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A novel method for isolating spermatid nuclei from cytoplasm prior to ROSI in the mouse

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Summary

In the current widely used round spermatid injection (ROSI) protocol for the mouse, the spermatid nucleus is separated from most of the cytoplasm before ROSI by drawing a spermatid in and out of a pipette. This results in the highest rate of normal fertilization. However, this separation method is not always consistent and can be time-consuming. An alternative separation method that cuts away the cytoplasm using the tip of an injection pipette was developed. After removing the cytoplasm, ROSI was performed following both post- and pre-activation protocols and development *in vitro* and *in vivo* were examined. The new method consistently removed the bulk of the cytoplasm, as shown by quantifying mitochondria. ROSI without the cytoplasm resulted in significantly higher rates of fertilization than ROSI with the cytoplasm into either post- or pre-activated oocytes. Furthermore, the offspring production rates of ROSI without the cytoplasm were also high (50% and 49% for the post- and pre-activation protocols, respectively). This new method for separating the cytoplasm is an alternative way of producing offspring using ROSI.

Keywords: Gamete biology, ROSI, Spermatid

Introduction

Viable offspring have been obtained after round spermatid injection (ROSI) in the mouse (Ogura *et al*., 1994; Kimura & Yanagimachi, 1995), rat (Hirabayashi *et al*., 2002), hamster (Haigo *et al*., 2004), rabbit (Sofikitis *et al*., 1994), and human (Tesarik *et al*., 1995). In mouse, the highest rate of normal fertilization occurs when the spermatid nucleus is freed from most of the surrounding cytoplasm, i.e. it is 'denuded', before ROSI into a previously activated oocyte (Kimura & Yanagimachi, 1995). Denudation was originally achieved by simply drawing a round spermatid in and out of an injection pipette repeatedly until the plasma membrane was completely ruptured and the spermatid nucleus was almost completely separated from the cytoplasm (Kimura & Yanagimachi, 1995). However, this 'repeatedly drawing method' does not always result in separation and may take a long time. Further, this method of 'tearing off' the cytoplasm may damage the spermatid nucleus.

Compared with intracytoplasmic sperm injection (ICSI), the rates of fertilization and offspring production after ROSI are lower for unknown reasons (Kimura & Yanagimachi, 1995; Kishigami *et al*., 2004). Although spermatozoa generally retain a small droplet of cytoplasm after spermiation as a functional cellular unit (reviewed in Cooper & Yeung, 2003), the caudal part of the cytoplasm of the mature spermatid is pinched off as the residual body during spermatozoa release from the seminiferous epithelium (Fawcett & Phillips, 1969). Therefore, after formation of the residual body, the bulk of the cytoplasm is discarded, including numerous mitochondria, the Golgi apparatus and lysosomes (Dietert, 1966). In mice, the spermatid nucleus is currently injected with some cytoplasm (Kimura & Yanagimachi, 1995). As a result, whole organelles and functional cellular units of transcription and translation are injected into recipient oocytes, which may cause unexpected embryonic development, as previously discussed with respect to injecting the Golgi apparatus (Moreno *et al*., 2000). Recently, it was also shown that pre-implantation

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embryos have a different gene expression pattern after ROSI compared with that after ICSI (Hayashi *et al*., 2003). Therefore, minimizing the amount of injected cytoplasm from spermatids might improve embryonic development.

We developed a new method that removes the bulk of the cytoplasm from round spermatids efficiently, and examined whether the round-spermatid nuclei isolated using this new method can support full-term development of mice after ROSI. Moreover, we examined how the injected cytoplasm affects embryo development *in vivo* and *in vitro*.

Materials and methods

Animals

BDF1 mice (C57BL/6 × DBA/2) were used to prepare spermatogenic cells and as oocyte donors. We used ICR females that were mated with vasectomized males of the same strain as surrogate mothers. All animals (obtained from SLC, Shizuoka, Japan) were maintained in accordance with the Animal Experiment Handbook of the Center for Developmental Biology.

