Zygote

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Effects of high calcium levels on the disturbed extrusion of the second polar body during *in vitro* fertilization in C3H/He mouse substrains

Y. Ohta-Takada[1,2,3,*], Y. Nagao[2] and S. Kito[3,†]

[1]Science Service Inc., Chiba, Japan; [2]University Farm, Faculty of Agriculture, Utsunomiya University, Tochigi, Japan and [3]Department of Technical Support and Development, National Institute of Radiological Sciences, National Institutes for Quantum and Radiological Science and Technology, Chiba, Japan

Summary

We previously reported that high concentrations (≥3.42 mM) of calcium during *in vitro* fertilization (IVF) disturbed the extrusion of the second polar body (PBII) in C3H/He inbred mice. In this study, the substrain specificity of this phenomenon was examined under 1.71–6.84 mM calcium concentration in ova from six C3H/He mouse commercially available substrains in Japan. PBII extrusion in ova from J substrains was not affected by calcium concentrations (<10% at any calcium level), but was grossly disturbed at high calcium levels in the ova of other substrains. This result has practical applications for the efficient production of normal zygotes by IVF, therefore contributing to the reduction in the numbers of donor animals for further zygote or embryo manipulation. Care must be taken in choosing IVF medium for particular strains and substrains.

Short Communication

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Author for correspondence:
Seiji Kito, Centre of Animal Research and Education, Nagoya University, Furo-cho Chikusa, Nagoya, 464–8601, Japan.
Tel: +81 52 747 6875. Fax: +81 52 747 6874.
E-mail: sk126@care.nagoya-u.ac.jp

*Present address: Yakult Central Institute, Tokyo, Japan.

†Present address: Centre of Animal Research and Education, Nagoya University, Nagoya, Japan.

Introduction

Assisted reproductive technologies (ARTs) such as *in vitro* fertilization (IVF) and cryopreservation have become important techniques for the efficient maintenance of animals and production of novel genetically modified animals (Thornton *et al*., 1999). According to the 3R Principles of animal welfare, successful IVF conditions resulting in normal fertilization and subsequent development to term lead to reduction of animal usage in animal-based investigations. However, IVF conditions in previous studies were not necessarily satisfactory for all inbred mice, including the 129, BALB/c and C3H strains (Byers *et al*., 2006; Vasudevan and Sztein, 2012). We previously showed that using medium with a high calcium concentration (5.13 mM) during sperm:ova co-incubation resulted in successful fertilization in BALB/cA mice (Kito and Ohta, 2008) but not in C3H/He mice (more specifically C3H/HeNrs) in which such medium induced disturbed PBII extrusion in 30–50% of ova (Ohta *et al*., 2016). Although digynic triploid embryos developed to blastocysts that were indistinguishable from normal diploid embryos, they never developed into newborns. Therefore, conditions leading to abnormal fertilization should be avoided for efficient use of animals and gametes.

C3H/He inbred mice are used as a general-purpose strain in several biomedical research areas. In addition, C3H/He mice have various substrains (Whitmore and Whitmore, 1985). Therefore, it is important to check the phenotype of C3H/He substrains in ART such as IVF. To examine the substrain specificity of disturbed PBII extrusion induced by high calcium concentrations, we compared the disturbance frequencies under 1.71–6.84 mM calcium concentrations among six C3H/He mouse substrains commercially available in Japan.

Materials and Methods

The six substrains examined were C3H/HeJJcl, C3H/HeNJcl (CLEA Japan, Tokyo, Japan), C3H/HeSlc, C3H/HeJYokSlc, C3H/HeNSlc (Japan SLC, Shizuoka, Japan) and C3H/HeNCrlCrlj (Oriental Yeast, Tokyo, Japan). Mice were maintained in a conventional facility at 23 ± 2°C and 50 ± 10% humidity under a light regimen of 12L:12D conditions (lights on at 07:00 am). All animals were housed and maintained as previously described (Ohta *et al*., 2016).

