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A novel method for detection of chromosomal integrity in cryopreserved livestock spermatozoa using artificially fused mouse oocytes

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Running head: Chromosome analysis using fused mouse egg

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Capsule

Sperm injection into fused mouse oocytes could be an alternative method for chromosomal analysis in livestock spermatozoa.

Abstract

Purpose: The aim of the present study was to investigate the effect of mouse oocyte volume on the efficiency of chromosomal analysis in livestock spermatozoa.

Methods: Oocytes were injected with bull, ram, boar and dog sperm heads, and then fused with enucleated mouse oocytes.

Results: The increment of oocyte volume increased the rates of morphologically normal oocytes after sperm injection, which induced much higher rates of overall chromosome detection in bull, ram and dog spermatozoa. The recipient oocyte volume did not affect the chromosomal integrity. Furthermore, in bull, the chromosomal integrity detected by fused mouse oocytes was similar to that derived from a homologous system. On the other hand, chromosomal plates of boar spermatozoa could not be detected despite the use of fused oocytes.

Conclusion: These data indicate that fused mouse oocytes improved the efficiency of chromosome detection in bull, ram and dog spermatozoa.

Keywords: chromosomal analysis, fused oocytes, intracytoplasmic sperm injection, livestock spermatozoa.

Introduction

To date, the techniques of reproductive engineering have been progressing. One of the most brilliant new achievements is intracytoplasmic sperm injection (ICSI), which was dramatically improved in the 1990s [1-3]. ICSI involves the direct injection of a single spermatozoon into an oocyte. However, ICSI oocytes obviously increased their chromosomal risks at the first mitotic division compared with oocytes derived from *in vitro* fertilization [4]. Sex-sorted [5], freeze-dried [6, 7] and xenogenetic [8] spermatozoa have also been used for ICSI. Therefore, it is necessary to investigate the normality of spermatozoa for ICSI at DNA and chromosome levels.

While the cytogenetic study of mouse spermatozoa has been extensively performed [9-13], the study of livestock spermatozoa is less developed. A low fertilizing capacity after *in vitro* maturation and the lipid contents in livestock oocytes are frequently hindrances for chromosomal analysis; as a result, research in this field has been delayed. In chromosomal analysis of human spermatozoa, since it is difficult to use homologous oocytes for research purposes, a heterologous fertilization system mediated by ICSI has been utilized using mouse oocytes [14-17]. On the other hand, mouse oocytes were shown to be unsuitable for livestock spermatozoa and were frequently deformed after injection with livestock spermatozoa [18, 19]. This may be attributed to the volume of the mouse oocyte, which has a smaller-volume ooplasm (70-80 μm in diameter) than livestock oocytes (100-120 μm in diameter).

As described above, an effective method of chromosome analysis in livestock spermatozoa should be rapidly established. Mouse oocytes have not been utilized for chromosomal analysis of livestock spermatozoa, although there have been abundant studies in

mice [9-13]. Therefore, the present study was performed to investigate the effect of oocyte volume in mouse oocyte recipients of livestock spermatozoa on the efficiency of chromosomal analysis. Mouse oocytes were injected with bull, ram, boar and dog spermatozoa, and then fused electrically with other cytoplasts.

Materials and methods

Reagents and media

All chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) unless specifically stated. The culture medium of mouse oocytes after ICSI was Chatot-Ziomek-Bavister (CZB) [20] supplemented with 5.56 mM D-glucose and 4 mg/ml bovine serum albumin (fraction V; Sigma-Aldrich, St. Louis, MO, USA). Mouse oocyte collection and microinjection were performed in modified CZB supplemented with 20 mM Hepes-Na, 5 mM $NaHCO_3$, and 0.1 mg/ml polyvinyl alcohol (cold water soluble; Sigma-Aldrich) in place of bovine serum albumin (H-CZB). Mouse spermatozoa were collected in modified Toyoda-Yokoyama-Hoshi (TYH) medium [21] supplemented with 20 mM Hepes-Na, 5 mM $NaHCO_3$, and 0.1 mg/ml polyvinyl alcohol in place of bovine serum albumin (H-TYH). The pH values of both H-CZB and H-TYH were adjusted to approximately 7.4.

Animals

All animals were purchased from CLEA Japan, Inc. (Tokyo, Japan). B6D2F1 mice were used to collect oocytes and spermatozoa. All experiments were performed according to the Guiding Principles for the Care and Use of Research Animals of Obihiro University of Agriculture and Veterinary Medicine.

