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Original article

Chemically enucleated mouse oocytes: ultrastructure and kinetics of histone H1 kinase activity

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Abstract — The objective of the study was to characterize the ultrastructure changes and biochemical mechanisms underlying the expulsion of the entire chromosome complement in chemically enucleated mouse oocytes. The ultrastructural studies demonstrated that the morphology of cytoplasts produced by etoposide-cycloheximide treatment were indistinguishable from intact metaphase I and II oocytes. Moreover, polar bodies formed by chemical enucleation were in almost all cases completely separated from the parent cytoplast and differed from normal polar bodies only in their chromatin content morphology and because they contained a slightly higher number of cytoplasmic organelles. The mode of polar body formation, however, in normal and chemically enucleated oocytes differs substantially: spindle involvement is important for normal polar body extrusion but plays no part in the protracted expulsion of chromosomes during chemical enucleation. After etoposide-cycloheximide treatment, histone H1 kinase activity remains high for the ensuing 6–8 h before declining gradually to basal levels 14 h after treatment. The expulsion of the polar body occurred only after the slowly declining H1 kinase activity reached basal levels. The activity of this kinase rose sharply to reach maximal levels within 4 h when the enucleated oocytes were removed from the inhibitor-supplemented medium and placed in normal medium. The findings in this paper indicate that cytoplasts produced by chemical enucleation are morphologically normal, thus suggesting that these enucleated cells are suitable for cloning studies. Although effective in mouse oocytes, we postulate that certain modifications to the enucleation technology are necessary before a reliable non-invasive protocol for ungulate oocytes will be available. © Inra/Elsevier, Paris

oocyte / enucleation / ultrastructure / histone H1 kinase

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Résumé — Énucléation chimique des ovocytes de souris : ultrastructure et cinétique de l'activité histone H1 kinase. L'objectif de l'étude est de caractériser les changements ultrastructuraux et les mécanismes biochimiques qui sous-tendent l'expulsion de l'appareil chromosomique dans des ovocytes chimiquement énuclés. La morphologie des cytoplastes produits par le traitement etoposide-cycloheximide est indistinguable d'ovocytes intacts en métaphase I ou II. Les globules polaires (GP) formés par énucléation chimique sont dans presque tous les cas complètement séparés de l'ovocyte et diffèrent de GP normaux seulement par la morphologie de la chromatine et le nombre faiblement supérieur d'organites. Cependant, la formation du GP dans les ovocytes normaux et énucléés chimiquement diffère : le fuseau ne joue aucun rôle dans l'énucléation chimique, plus étalée dans le temps. Contrairement aux témoins, les ovocytes traités conservent une activité H1 kinase forte pendant 6–8 h suivie d'une décroissance graduelle jusqu'à un niveau basal à 14 h, après laquelle se produit seulement l'émission du GP. L'effet du traitement sur l'activité kinase H1 est annulé 4 h après retour dans le milieu normal. Les cytoplastes produits par énucléation chimique ont donc une morphologie normale et semblent utilisables pour des expériences de clonage. Des modifications de la technique seraient probablement nécessaires pour l'adapter aux bovins. © Inra/Elsevier, Paris

ovocyte / énucléation / ultrastructure / histone H1 kinase

1. INTRODUCTION

Considerable success in mammalian embryo cloning and in cloning by somatic cell nuclear transfer has recently been achieved [4, 5, 16, 21, 25–27]. While embryo cloning gives very good results [18], the overall efficiency is extremely poor when somatic cells are used as karyoplasts. Thus, it is evident that for further improvement in nuclear transplantation technology additional research is necessary on nucleo-cytoplasmic interactions, the mechanisms of egg activation and embryonic genome activation and imprinting [13]. Moreover, the solution to some technical aspects of cloning may significantly simplify and improve this procedure. One of the most potentially damaging steps in nuclear transplantation is the removal of the chromosome complement for the production of cytoplasts [3]. Typically, cytoplasts are produced by the aspiration of the polar body (PB) and the adjacent cytoplasm containing the second metaphase plate. This step is complicated when aged oocytes with disintegrated first PBs are used. Moreover, the inevitable decrease of the cytoplasmic volume and the removal of important nuclear-associated proteins may also occur as a result of enucleation. Furthermore, recent results indicate that mammalian oocytes are polarized in a manner similar to that in lower vertebrates [2]. For all these reasons various alternative approaches to enucleation have been developed in an attempt to produce optimal cytoplasts. Oocyte centrifugation with subsequent enucleation has been advocated for the production of cytoplasts convenient for cloning. This procedure results, however, in a significant cytoplasmic volume decrease [23]. An alternative approach has been suggested by Bordignon and Smith [3]. Metaphase II (MII) oocytes were activated and at the time of the second PB extrusion the oocytes were enucleated so that only the anaphase-telophase II (AI, TI) spindles with the associated chromosomes were removed, leaving the original oocyte cytoplasmic volume almost unchanged. Another