Oocyte collection

Mature oocytes were collected from the oviducts of 8- to 12-week-old BDF1 females that had been induced to superovulate with 5 IU of pregnant mare serum gonadotropin (PMSG), followed by 5 IU of human chorionic gonadotropin (hCG) 48 h later. Oocytes were collected from the oviducts approximately 16 h after hCG injection, placed in CZB medium (Chatot *et al*., 1989) supplemented with 5.56 mM D-glucose and 5 mg/ml bovine serum albumin (BSA, fraction V), and treated with 0.1% hyaluronidase until the cumulus cells dispersed. The oocytes were then placed in synthetic oviductal medium enriched with potassium (KSOM) containing non-essential and essential amino acids (KSOM AA; Specialty Media, Phillipsburg, NJ) supplemented with 1 mg/ml BSA, covered with paraffin oil (Nacalai Tesque, Kyoto, Japan), and stored at 37 °C (5% CO_2/air).

Preparing round spermatids and vital labelling

To collect spermatogenic cells, the seminiferous tubules of the testes from the same BDF1 male were minced, as described previously (Ogura *et al*., 1994), except that the cells were suspended in HEPES-buffered CZB medium (CZB-H). For ROSI, a 1 µl aliquot of the spermatogenic cell suspension was mixed with ~10 µl of HEPES-CZB medium containing 12% (w/v) polyvinylpyrrolidone (PVP, M_r 360 kDa; Wako, Japan) in a micromanipulation chamber. For vital labelling, collected spermatogenic cells were incubated in CZB-H medium containing 500 nM MitoTracker Green FM (Molecular Probes, Eugene, OR) and 2 µg/ml Hoechst 33342 at room temperature (24–26 °C) for 1 h, and transferred to a PVP drop after washing twice with CZB-H. For the quantitative analysis of the separated cellular parts of round spermatids, fluorescent images using MitoTracker and Hoechst staining were subjected to densitometric analysis using the program Image-J from the National Institutes of Health (available on the Internet at http://rsb.info.nih.gov/ij/).

Oocyte activation and microinsemination with round spermatids

ROSI was carried out using a previously described protocol (Kimura & Yanagimachi, 1995), except that our experiments were performed at room temperature (Kishigami *et al*., 2004). Briefly, for ROSI, a round spermatid, which was characterized by its small size (~10 µm) and centrally located, distinct nucleolus, was injected into a single oocyte. In the pre-activation protocol, collected oocytes were activated by treatment with Ca^{2+}-free CZB containing 5 mM $SrCl_2$ for 20 min (Bos-Mikich *et al*., 1995) and transferred into KSOM medium at 37 °C under 5% CO_2 in air. Round spermatids were injected into 12–15 activated oocytes between 50 and 80 min after oocyte activation. All the procedures were performed at room temperature (24–26 °C). The injected oocytes were then kept at room temperature for 10 min before they were cultured in KSOM medium at 37 °C in a CO_2 incubator. In the post-activation protocol, 12–15 unactivated MII oocytes were injected within 20 min and, after 10 min on the microscope stage, were activated in a similar manner.

Examining pronucleus formation and embryo culture

Six hours after ROSI, the number of pronuclei in the injected oocytes was examined using an inverted microscope with a relief contrast condenser (IX71; Olympus, Japan). A full-grown pronucleus in close proximity to the second polar body was assumed to be a female pronucleus. 'A normal male pronucleus' was determined by both its size (at least as large as the female one) and the presence of clear nucleoli. Thus, oocytes with two large pronuclei and one second polar body were considered 'fertilized normally' and as 'normal zygotes'. At this time, only normal zygotes were transferred into new drops of KSOM medium and cultured continuously for 24 or 96 h to examine their development *in vivo* and *in vitro*.