Preparation of oocytes and spermatozoa for ICSI

B6D2F1 female mice, 7-11 weeks of age, were superovulated by i.p. injection of 10

IU eCG (Asuka Pharmaceutical, Tokyo, Japan) followed by injection of 10 IU hCG (Asuka Pharmaceutical) 48 h later. The oocytes recovered from oviducts between 14 and 16 h after hCG injection were denuded of their cumulus cells by treatment with 0.1% (w/v) bovine testicular hyaluronidase (Sigma-Aldrich) in H-CZB. The denuded oocytes were repeatedly rinsed in CZB medium and kept at 37°C under 5% CO_2 in the same medium until ICSI.

Mouse spermatozoa were collected from the cauda epididymis of male mice, 7-12 weeks of age. To verify the reliability of fused oocytes in chromosomal analysis, a part of spermatozoa were treated with a mutagenic compound, methyl methanesulfonate (MMS; 100 μg/ml in H-TYH for 2 h; Nacalai Tesque, Kyoto, Japan) [22, 23] and then were washed twice by centrifugation at 300 × g for 5 min in H-TYH. Furthermore, frozen-thawed livestock spermatozoa were also used for the experiment: commercially available frozen Holstein bull, Suffolk ram and Duroc boar semen frozen with Tris-based egg-yolk buffer [24] and Labrador Retriever dog semen frozen with a synthetic semen extender, AndroMed (Minitüb, Tiefenbach, Germany) [19]. These frozen semen were thawed in a water bath at 37°C and were washed by centrifugation at 300 × g for 5 min in H-TYH.

ICSI procedures

Before sperm injection, a batch of 15 oocytes was transferred into a droplet (5 μl) of H-CZB in an ICSI chamber covered with paraffin oil (Merck Japan, Tokyo, Japan). Concurrently, a small amount (1-2 μl) of the sperm suspension was transferred into a droplet (5 μl) of H-TYH containing 10-12% polyvinyl pyrrolidone (PVP; molecular weight: 360000; Nacalai Tesque) in the same chamber. A spermatozoon was aspirated into the injection pipette tail first, and the tail was cut by applying a few piezopulses. The tail-cut spermatozoon was

individually injected into a mouse oocyte according to the method of Kimura and Yanagimachi [2]. The series of experiments for ICSI was finished within 60 min of sperm preparation. In addition, bull spermatozoa were also injected into bovine oocytes that were matured *in vitro* as described in a previous study [25]. The sperm injection procedure was performed in the same manner. The injected oocytes were washed with CZB and transferred into a droplet (30 μl) of the same medium covered with paraffin oil at 37°C under 5% CO_2 in air for cultivation.

Oocyte fusion

Before starting cultivation, a portion of the ICSI oocytes were fused with enucleated mouse oocytes [26] using the electrofusion method described by Wakayama *et al.* [27] with slight modification. Briefly, ICSI and enucleated oocytes, from which the zona pellucida was removed in 0.5% protease (Kaken Pharmaceuticals, Tokyo, Japan) in Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate buffered saline, were treated in 1% phytohemagglutinin (Gibco-BRL, Grand Island, NY, USA) in H-CZB for 1 min and were paired in H-CZB using a micromanipulator. The paired oocytes were placed into fusion medium consisting of 0.3 M mannitol, 0.1 mM $MgSO_4$ and 0.1% PVP in a fusion chamber with electrodes at 1-mm gaps. Oocyte fusion was induced by applying an alternating current (1 MHz for 1-2 s) followed by a direct current (150 V for 20 μs) using a cell fusion generator (LF101; NepaGene, Chiba, Japan). Thereafter, the oocytes were transferred into a droplet of CZB to start cultivation. An ICSI oocyte was fused with nine enucleated oocytes at maximum and, for example, oocytes enlarged to double- and triple-volume through the fusion procedure were defined as 2-egg and 3-egg oocytes, respectively (Fig. 1). To confirm the effect of the oocyte volume, two groups

were designed; one was sham fusion that 1-egg oocyte was injected with a spermatozoon and subjected with only fusion procedure, and another was 2-egg × 2-sperm that 2-egg oocyte was injected with two spermatozoa.

Chromosome preparation of one-cell oocytes.

