnancy was identified by the presence of a vaginal plug. Pregnant mice were killed by cervical dislocation at 16, 24, 28, 32, and 36 hours post-hCG injection, and *in-vivo* fertilized zygotes were

collected from the oviduct ampullae. The cumulus cell masses surrounding the zygotes were removed by brief exposure to 300 IU/ml of hyaluronidase in M2 medium (Sigma-Aldrich Corp., St. Louis, MO, USA). At least 3 samples (100 cumulus cell free zygotes/sample) were collected at each stage. The samples were frozen at -70 °C until use.

Western blot analysis. The zygotes were washed in ice-cold phosphate buffered saline (PBS) by centrifugation, and subsequently lysed in lysis buffer (RIPA, ProMab Biotechnologies, CA, USA). Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE [Sigma-Aldrich Corp., St. Louis, MO, USA]), and transferred into a nitrocellulose membrane (Pierce Biotechnology, Rockford, Illinois, USA). The membranes were blocked in 5% non-fat milk for 2 hours at 37°C. After washing 3 times with phosphate-buffered saline/tween (PBST), 10 minutes each, the membranes were incubated with anti-PLK1 (Abcam, Cambridge, UK) or anti-GAPDH (ProMab Biotechnologies, Richmond, CA, USA) antibody for 2 hours at 37°C. After washing thrice with PBST for 10 minutes each, the membranes were incubated with goat anti-rabbit immunoglobulin G (IgG)-horseradish peroxidase (HRP) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or goat anti-mouse IgGAM-HRP antibody (Invitrogen, California, USA) for one hour at 37°C. The membranes were then washed 3 times with PBST, 10 minutes each, and the protein bands of interest were analyzed by Gel-Pro Analyzer 4.0 (Media Cybernetics, Inc., Bethesda, MD, USA).

Transfection of mouse zygotes. The cumulus-cell-free-zygotes were exposed to an acidified medium (Sage, Cooper Surgical Co., Trumbull, CT, USA) briefly to remove the zona pellucida (ZP) surrounding the zygotes. The ZP-free zygotes were washed with M2 medium 3 times, 10 minutes each. The zygotes were then maintained in M16 medium at 37°C in a humidified atmosphere of 5% CO_2 until use. After that, small interfering ribonucleic acid (siRNA) was transfected into the zygotes (PLK1 siRNA: sense 5'-CAG GCA AGA UCG UGC CUA ATT-3', antisense 5'-UUA GGC ACG AUC UUG CCU GCG-3'; control siRNA (negative control): sense 5'-UUC UCC GAA CGU GUC ACG UTT-3', antisense 5'-ACG UGA CAC GUU CGG AGA ATT-3'). For each transfection sample, oligomer-Lipofectamine™ 2000 complexes were prepared according to the instruction of the reagent. The oligomer-Lipofectamine™ 2000 complexes were added to each well containing cells and medium. While gently shaking, the plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 for 9 hours. The zygotes were collected and washed in M2 medium 3 times, 10 minutes each, and the effects of the siRNA transfection were analyzed by Western blot analysis. There are 4 groups in this experiment: 1) PLK1 siRNA - the zygotes transfected with PLK1 siRNA, 2) siRNA control - the zygotes transfected with control siRNA, 3) mock transfection without siRNA – the zygotes transfected without siRNA and 4) only ZP removal (no transfection) - the zygotes without ZP that were not treated by RNA interfere.

Mouse embryo monitoring. After transfection, the zygotes were cultured overnight in M16 medium at 37°C in a humidified atmosphere of 5% CO_2. The number of one-cell embryos, 2-cell embryos, and the cleavage rate were then evaluated. Zygotes showing degenerative signs were not included in the final analysis.

We used one-way analysis of variance (ANOVA) and independent-samples t test for statistical analysis. P value less than 0.05 was considered statistically significant.