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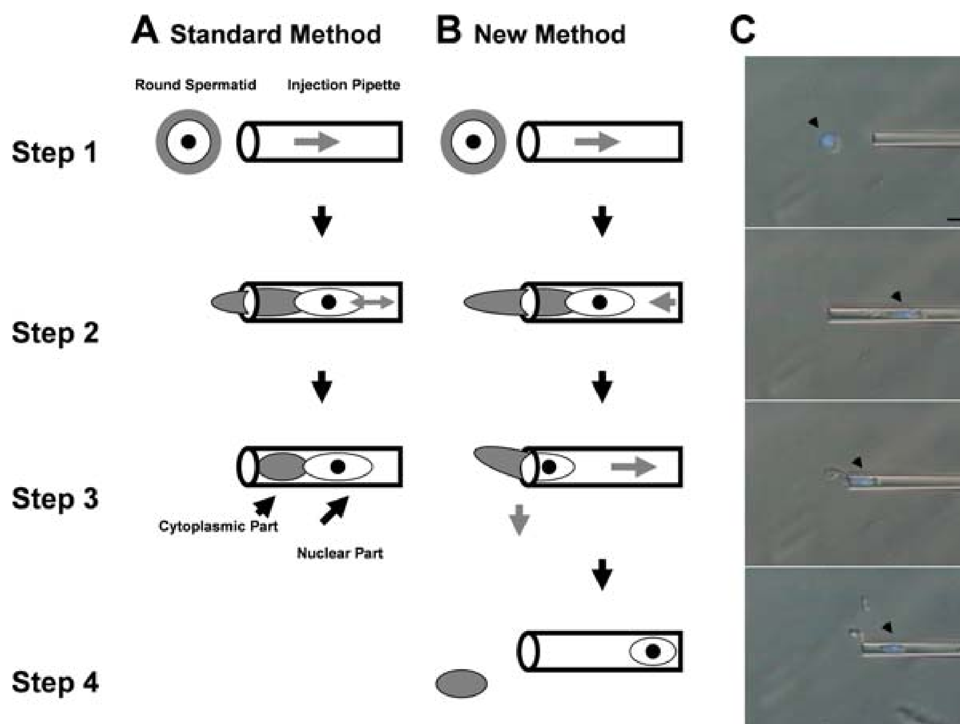

Figure 1 A novel procedure for removing the cytoplasm from round spermatids for ROSI. In the standard procedure (*A*), the nucleus (arrowhead in *C*) is separated from the cytoplasm by repeated pipetting (Kimura & Yanagimachi, 1995). The new procedure (*B* and *C*) uses the tip of an injection pipette (4–5 μm i.d. at the tip) to cut off the cytoplasm. When a round spermatid is gently drawn into the injection pipette, the boundary between the nucleus and cytoplasm is clearly visible (Step 2, *B* and *C*). After moving the spermatid so that the boundary is at the tip of the pipette (Step 3, *B* and *C*), the pipette is moved vertically while aspirating the nucleus (Step 4, *B* and *C*). (*C*) Each step is shown using round spermatids stained with Hoechst 33342 to demonstrate the nuclear part. Scale bar represents 10 μm.

Embryo transfer

Zygotes that were normal after ROSI were cultured for 24–30 h in KSOM medium until the 2-cell stage. Then, 8–10 2-cell embryos were transferred to each oviduct of surrogate females on day 1 of pseudopregnancy.

Statistical analyses

Offspring production and *in vitro* development were analysed using the chi-squared test with Yates' correction for continuity. A value of $p < 0.01$ was considered statistically significant.

Results

A novel procedure for removing the cytoplasm of round spermatids for ROSI

To separate the round spermatid nucleus from the bulk of the cytoplasm efficiently and consistently, a new separation method was developed that used the tip of an injection pipette as shown in Fig. 1. We were able to prepare isolated spermatid nuclei from the cytoplasm for ROSI in 5.4 ± 1.5 s per nucleus in contrast to 7.6 ± 2.2 s per nucleus for the 'repeatedly drawing in and out' method ($n = 50$, respectively). This new method quickly separates the nucleus of the round spermatid for ROSI. After cutting the round spermatid at the boundary, only the nuclear part is used for 'ROSI without cytoplasm'. In control experiments using 'ROSI with the cytoplasm', a round spermatid was drawn in and out of the pipette gently to break the plasma membrane, but without completely separating the cytoplasmic part, to minimize the potential damage to the nucleus.

Characterization of the separated cellular parts using organelle-specific vital dyes

The staining of round spermatid mitochondria using MitoTracker has been reported in mice (Cummins *et al*., 1998) and rhesus monkeys (Sutovsky *et al*., 1999). In this study, MitoTracker probes consistently revealed the polarization of round spermatid mitochondria depending on their developmental stage (Fig. 2C). After separating round spermatids into two cellular parts, each part in the PVP drop was clearly characterized as the nuclear or cytoplasmic part based on the Hoechst and MitoTracker staining patterns (Fig. 2E, F).