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Abbreviations: GV: germinal vesicles; GVBD: germinal vesicle breakdown; MI (AI, TI): metaphase I (anaphase, telophase); MII: metaphase II; PB: polar body; ETO: etoposide; CHXM: cycloheximide; MPF: maturation promoting factor; CCA: chromosone condensation activity.

promising procedure is that of non-invasive chemical enucleation [8]. Unfortunately, this approach, while suitable for murine oocytes, has not proved reliable in oocytes of ungulates. Moreover, the phenomenon of non-invasive enucleation is interesting from a more fundamental cell cycle point of view. First, we shall show that chemical enucleation induces delayed PB expulsion with the whole oocyte chromosome complement being involved, second, the polar body is extruded in the absence of spindle involvement and third, the technology is only fully effective in some species [9]. In mice, however, the cytoplasts obtained by a non-invasive approach are very suitable for cloning studies, i.e. the remodelling of transplanted nuclei [11]. Clearly, the successful application of the non-invasive approach for the enucleation of ungulate oocytes would greatly facilitate the production of clones and also help us to expand the theoretical research in this field. As mentioned earlier, even in the mouse some aspects of chemical enucleation are still unclear. In the present study, ultrastructural studies have been carried out to compare etoposide-cycloheximide-induced chromosome expulsion with natural expulsion of homologs at metaphase I (MI) and sister chromatids at MII. Biochemical studies were also undertaken to correlate changes in histone H1 kinase activity with chromatin expulsion.

2. MATERIALS AND METHODS

2.1. Oocyte culture

The oocytes were obtained from large antral follicles of PMSG (5 IU)-stimulated ICR mice injected 44–48 h previously. Their cumuli were removed by pipetting and only those oocytes with germinal vesicles (GV) were cultured in M199 containing Na-pyruvate (0.22 mM), gentamicin (25 μg·mL^{-1}) and BSA (4 mg·mL^{-1}) in a 5 % CO_2 atmosphere in air at 37 °C. Under our culture conditions germinal vesicle breakdown (GVBD) occurred within 60 min and those oocytes still containing GV were discarded; metaphase to anaphase I transition was detected between 8–9 h and after 10 h in culture, MII were typically detected.

2.2. Oocyte enucleation

The oocytes were enucleated exactly as described by Fulka Jr and Moor [8]. After 6 h in culture, the oocytes were transferred into M199 supplemented with etoposide (ETO; 50 μg·mL^{-1}) and cultured for another 2 h before cycloheximide was added (CHXM; 50 μg·mL^{-1}). Oocytes in this medium were cultured for up to 12–14 h before fixation for electron microscopy or retransfer to normal medium.

2.3. The induction of metaphase-anaphase I (II) exit

The MI oocytes (8 h in culture) were incubated in medium with CHXM and cultured in it for up to 6 h [6]. The exit from MII was induced by electric pulses (1 kV, 100 μs).

2.4. Electron microscopy

Enucleated oocytes collected after 3 h in culture in normal medium (n = 20), oocytes extruding the first PB 3 and 6 h after CHXM treatment (n = 6), MII oocytes (n = 6) and electrically activated oocytes (3 and 6 h) were fixed for 60 min in 2.5 % glutaraldehyde and 0.06 M·L^{-1} cacodylate buffer, pH 7.2; they were postfixed in 1 % OsO_4 and dehydrated in ethanol before embedding in Epon. Sections were examined in a JEOL JEM CX II 100 electron microscope [12].