Results. ***Expression of PLK1 during the first cleavage of zygotes.*** Western blot analysis showed that the PLK1 expression increased gradually during the first cleavage of zygotes (**Figures 1a & b**). There was a low level of PLK1 protein expression at the sixteenth-hour post-hCG injection, afterwards, the expression of PLK1 increased gradually. From 32-36 hours post-hCG injection, there was a high level of PLK1 protein expression in the zygotes, which was significantly higher than that of the other groups (16, 24, and 28 hours, p=0.000).

The influence of removing ZP on the first cleavage rate. The first cleavage rates between the zygotes with and without ZP were evaluated to determine whether removing the ZP had an influence on the cleavage rate. Zygotes showing degenerative signs were not included. The average cleavage rate was 64.1 ± 1.6% for the group without ZP, and 63.1 ± 1.5% for the group with ZP (**Table 1**). The cleavage rates of the 2 groups were similar. These results indicate that removing ZP had no influence on the first cleavage rate.

Effects of PLK1 knockdown on the first cleavage rate of zygotes. To analyze whether PLK1 is important to the first cleavage of zygotes, PLK1 expression was knocked down in the zygotes by using PLK1 siRNA. After PLK1 siRNA transfection, the expression level of PLK1 in zygotes at the 34 hour post-hCG injection was significantly less than that of the siRNA-control, mock transfection, and only ZP removal groups. There was no significant difference between the mock transfection group and the only ZP removal group. Thus, the transfection reagents had no influence on the zygotic cleavage, and PLK1 siRNA significantly reduced the expression of PLK1 in zygotes (**Figures 2a & b**). After transfection, the zygotes were cultured in M16 medium at 37°C in a humidified atmosphere of 5% CO_2. The

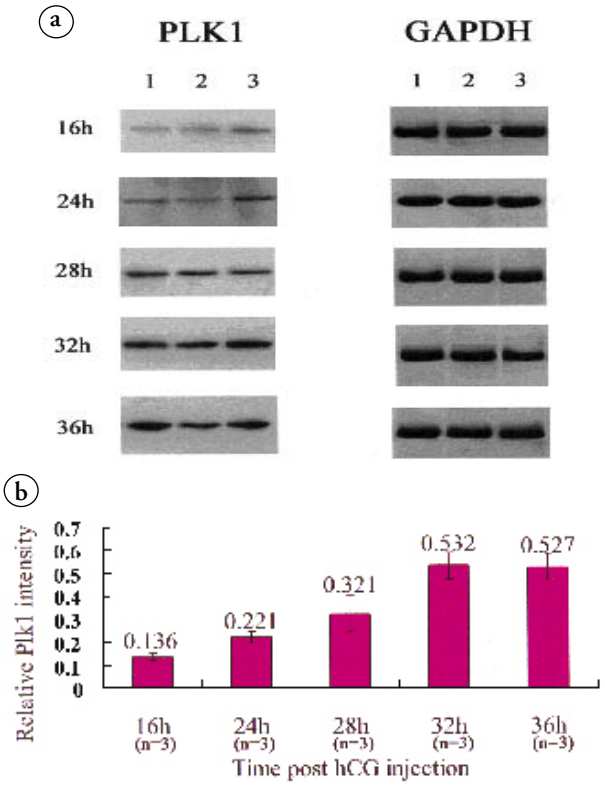

Figure 1 - The polo-like kinase one (PLK1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression during the first cleavage of zygotes. **a**) Western blot results at various times (16-36 hours post-hCG injection); 1, 2, and 3 were the 3 samples in each group. The GAPDH was used to normalize the amount of PLK1 expression. **b**) Relative PLK1 expression was determined by densitometry. The relative PLK1 intensity was increased during the first cleavage of zygotes. The values are mean standard deviations (n=3) for various times. The comparison was made by one-way analysis of variance: 32 versus 16 hrs, 32 versus 24 hrs, 32 versus 28 hrs, p=0.000; 32 versus 36 hrs, p=0.938.