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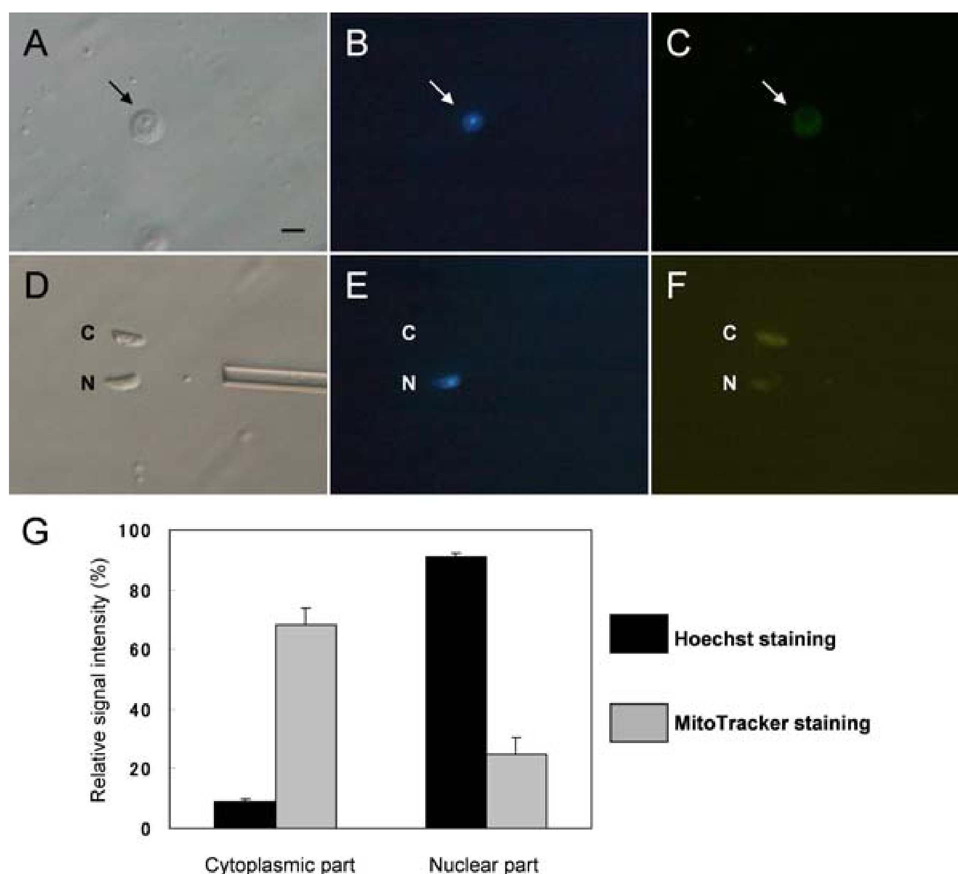

Figure 2 Quantitative analysis of cytoplasm removal. Following the new procedure shown in Fig. 1, a round spermatid (arrow) is recognized by its small size and centrally located chromatin mass (*A*) and is easily separated into two parts with similar volumes (*D*). Staining a round spermatid with Hoechst 33342 and MitoTracker revealed a large nucleus with an emphatically stained chromatin mass (*B*). By contrast, the mitochondria were dispersed throughout the entire cell, with more in the acrosomal region (*C*). After separation, > 90% of the Hoechst signal was consistently detected in one part (*E* and *G*), while about 70% of the MitoTracker signal was in the opposite part (*F* and *G*). The percentage (mean ± SEM, total number of round spermatids examined = 15) is based on the relative signal strength of each part (*G*). Note the nuclear (N) and cytoplasmic (C) parts. Scale bar represents 10 μm.

A quantitative analysis of these fluorescent images indicated that an average of 68% of mitochondria remained with the cytoplasmic part (Fig. 2G). On the other hand, 87% ± 4.6% of mitochondria ($n = 13$) stayed with the nuclear part after gently drawing in and out for 'ROSI with the cytoplasm'. This separation pattern was also confirmed using LysoTracker DND-99, another vital dye, which labels acidic organelles such as lysosomes (S. Kishigami, unpublished). These observations show that separation using this new method consistently removes more cytoplasmic organelles.