Six hours after ICSI, deformed oocytes were discarded since these oocytes never reached the first mitotic stage [18, 19], and morphologically normal oocytes were transferred to CZB containing 0.02 μg/ml vinblastine sulfate to inhibit the first cleavage division. At 19-21 h after ICSI, they were maintained in hypotonic solution consisting of equal volumes of 1% (w/v) sodium citrate and 30% (v/v) fetal calf serum (Gibco-BRL) for 10 min at room temperature. These samples were prepared by the gradual-fixation/air-drying method [28]. The slides were conventionally stained with 2% Giemsa (Merck) in buffered saline (pH 6.8) for 10 min.

Statistical analysis

Each experiment was repeated at least three times. The data were analyzed by an analysis of logistic regression following a binomial distribution using the model: $\ln(\alpha/1-\alpha) = \beta + \text{main factor (oocyte volume)}$, where α = frequency of positive outcome and β = the intercept, using Statistical Analysis System (SAS; SAS Institute, Cary, NC, USA) software. If the main factor had a significant effect, comparisons among the subgroups were performed using the 95% confidence interval of the odds ratio.

Results

In the mouse homologous system, 3-egg oocytes as well as 1-egg oocytes successfully developed to the first mitotic metaphase at 19-21 h after ICSI, even when spermatozoa were treated with MMS (Table 1). Arrested eggs had a decondensed sperm head (DSH), prematurely condensed chromosomes (PCC) or a pronucleus (PN) in the ooplasm. The chromosomal analysis revealed that the percentage of eggs with normal chromosome complements in the 1-egg oocytes was similar to that in the 3-egg oocytes. Although structural chromosome aberrations were frequently induced when spermatozoa were treated with MMS, there was no significant difference in the percentage of chromosomally normal eggs between the 1-egg and 3-egg oocytes. Thus, the fusion procedure and the increment of oocyte volume did not affect the fertilizing capacity of ICSI eggs and their chromosomal integrity.

The results of fertilization and chromosomal analysis of ICSI oocytes injected with bull spermatozoa are summarized in Table 2. The rates of successfully fused oocytes were 93.2 and 95.9% in the 2-egg and 3-egg oocytes, respectively. Mouse 1-egg oocytes injected with bull spermatozoa frequently deformed (70.5%; 55/78; Fig. 2a). The increased volume of recipient ooplasm increased the rate of morphologically normal oocytes (85.3-97.8%). In 3-egg ooplasm, bull spermatozoa developed to PN with normal morphology (Fig. 2b-e). On the other hand, the fusion procedure did not affect the morphology of ICSI oocytes (sham fusion, 23.3%), and when two spermatozoa were injected into the 2-egg oocytes, the rates of morphologically normal oocytes decreased to 37.5%, which was similar to the 1-egg group. At 19-21 h after ICSI, the proportion of ICSI eggs with mitotic chromosomes (Fig. 3a)

remarkably ($P<0.05$) improved to 76.8% from 30.4% in an oocyte volume-dependent manner. In the 3-egg oocytes, the rate of mitotic chromosomes was also higher ($P<0.05$) than that of bovine oocytes injected with bull spermatozoa (48.3%). However, the oocyte volume for a recipient did not affect the chromosomal integrity (50.0 to 78.2%).

Table 3 represents the results of ICSI oocytes injected with ram, boar and dog spermatozoa. The volumes of recipient oocyte affected the morphology of ICSI oocytes injected with these spermatozoa. However, a 10-fold mouse ooplasm (10-egg) as a recipient was needed to prevent deformation for a boar spermatozoon. Furthermore, even if the oocyte deformation was overcome using the 10-egg oocyte, these oocytes never reached the first mitotic stage. As a consequence, chromosome analysis in boar spermatozoa could not be performed. On the other hand, ICSI oocytes injected with dog spermatozoa had significantly ($P<0.05$) higher rates of mitotic chromosomes (86.7-88.4%) in the fusion group compared with the non-fused group (1-egg, 68.1%), while there was no significant difference in the proportions of mitotic chromosomes (63.4 to 80.7%) in ram spermatozoa for different oocyte volumes (Fig. 3b,c). As in the case of bull spermatozoa, the chromosomal integrity in ram and dog spermatozoa did not depend on the volume of recipient oocytes. Through the experiments in all species, almost all chromosomal aberrations were structural anomaly; the rates of aneuploidy and diploidy were less than 5%.

Discussion

The present results clearly demonstrated that the volume of recipient mouse oocytes was an important factor in overcoming oocyte deformation that was otherwise common when livestock sperm heads were injected. In enlarged (fused) mouse ooplasms, bull, ram and dog sperm heads decondensed, formed PN and reached the first mitotic stage with acceptable frequency. When these oocytes were fixed, analyzable chromosome plates were effectively observed. In bull, the results of chromosomal integrity obtained from the fused mouse oocytes were similar to that in a homologous system. These findings suggest that the increased volume of mouse oocytes by electrofusion is a breakthrough among efforts to obtain chromosome spreads in bull, ram and dog spermatozoa.