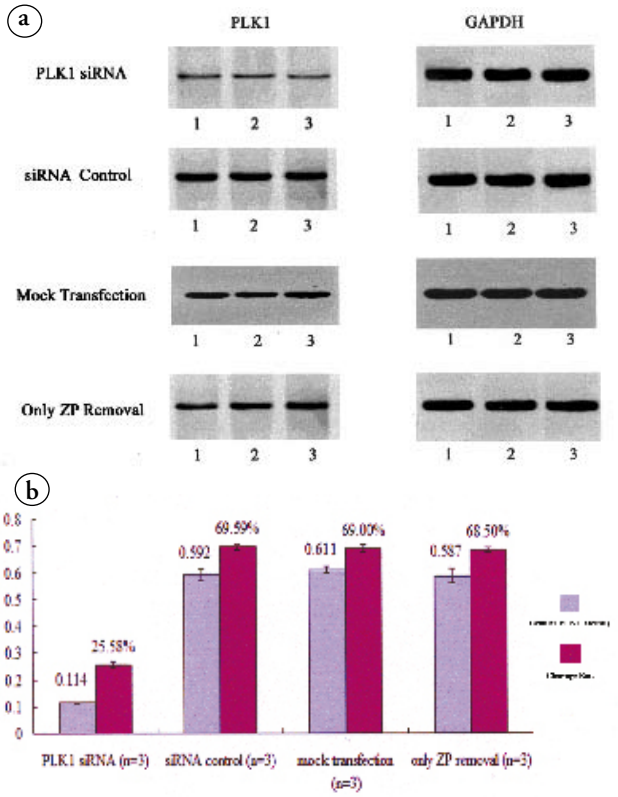

Figure 2 - Relative polo-like kinase one (PLK1) intensity and first cleavage rates after treatments. **a**) Western blot results. 1, 2, and 3 were the 3 samples from each group. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize the amount of PLK1. **b**) Relative PLK1 intensity (blue bars) and cleavage rates (red bars) decreased significantly after PLK1 siRNA treatment. The values were mean standard deviation (n=3). The comparison was made by one-way analysis of variance: PLK1 siRNA versus siRNA control, PLK1 siRNA versus mock transfection, and PLK1 siRNA versus only ZP removal, p=0.000; siRNA control versus only ZP removal, mock transfection versus only ZP removal, p>0.05.

next morning, the number of one-cell embryos, 2-cell embryos, and the cleavage rate were evaluated (**Table 2, Figure 2b**). Zygotes showing degenerative signs were not included. The first cleavage rate of the PLK1 siRNA group (24.4%) was significantly less than that of the other groups (p=0.000). And there was no significant difference between the mock transfection and the only ZP removal groups (p=0.599) (**Table 2, Figure 2b**).

Discussion. The PLK1 is a key regulator of progression through mitosis and cell division in the eukaryotic cells. Previous studies revealed that the kinase plays multiple roles during mitosis, including entry into and exit from the M phase, centrosome maturation, bipolar spindle assembly, activation of the APC/C, and cytokinesis.[18] Recently, the dynamics of localization and enzyme activity of PLK1 were examined during the mouse oocyte maturation. At the germinal vesicle stage, PLK1 is diffusely distributed into the cytoplasm, enriched in the nucleus, and concentrated to the spindle poles during prometaphase. It is then relocated to the equatorial plate at anaphase and associated with the midbody at telophase.[19] The PLK1 is concentrated between the male and female pronuclei after fertilization. The amount of PLK1 protein remains stable during meiotic maturation; it is suggested to be a pivotal regulator of microtubule organization during mouse oocyte meiosis and fertilization.[20] The PLK1 has similar characteristic in rat oocytes.[21] In human oocytes, PLK1 is localized in the spindle midzone at anaphase 1 and telophase 1.[22] Although PLK1 plays multiple roles in the eukaryotic cell cycle and meiosis regulation, the role of PLK1 in the first cleavage of one-cell embryo remains unclear.