Fertilization and *in vitro* development after ROSI using the new procedure

Although pre-activated oocytes are thought to be better recipient oocytes for ROSI, both pre- and post-activation protocols can be used to produce offspring using ROSI (Kimura & Yanagimachi, 1995; Ogura *et al.*, 1999). After removing the cytoplasm, ROSI was performed following both protocols to examine the fertilization rate. As expected from a previous study (Kimura & Yanagimachi, 1995), removal of the cytoplasm had a significant effect on normal male pronucleus formation following either post- or pre-activation protocols, with fertilization rates of 45% versus 60% (with and without the cytoplasm, respectively) in the post-activation protocol, and 38% versus 56%, respectively, in the pre-activation protocol (Table 1). Although the fertilization rates after ROSI without the cytoplasm were similar in these two protocols, the type of abnormal fertilization differed. Small male pronuclei only developed in the pre-activation group (Table 1), as previously described by Kimura & Yanagimachi (1995).

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Table 1 Fertilization after ROSI

Injection protocol	ROSI method	No. of injected oocytes	No.(%) of surviving oocytes	No.(%) of normal fertilizations	No. (%) of abnormally fertilized eggs with: 2PN small mPN	2PN extra 2nd Pb	1PN –	1PN extra 2nd Pb	others[a]
Post-activation	With cytoplasm	186	164 (88)	73 (45)[b]	0	17 (10)	38 (23)	7 (4)	29 (18)
	Without cytoplasm	149	136 (91)	82 (60)[c]	0	13 (10)	21 (15)	2 (1)	18 (13)
Pre-activation (45–80 min)	With cytoplasm	290	286 (99)	109 (38)[d]	6 (2)	7 (2)	122 (43)	1 (0.3)	41 (14)
	Without cytoplasm	275	268 (97)	151 (56)[e]	16 (6)	4 (1)	75 (28)	3 (1)	19 (7)

2PN, two pronuclei; 1PN, one pronucleus; small mPN, failure of the male pronucleus to expand; Extra 2nd Pb, two second polar bodies (Kimura and Yanagimachi *et al.*, 1995).
[a] Others include eggs with 3 PN, eggs without a second polar body, eggs that failed to form any pronuclei, and eggs with an abnormal second polar body.
[b–e] Significant χ^2 comparisons b versus c, and d versus e, $p < 0.01$.

Table 2 Effect of cytoplasm removal on *in vitro* development

Injection protocol	ROSI method	No. of normal zygotes cultured	After 24 h culture: No. at 2-cell stage (%)	After 72 h culture: No. of morulae/ blastocysts (%)	After 96 h culture: No. of expanded blastocysts (%)[a]
Post-activation	With cytoplasm	57	54 (95)	49 (86)	40 (70)
	Without cytoplasm	74	73 (99)	65 (88)	48 (65)
Pre-activation (45–80 min)	With cytoplasm	55	54 (98)	46 (84)	35 (64)
	Without cytoplasm	57	53 (100)	45 (85)	37 (70)

The samples were cultured separately depending on the number of pronuclei 6 h after injection.
[a] No significant differences were observed between these four experimental groups.

Table 3 Effect of cytoplasm removal on *in vivo* development

Injection protocol	ROSI method	Normal zygotes	No. of transferred 2-cell embryos (%)	No. of recipient females	No. of pups	Offspring production rate per transferred embryo (%)[a]
Post-activation	With cytoplasm	93	92 (99)	13	45	48.9
	Without cytoplasm	112	110 (98)	17	55	50.0
Pre-activation (45–80 min)	With cytoplasm	87	85 (98)	12	35	41.2
	Without cytoplasm	104	101 (97)	15	49	48.5

The samples were cultured separately depending on the number of pronuclei 6 h after injection.
[a] No significant differences were observed between these four experimental groups.