2.5. Histone H1 kinase assay

Groups of ten oocytes collected during normal maturation or after different treatments (see Results) were transferred into buffer (25 mM HEPES, 80 mM β-glycerol-phosphate, 100 μM Na-vanadate, 15 mM P-nitrophosphate, 10 mM EGTA, 10 mM $MgCl_2$ $6H_2O$, 1 mM DTT, protease inhibitors [Complete Mini, EDTA-free, Boehringer, Mannheim, Germany] 100 nM ATP, 2.2 μm protein kinase A inhibitor peptide and histone [type III-S]) and stored at –70 °C before being used for H1 kinase assay essentially as described by Moos et al. [20]. When the assay

was performed (γ-^{32}P) ATP (sa 3 000 Ci/mM; Amersham, Zlín, Czech Republic) was added at a final concentration of 100 μCi. A fresh assay buffer (10 μL) was added to the mixture and tubes were incubated for 40 min at 37 °C. The reaction was terminated by spotting the aliquots (20 μL) onto P81 Whatman phosphocellulose discs which were immersed immediately into 500 mL of 0.75 mM phosphoric acid. After extensive washing, dehydration and drying, the discs were transferred into 5 mL Instagel (Canberra, Packard) and subjected to liquid scintillation counting by using Tri-Carb 1600A LSC (Canberra Packard, USA). Each experiment was repeated at least three times. Unless otherwise stated all chemicals were purchased from Sigma, Prague, Czech Republic.

3. RESULTS

3.1. The ultrastructure of chemically enucleated oocytes

In all, we subjected 20 enucleated oocytes together with six MI and six MII control oocytes to ultrastructural analysis. Where possible each oocyte was specifically oriented during embedding to ensure that both the oocyte cytoplasm and PB were present in the same section. Detailed comparisons were made both of the cytoplasmic components and PB of chemically enucleated and control oocytes. The oocytes during the metaphase-anaphase I (II) transition had evenly distributed organelles (mitochondria, Golgi complex, endoplasmic reticulum and cytoplasmic vesicles). Cortical granules were localized beneath the oocyte surface which was homogeneously covered with microvilli. The only exception was the area where the metaphase (I, II) spindle is located. This area does not contain organelles and the membrane just about the spindle was devoid of microvilli.

No cytoplasmic abnormalities were detected in enucleated oocytes when compared to MI or MII cells. Thus, we may conclude that the ETO and ETO-CHXM treatments did not damage the eggs. Furthermore, no contact between the cytoplasts and the PBs was observed in almost all cases (n = 19) and the PB was completely separated (*figure 1*). The only exception was the oocyte shown in *figure 2*. Here we detected the thin bridge connecting the cytoplast and PB. However, higher magnification indicates that even in this case a new junction connection has been established after the PB was completely extruded (arrow). The

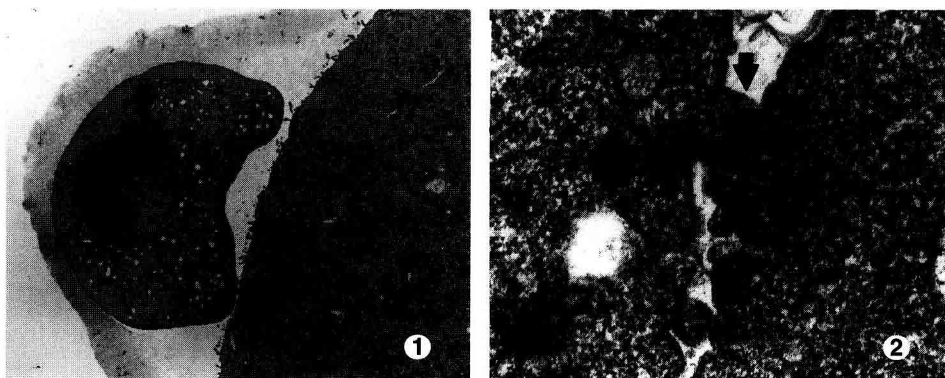

Figure 1. Non-invasive enucleation of mouse oocytes; the polar body contains an entire chromosome complement. Note the degeneration of chromatin (arrow) and the abundance of organelles in the polar body (× 500).
Figure 2. In a single case a new junction (arrow) has been established between the cytoplast and the polar body (× 30 000).

chromatin localized in PBs typically showed certain signs of degeneration (*figure 1*). This degeneration may reflect the degradation of PB. In one case (*figure 3*), an unusual situation was observed. Here the heavily condensed chromatin was surrounded with the double-layered nuclear envelope (*figure 4*). Typically, PBs in chemically enucleated oocytes evidently contained much more cytoplasmic organelles than the first and the second PB (*figures 5* and *6*).