It has been reported that the disruption of the acrosomal membrane improves embryonic development after ICSI in mice [29] and rats [30]. Direct injection of the enzymes (i.e. trypsin or hyaluronidase) into mouse oocytes results in defects of the oocyte morphology [18]. Furthermore, mouse oocytes become deformed according to the number of sperm heads (i.e. amount of acrosome enzymes) injected into an oocyte [18]. The present results revealed that enlarged (fused) mouse oocytes rarely became deformed with the injection of livestock sperm heads and allowed for the dilution of acrosomal enzymes within the ooplasm. Thus, it is likely that the deformation in mouse oocytes injected with livestock spermatozoa is the result of the concentration of acrosomal enzymes in a small volume of ooplasm. Alternatively, spermatozoa demembranized by pre-treatments also seemed to be suitable for chromosomal analysis. However, since the sperm pre-treatment impaired sperm chromosome integrity at the first mitotic stage in mice [31], we propose that the method using fused mouse oocytes as

shown in the present study is more appropriate for chromosomal analysis of livestock spermatozoa.

The results for boar spermatozoa were an exception among the species used in this study, as the ICSI oocytes injected with boar sperm heads needed a 10-fold mouse ooplasm (10-egg) to prevent deformation, about 2.13-fold larger in volume than a porcine oocyte (Table 3). This suggests that the porcine acrosomal enzymes are more active than those of other livestock spermatozoa. Therefore, it was possible to interpret that the acrosomal enzymes that were brought into the oocytes by the sperm heads had arrested fertilization events, as was shown in previous studies [18, 19], even if the oocyte deformation was limited by the enlarged ooplasm. As a consequence, ICSI oocytes injected with boar sperm heads could never reach the first mitotic stage. The specific character of the acrosomal enzymes of boar spermatozoa responsible for this result remains to be understood, although there are some reports describing the effect of acrosomal contents in boar spermatozoa on fertilization events following ICSI [32, 33].

As shown in Tables 2 and 3 and Fig. 2b-e, fertilization events using fused mouse oocytes and bull or dog spermatozoa normally progressed. The remodeling of sperm nuclei during fertilization involves various factors. For example, the sperm-specific protein protamines were replaced by oocyte-supplied histones, and the histones were deacetylated by histone deacetylases stored in the ooplasm as fertilization progressed (reviewed by McLay and Clarke [34]). Thus, the increment of oocyte volume could increase these cytoplasmic factors to support the remodeling of sperm chromatin during fertilization. However, it should be noted that there was a species (i.e. ram) without the positive effect of oocyte fusion on the fertilization results after ICSI. Namely, ram sperm heads frequently developed to the first

mitotic stage even with non-fused mouse oocytes (1-egg), suggesting that the remodeling of ram sperm chromatin was completed using only the cytoplasmic factors stored in a 1-egg ooplasm. In this respect, the sperm requirements for remodeling factors in ooplasm are likely to be species-specific. Furthermore, it could not be excluded that different activity of maturation promoting factor and mitogen-activated protein kinase in mouse oocyte affected sperm chromatin remodeling in different species, remaining to be investigated in future study.

The present method using fused mouse oocytes resulted in highly detectable paternal chromosomes in all species except for boar. Chromosomal integrity in livestock spermatozoa analyzed by the present method was relatively low compared with mouse (91.6% [4]) and human (95.7% [16]) spermatozoa for undetermined reasons. However, bull sperm heads injected into bovine oocytes had a similar incidence of chromosomal aberrations (Table 2). Therefore, it is possible to interpret that the present method using fused mouse oocytes can successfully detect the chromosomal integrity of livestock spermatozoa. Sperm DNA is repairable during fertilization [35, 36], this repair involves topoisomerase II in the ooplasm [37]. Although there was no effectiveness of oocyte volume on chromosomal integrity in this study, the present method might provide a valuable tool for investigating the repair mechanisms of chromosomal aberrations during fertilization.

In conclusion, the present results clearly demonstrated the positive effect of fused mouse oocytes on the visualization of chromosomal spreads in bull, ram and dog spermatozoa. Since chromosomes of boar spermatozoa were never detected in mouse ooplasm, a more effective method of detecting their chromosomes and a better understanding of the effect of their acrosomal contents on fertilization events remain to be determined.