In vitro and *in vivo* development after ROSI

Normal zygotes after ROSI were cultured continuously to examine their development. Ninety-six hours after fertilization, the rate of development into an expanded-blastocyst after ROSI without the cytoplasm was comparable to that of ROSI with the cytoplasm regardless of the protocol (Table 2). Next, the ability of these normal zygotes to develop to term after ROSI without the cytoplasm was also examined by transferring 2-cell embryos from normal zygotes into surrogate mothers. The rates of offspring production with ROSI without the cytoplasm (50% and 49%, for the post- and pre-activation protocols, respectively) were slightly higher than for ROSI with the cytoplasm (49% and 41%, respectively, Table 3) but not significantly different. This new method for separating spermatid nuclei from the cytoplasm results in normal *in vitro* and *in vivo* development after ROSI in the mouse.

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Discussion

A new separation method was developed to separate the cytoplasm from the spermatid nucleus more quickly. This technique successfully removed a constant amount of cytoplasm, as shown by mitochondria quantification (Fig. 2), and led to a significantly higher rate of fertilization following either the pre- or post-activation protocols compared with ROSI with cytoplasm (Table 1). Furthermore, the separated nuclear part resulted in rates of full-term development similar to those for whole spermatids (Table 3). In summary, as compared with ROSI with the cytoplasm, ROSI without the cytoplasm resulted in a significantly better overall performance defined as the offspring production rate calculated based on the number of oocytes surviving after injection. These data indicate that our new method can be used to produce offspring as an alternative to the 'repeatedly drawing in and out' method. The offspring production rates of 50% in the post-activation protocol and 49% in the pre-activation protocol are comparable to or higher than those previously reported using 'repeatedly drawing in and out' (28%, Kimura & Yanagimachi, 1995; 45%, Shamanski *et al*., 1999; 21%, Hayashi *et al*., 2003), suggesting that our new method causes less damage during cytoplasmic separation. In addition, no overt abnormalities were observed in the mice produced using this method.

It is known that spermatids injected into or electrically fused with oocytes show low fertilization rates for unknown reasons (Ogura *et al*., 1993; Kimura & Yanagimachi, 1995). The highest reported rate of normal fertilization (77%) was with ROSI using 'denuded' round spermatids following the pre-activation protocol, regardless of the presence of the spermatid cytoplasm (Kimura & Yanagimachi, 1995). This suggests that the injected cytoplasm from round spermatids does not lower the rate of normal fertilization *per se*. Therefore, the rate of normal fertilization after ROSI without cytoplasm (56%) that we obtained was probably caused by the separation of the cytoplasmic part of the cell rather than by its removal. Our relatively low rate of normal fertilization might have resulted from resealing of the nucleus by the cytoplasmic membrane after separation.

Although using our new separation method with the post-activation protocol produced offspring, it did not significantly improve the success rate of ROSI (49% versus 50%). This suggests that injected cytoplasm from round spermatids including organelles does not affect the subsequent embryonic development of normal fertilized zygotes after ROSI. However, it is still possible that the remaining cytoplasm surrounding the separated spermatid nucleus lowers the success rate of ROSI. For example, mRNA from round spermatids, which results in differential gene expression in the embryo after ROSI (Hayashi *et al*., 2003), might remain in the separated spermatid nuclei. It has been suggested that mRNA delivered to the oocytes by spermatozoa contributes to early development (Ostermeier *et al*., 2004). Future study should examine the effect of injected RNA on the success rate of ROSI.

What is the appropriate protocol for ROSI in mice? A previous attempt to improve ROSI by isolating and storing round spermatid nuclei was unsuccessful, even with a nuclear isolation medium that was effective for storing nuclei (Suzuki *et al*., 1998). At present, previously activated oocytes are thought to be the best recipients for ROSI after the 'denudation' of round spermatids (Kimura & Yanagimachi, 1995). However, we showed that isolated nuclei could support full-term development with high offspring production rates following either the post- or pre-activation protocols (50% versus 49%, respectively). In addition, there was no significant difference between the overall performance with each protocol (30% versus 27%, for the post- and pre-activation protocols, respectively). This seems to be consistent with our observation that offspring can be produced at comparable rates by ICSI using either the pre- or post-activation protocols (Kishigami *et al*., 2004). In conclusion, our new separation method allows us to increase the success rate of ROSI in mice through the improvement of the fertilization rates following either pre- or post-activation protocols.

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