The precise mechanism(s) leading to PB extrusion in chemically enucleated oocytes are not yet understood. In normal oocytes both the chromosomes and spindle play an active role in chromatin expulsion and PB formation in anaphase-telophase I and II. Our results indicate that the spindle is not an important component of the chromatin expulsion machinery during ETO-CHXM-induced chemical enucleation. The morphological observations, rather, suggest that

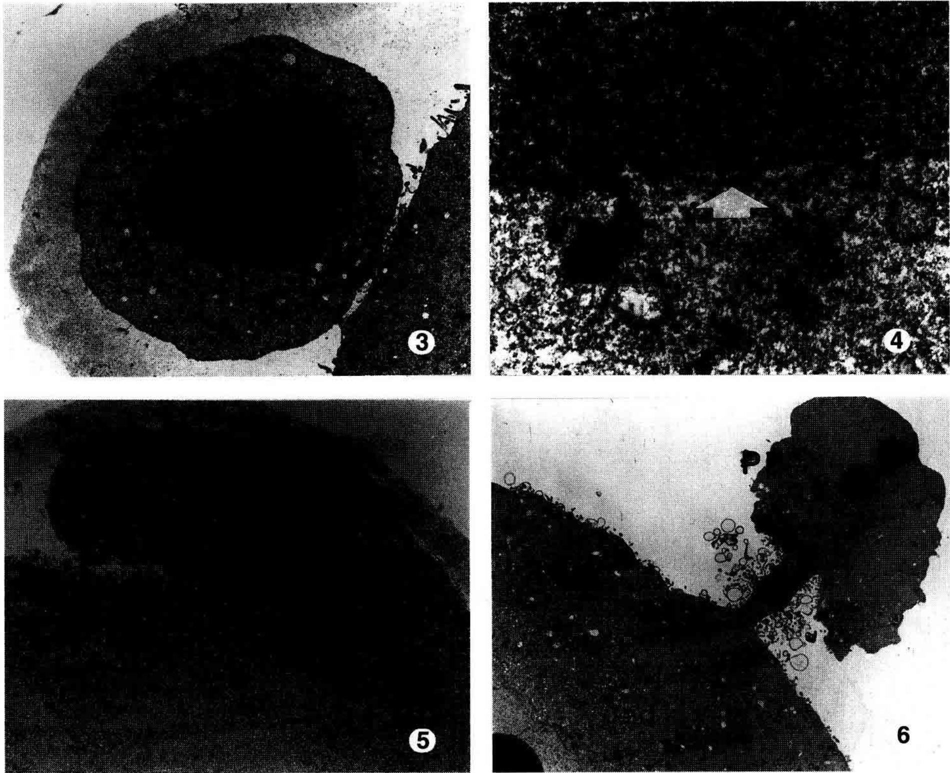

Figure 3. Newly formed membrane around the chromatin in the polar body. The chromatin has not degenerated in this instance (arrow) (× 5 000).
Figure 4. Fine detail of the double-layered membrane (arrow) enclosing the condensed chromatin in the polar body (*figure 3*) (× 60 000).
Figure 5. Extrusion of the first polar body during normal mouse oocyte maturation. The first polar body contains much fewer organelles than the polar bodies in chemically enucleated oocytes (× 5 000).
Figure 6. Detailed structure of the second polar body. Note the relatively low abundance of organelles in the polar body cytoplasm (PB). Nuclei are visible in the polar body and in the oocyte cytoplasm (× 3 000).

a simple bulge containing the entire chromatin complement is gradually isolated by the constriction of the membrane at its base. Unlike the rapid expulsion of chromosomes at telophase (> 30 min), chemically induced expulsion is very slow and is completed only 14–16 h after the addition of ETO and CHXM.