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Table 1. Fertilizing capacity and chromosomal integrity of mouse spermatozoa in mouse oocytes of different volumes.

Sperm	Oocyte	No. of eggs fixed†	Status of paternal nuclei (%)			No. of eggs analyzed	No. (%) of normal chromosome
			DSH or PCC	PN	Mitotic chromosomes		
Mouse	1-egg	70	1 (1.4)	2 (2.9)	67 (95.7)	67	62 (92.5) [a]
	3-egg	69	2 (2.9)	1 (1.4)	66 (95.7)	66	57 (86.4) [a]
Mouse	1-egg	61	1 (1.6)	3 (4.9)	57 (93.4)	57	25 (43.9) [b]
(MMS-treated)	3-egg	55	0 (0)	0 (0)	55 (100)	55	27 (49.1) [b]

a-b: Within the same column, values without a common superscript differ significantly ($P<0.05$)

† Oocytes were fixed 19–21 h after ICSI.

MMS, methyl methanesulfonate; DSH, decondensed sperm head; PCC, prematurely condensed chromosome; PN, pronucleus.

Table 2. Fertilizing capacity and chromosomal integrity of bull spermatozoa in mouse oocytes of different volumes.

Sperm	Oocyte (relative volume)*	No. of eggs examined**	No. (%) of normal shape oocytes†	No. of eggs fixed‡	Status of paternal nuclei (%)			No. of eggs analyzed	No. (%) of normal chromosome
					DSH or PCC	PN	Mitotic chromosomes		
Bull	1-egg (0.32)	78	23 (29.5) [a]	23	11 (47.8) [a]	5 (21.7) [a]	7 (30.4) [a]	6	3 (50.0)
	2-egg (0.62)	109	93 (85.3) [b]	81	24 (29.6) [ab]	16 (19.8) [a]	41 (50.6) [a]	40	26 (65.0)
	3-egg (0.93)	185	181 (97.8) [c]	168	32 (19.0) [b]	7 (4.2) [b]	129 (76.8) [b]	124	97 (78.2)
	Sham fusion	73	17 (23.3) [a]	N.D.	-	-	-	N.D.	-
	2-egg × 2-sperm	48	18 (37.5) [a]	N.D.	-	-	-	N.D.	-
	Bovine (1.00)	N.D.	-	87	30 (34.5) [a]	15 (17.2) [a]	42 (48.3) [a]	40	27 (67.5)

a-c: Within the same column, values without a common superscript differ significantly ($P<0.05$)

* Relative volume compared with bovine oocyte

** No. of successfully injected and fused oocytes

† The oocytes were observed at 6 h after ICSI.

‡ Only morphologically normal oocytes were fixed 19–21 h after ICSI.

N.D., not determined.

DSH, decondensed sperm head; PCC, prematurely condensed chromosome; PN, pronucleus.

Table 3. Fertilizing capacity and chromosomal integrity of ram, boar and dog spermatozoa in mouse oocytes of different volumes.

Sperm	Oocyte (relative volume)*	No. of eggs examined**	No. (%) of normal shape oocytes†	No. of eggs fixed‡	Status of paternal nuclei (%)			No. of eggs analyzed	No. (%) of normal chromosome
					DSH or PCC	PN	Mitotic chromosomes		
Ram	1-egg (0.23)	70	22 (31.4) [a]	21	4 (19.0)	1 (4.8)	16 (76.2)	16	8 (50.0)
	2-egg (0.44)	68	43 (63.2) [b]	41	13 (31.7)	2 (4.9)	26 (63.4)	26	15 (57.7)
	3-egg (0.67)	57	57 (100) [c]	57	11 (19.3)	0 (0)	46 (80.7)	46	30 (65.2)
Boar	1-egg (0.24)	35	7 (20.0) [a]	7	3 (42.9)	4 (57.1)	0 (0)	N.D.	-
	3-egg (0.69)	40	16 (40.0) [ab]	15	11 (73.3)	4 (26.7)	0 (0)	N.D.	-
	6-egg (1.45)	32	15 (46.9) [b]	15	10 (66.7)	5 (33.3)	0 (0)	N.D.	-
	10-egg (2.13)	17	16 (94.1) [c]	12	8 (66.7)	4 (33.3)	0 (0)	N.D.	-
Dog	1-egg (0.24)	86	57 (66.3) [a]	47	13 (27.7)	2 (4.3)	32 (68.1) [a]	30	22 (73.3)
	2-egg (0.45)	59	53 (89.8) [b]	43	5 (11.6)	0 (0)	38 (88.4) [b]	38	28 (73.7)
	3-egg (0.68)	49	48 (98.0) [b]	45	5 (11.1)	1 (2.2)	39 (86.7) [b]	39	30 (76.9)

a-c: Within the same column in each species, values without a common superscript differ significantly ($P<0.05$)

* Relative volume compared with each homologous oocyte

** No. of successfully injected and fused oocytes

† The oocytes were observed at 6 h after ICSI.