Due to ethical issues, fresh human fertilized one-cell embryos cannot be used for detailed studies. Accordingly,

Table 1 - The influence of removing ZP on the first cleavage rate.

Replicates	Without ZP[a] n (%)	With ZP[b] n (%)
Replicate 1	98 (65.3)	107 (62.6)
Replicate 2	114 (62.3)	102 (64.7)
Replicate 3	105 (64.8)	118 (61.9)
Mean ± SD	64.1 ± 1.6%	63.1 ±1.5%

The comparison carried out by independent-sample t test: a versus b; p=0.451, ZP - zona pellucida, SD - standard deviation

Table 2 - First cleavage rate of each group.

Replicates	PLK1 siRNA[a]	siRNA control[b]	Mock transfection[c]	Only ZP removal[d]
	n (%)			
Replicate 1	496 (24.4)	308 (70.8)	317 (69.4)	310 (67.7)
Replicate 2	320 (25.6)	313 (69.3)	324 (70.2)	311 (69.1)
Replicate 3	329 (26.7)	332 (68.7)	341 (67.5)	338 (68.6)
Mean ± SD	25.6 ± 1.2%	69.5 ±1.1%	69.0 ± 1.4%	68.5 ± 0.7%

The comparison was made by one-way analysis of variance: a versus b, a versus c, a versus d, p=0.000; b versus c, p>0.05; b versus d, p>0.05; c versus d, p>0.05. 1, 2, and 3 were the 3 replicates of an experiment examining cleavage rate after treatment. PLK1 siRNA - polo-like kinase - small interfering ribonucleic acid, ZP - zona pellucida

we used mouse one-cell embryos in this study. In mice, fertilization takes place at the twelfth hour post-hCG injection and the M phase lasts from 32-35 hours post-hCG injection.[17] In this study, the time PLK1 reached its highest level was exactly coincident with the first M phase of zygotic cleavage, suggesting that the expression of PLK1 reaches its peak in the first M phase of zygotic cleavage. This indicates that PLK1 plays an important role in the process of the first mitosis of the one-cell embryos.

Tong et al[20] reported that the amount of PLK1 protein decreases gradually after fertilization in mouse one-cell embryos. Fan et al[21] reported similar results in rats. In their studies, the quantity of PLK1 protein was detected from 2-8 hrs after fertilization, not the entire process of the first mitosis of the zygotes. In the present study, we analyzed the PLK1 levels from 16-36 hrs post-hCG injection, which covers nearly the entire process of the first mitosis of one-cell embryos. To improve the efficiency of the siRNA transfection, we removed the ZP of the zygotes. The ZP is composed of a small number of glycoproteins,[23] in addition to mediating the interaction with sperm, it is also involved in the prevention of polyspermy and the protection of the developing embryo prior to implantation.[24-26] A functional ZP is critical for both fertilization and early stages of embryo development. In this study, we compared the cleavage rates between the zygotes with and without ZP, and found that removing the ZP did not influence the first cleavage rate of zygotes.

The role of PLK1 in the first cleavage rate of zygotes was further demonstrated in this study by using PLK1 siRNA. In this part, we demonstrated that PLK1 siRNA can significantly reduce the expression of PLK1 in zygotes. Our data suggest that zygotes without PLK1 are not able to divide successfully, and PLK1's deficiency can cause one-cell embryo cleavage arrest.

In this study, we used mouse one-cell embryos instead of human zygotes. If we investigate the role of PLK1 in human zygotes in the future, it will be more helpful to solve clinical problems. The first cleavage of a fertilized egg is an important process in mammalian embryology, and its arrest is one of the major problems of human assisted reproductive technology centers. Our results may provide a new avenue of research to overcome this problem.

Acknowledgment. *The authors gratefully acknowledge all the staff at the Human Reproductive Medicine Centre of Tongji Hospital, Wuhan, China for their excellent technical assistance.*

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