3.2. Different modes of H1 kinase degradation determine the rates of chromosome expulsion during telophase and after chemical enucleation

The levels of histone H1 kinase activity at different meiotic stages of normal mouse oocyte maturation (GV, MI and MII), in MI oocytes treated with cycloheximide, in MII oocytes parthenogenetically activated and in ETO-CHXM-treated oocytes, are shown in *figure 7*. Both MI- and ETO-treated oocytes contained comparable levels of H1 kinase after 8 h of culture. In untreated oocytes the exit from MI to AI was accompanied by a significant decrease in H1 kinase activity. High levels of H1 kinase were restored in MII oocytes. When MI oocytes were treated with CHXM, histone H1 kinase activity decreased concomitantly with first PB extrusion (2–3 h after addition of CHXM). Thereafter, levels of H1 kinase remained low and a new nucleus was formed (6 h after treatment). A similar situation was observed after electroactivation of MII oocytes when a rapid fall in H1 activ-

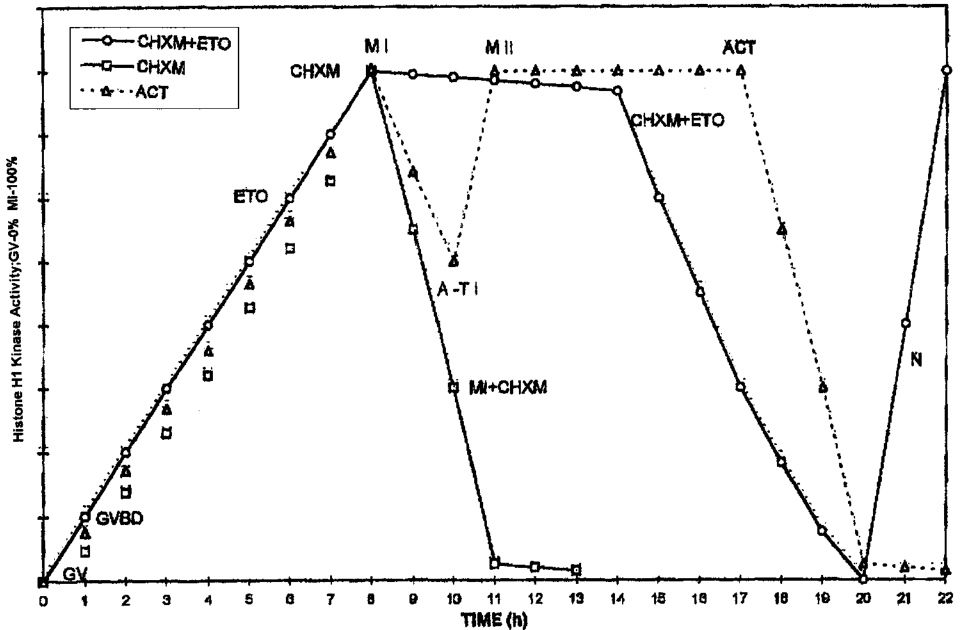

Figure 7. Histone H1 kinase activity in mouse oocytes during normal maturation or after different inhibitor protocols. The following cell cycle stages and inhibitor combinations can be seen: germinal vesicle (GV), metaphase I (MI), anaphase-telophase (A-TI), metaphase II (MII) normal maturation. GVBD: germinal vesicle breakdown; MI + cycloheximide (CHXM): MI oocytes (8 h) were subsequently cultured in CHXM-supplemented medium; ACT: MII oocytes were activated by electric pulses; CHXM-etoposide (ETO): maturing oocytes (6 h) were cultured in ETO-supplemented medium for 2 h and subsequently in ETO-CHMX-supplemented medium (enucleation); N: enucleated oocytes (CHXM-ETO) were cultured in normal medium.

ity was followed a few hours later by the formation of the pronucleus. The finding that the normal sharp decrease in H1 kinase activity at anaphase was not detected in ETO-CHXM-treated oocytes is, however, of the greatest interest. The histone H1 levels (3 h after the beginning of ETO-CHXM treatment) were similar to those in normal MI-staged oocytes. Thereafter, high levels of histone H1 kinase activity persisted in chemically enucleated oocytes for up to 6–8 h before gradually declining to basal levels 12–14 h after the initiation of treatment (~18 h after the induction of maturation). Our finding that chromosome complement extrusion occurs only when the H1 kinase levels decrease to basal levels is central to the understanding of chromosome expulsion by ETO and CHXM. H1 kinase levels rose again to maximal metaphase levels when enucleated oocytes were removed from ETO-CHXM inhibition and cultured in normal medium.

In summary our combined results show that chromosome extrusion in both normal anaphase-telophase and after chemical enucleation results in the formation of PBs. Unlike the rapid spindle-dependent extrusion of homologs or sister chromatids in anaphase-telophase I and II, respectively, chemically induced chromosome expulsion is both slower and independent of spindle involvement. Evidence from combined biochemical and morphological studies indicates that the decline in histone H1 kinase activity during chemical enucleation is remarkably slow; extrusion of the PB occurs as the H1 kinase levels reach basal levels.