‡ Only morphologically normal oocytes were fixed 19–21 h after ICSI.

N.D., not determined.

DSH, decondensed sperm head; PCC, prematurely condensed chromosome; PN, pronucleus.

Figure legends

Fig. 1. Explanatory notes of artificially fused mouse oocytes. a) non-fused oocyte, 1-egg; b) double-volume oocyte, 2-egg; c) triple-volume oocyte, 3-egg. Bar = 50 μm.

Fig. 2. Morphological changes of ICSI oocyte (a) and injected bull sperm head (b-e). a) Normal (left) and deformed (right) 1-egg oocytes without zona pellucida. b-e) Phase-contrast microscopic images of bull sperm heads injected into 3-egg oocytes; b) condensed sperm head at 1 h, c and d) decondensed and recondensed sperm heads at 3 h, respectively, and e) male pronucleus with a large nucleolus at 6 h after ICSI. Bar = 10 μm.

Fig. 3. Chromosomal spreads of bull (a), ram (b) and dog (c) spermatozoon detected using triple-volume mouse oocytes (3-egg). Bar = 10 μm.

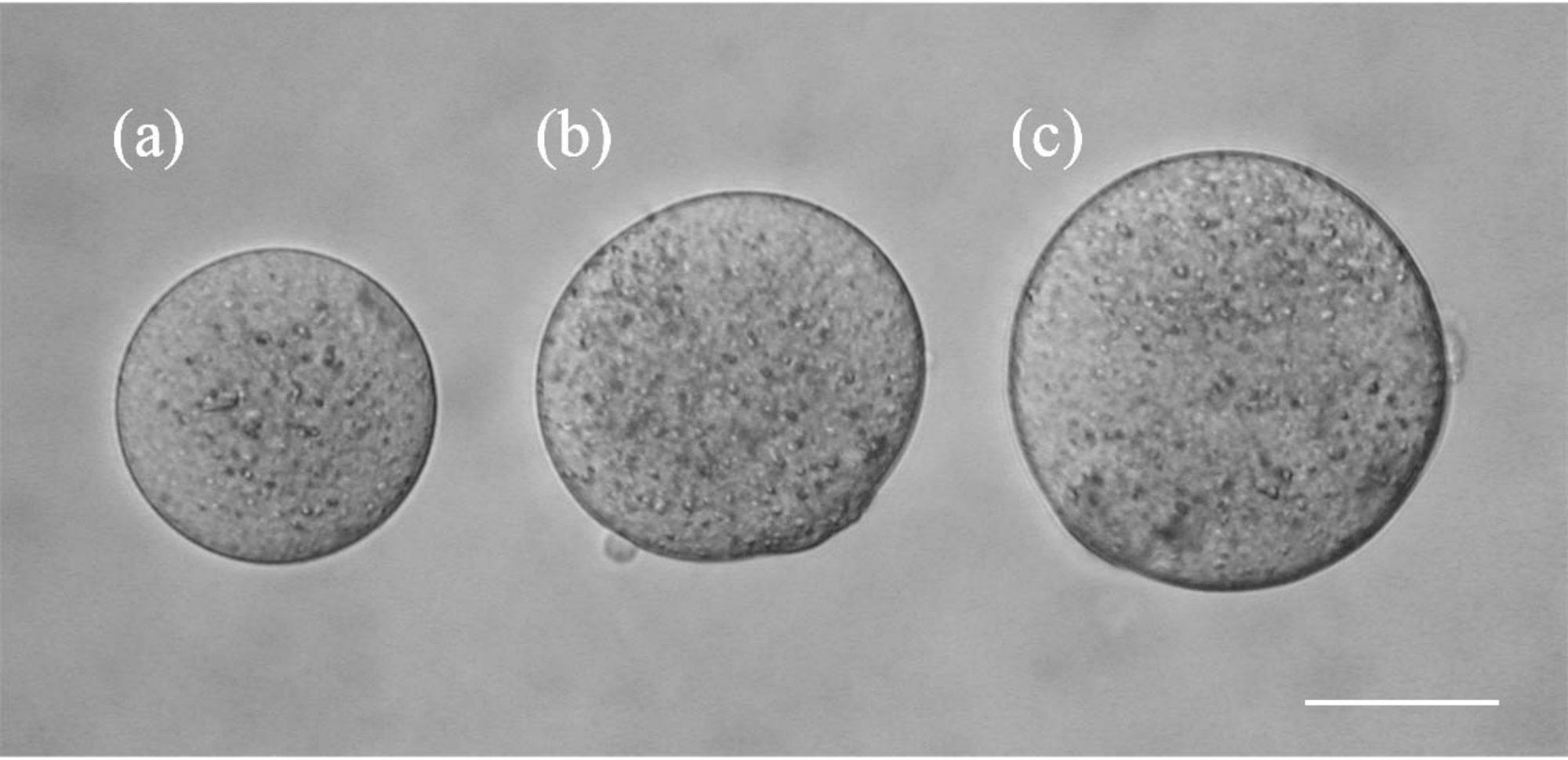

Fig. 1.

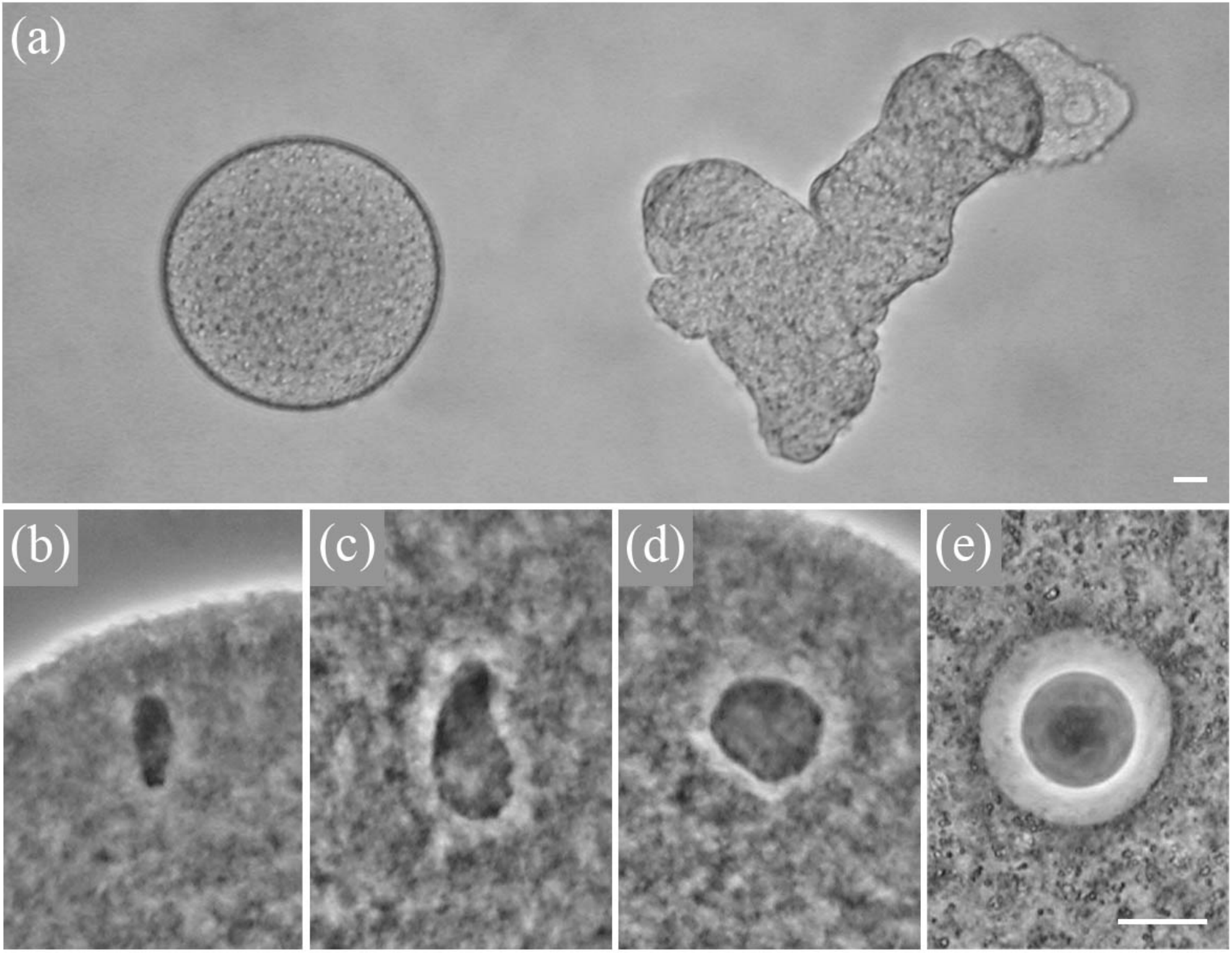

Fig. 2.

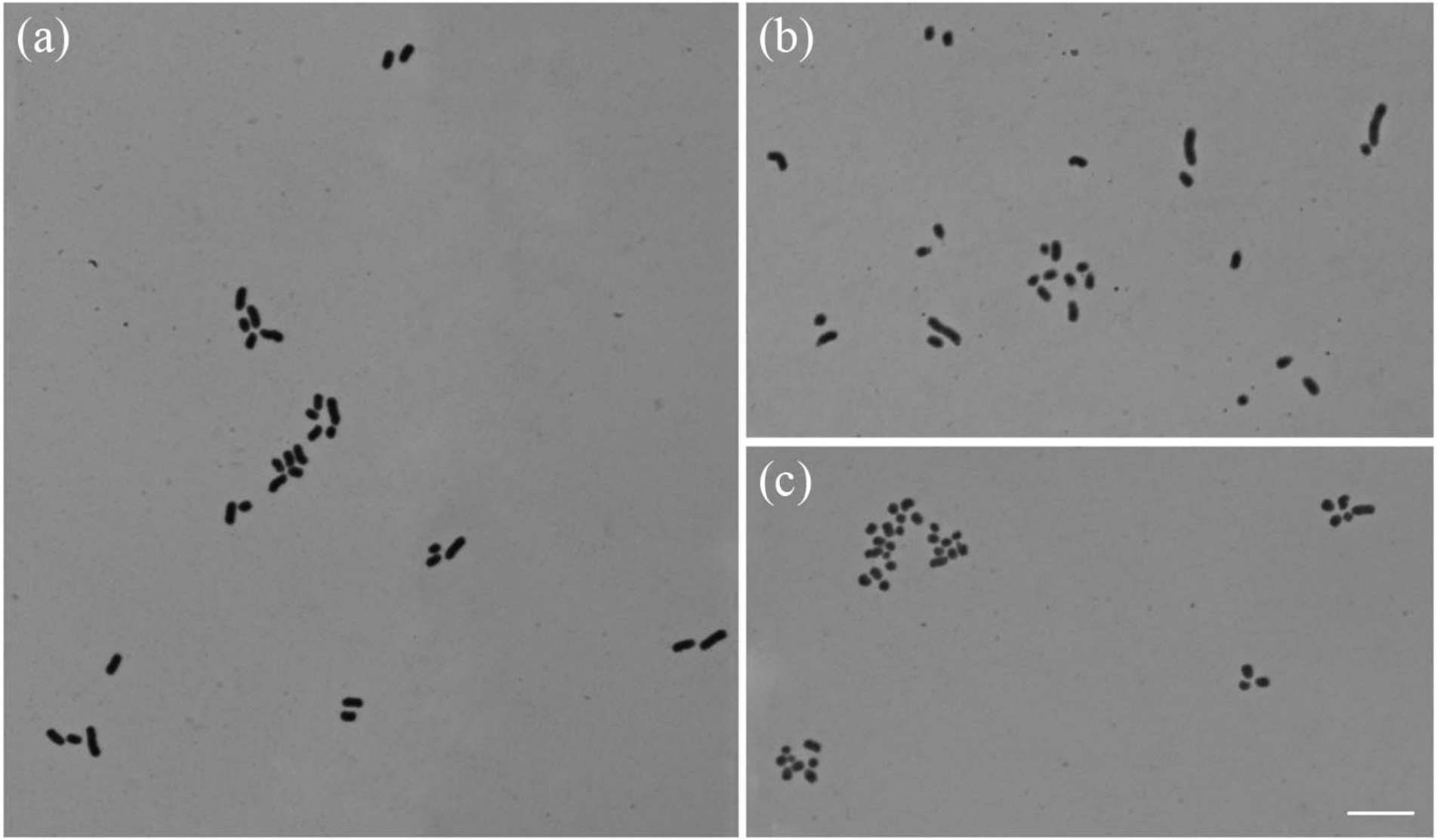

Fig. 3.