4. DISCUSSION

Chemical enucleation of oocytes depends on two processes: the prevention of chromosome segregation at anaphase I and the maintenance or enhancement of those processes involved in PB expulsion. Normal chromosome segregation involves the modification or degradation during early anaphase of so-called cohesion molecules, which bind homologs together during metaphase (see [1]). Since topoisomerase II is required for the modification of the cohesion proteins and the consequent release of homologs at anaphase, we used an inhibitor (ETO) of this released protein. Inhibiting topoisomerase II prevented chromosome separation but also yielded an oocyte in which histone H1 (MPF) kinase remained indefinitely at maximal metaphase levels, thus emphasizing that the separation of bivalents is a prerequisite for subsequent MPF degradation and PB expulsion. By inhibiting both topoisomerase II activity with ETO and all subsequent protein synthesis in the oocyte with CHXM, we were able to successfully induce expulsion of the entire chromosome complex after an extended period of delay [8]. The results presented in the present study provide the basis for understanding the enucleation process. Two key points emerge from our study. First, PBs formed by chemical enucleation appear similar to those formed at telophase, with the major exception that the spindle is not involved in the formation of the former but is important for the latter. Second, in both instances PBs only form when histone H1 (MPF) kinase activity is low. Thus, the rapid decline of MPF kinase activity during anaphase induces rapid PB formation in normal oocytes. In contrast, the slow decline in MPF kinase activity in ETO-CHXM-treated oocytes is accompanied by a corresponding long period before PB expulsion. The different rates of MPF kinase inactivation reflect two entirely different biochemical processes. It is well established that cyclin is rapidly degraded by ubiquitin-mediated proteolysis at the end of M-phase [14]. After chemical enucleation MPF-kinase inactivation is not only slow but appears to be of a temporary nature since there is a rapid and complete return to full MPF-kinase activity after transferring oocytes from medium containing inhibitors to inhibitor-free normal medium.

The biochemical changes that occur in chemically enucleated oocytes are of interest primarily because of their significance to the goal of producing perfect cytoplasts for nuclear transplantation. This objective is beset by a range of difficulties, which are much more apparent in commercially important animals than in mice. For example, precisely timed oocytes with well-formed PBs are relatively easily obtained after normal ovulation in mice but not in ungulates. To compensate for this difficulty, in vitro-matured oocytes are widely used for the production of cytoplasts in ungulates; this is a situation which should favour the use of the non-invasive enucleation approach. Unfortunately, our current system of ETO-CHXM-induced enucleation is only effective in approximately 40 % of cattle oocytes [19]. This difference in enucleation efficiency between mouse and bovine oocytes may be due to an inability to time meiotic events in ungulates with the same precision as is possible in the mouse. This could in turn result in the delivery of the chemical inhibitors at incorrect cell cycle stages. Moreover, the population of cultured oocytes in ungulates is much less homogeneous than that in the mouse. Thus, it is not surprising that rapidly and slowly maturing oocytes can be found in bovine in vitro culture systems [7]. A further factor that may influence the success of enucleation in mice and ungulates is the difference in orientation of the first metaphase plate between these species. In mouse oocytes the spindle is organized in a parallel orientation to the membrane while in sheep, pig and cattle a perpendicular orientation is normal [24]. After ETO treatment, however, no spindle is detected and only a single cluster of chromatin can be seen [10]. This probably argues against the theory that the orientation of the metaphase spindle position has an important effect on the success of chemical enucleation. It is well known that during oocyte ageing the second metaphase moves to the oocyte centre [15]. It is not known whether the same movement occurs in ETO and ETO-CHXM-treated oocytes. It is conceivable that this inward movement is rather rapid in treated ungulate oocytes with the possible result that the PB cannot be extruded because it is too far from the cortex region when the H1 kinase activity reaches basal levels. Thus, an alternative and more rapid method of inducing MPF destruction may be required for the efficient enucleation of ungulate oocytes by chemical enucleation. An approach favoured by us on theoretical grounds is that of ionomycin: 6-dimethylaminopurine (6-DMAP) as reported by Susko-Parish et al. [22]. However, experimental evidence demonstrates that this protocol leads to the retention of the PB in normal oocytes [17]. Nevertheless, further experiments are required to determine whether PB retention also occurs during non-invasive enucleation.

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