Thirty 10–12-week-old females and four to six 12–16-week-old males with >60% sperm motility were used for each substrain. Detailed procedures of IVF using cumulus-free ova were the same as those described previously (Kito and Ohta, 2008; Ohta *et al*., 2016). To control variability among females, equal numbers of ova (10–20 ova per drop) from individual females were distributed in human tubal fluid with 1.71, 2.57, 3.42, 5.14 or 6.84 mM calcium. At 5 h post insemination, fixed ova were examined for PBII extrusion and formation of male and female pronuclei (PNs). Fertilized ova were those that had incorporated sperm nuclei or male PN

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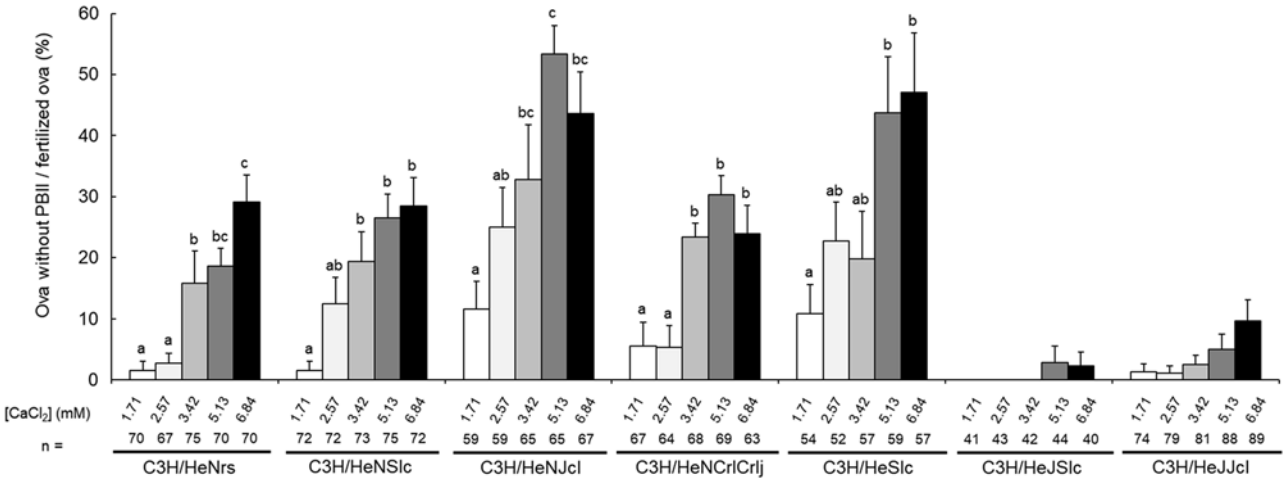

Figure 1. Percentages of ova with disturbed PBII extrusion out of fertilized ova in six C3H/He substrains. Data for C3H/HeNrs were extracted from table 1 in the publication by Ohta *et al.* (2016) with permission. n: Total number of fertilized ova examined. [a–c]Percentages with different superscripts within each substrain are significantly different ($P < 0.05$).

and ova with disturbed PBII were those with two female pronuclei instead of extruded PBII (Ohta *et al.*, 2016).

For each substrain, percentage data from four to six replicates, in which one male was used in each replicate, were transformed using arcsin transformation to analyse variance with a random block design, with each male assigned as a block (Ohta *et al.*, 2016; Kito and Ohta, 2008). Multiple comparisons within each substrain were made using Tukey's test at a probability of $P < 0.05$.

Results and Discussion

Fertilization was not affected by calcium concentration because more than 80% of the ova from all six substrains were fertilized at the various calcium concentrations. As shown in Fig. 1, only the two J substrains showed no significant increase in disturbed PBII extrusion at the various calcium concentrations (<10% of fertilized ova, $P > 0.05$). Ova of C3H/HeNJcl and C3H/HeSlc were extremely sensitive because more than 40% of fertilized ova were abnormal at calcium concentrations of 5.13 and 6.84 mM. We concluded that this phenomenon is specific for all the C3H/He substrains except for the J substrains (Fig. 1).

C3H/HeJ substrains are known to have a point mutation in Toll-like receptor 4 (Tlr4) alleles that causes cells with Tlr4 to become unresponsive to endotoxic lipopolysaccharide (Festing and Blackmore, 1971; Poltorak *et al.*, 1998). However, there is no report of detailed genetic analysis for genes other than Tlr4 in the C3H/He substrains. Genetic differences have been investigated in detail among the C57BL/6 substrains, including C57BL/6J and C57BL/6N (Simon *et al.*, 2013). In addition, phenotypic differences have been reported among the C57BL/6 substrains, especially in sperm freezing and *in vitro* fertilization (Liu *et al.*, 2009). Therefore, this study was performed to evaluate other phenotypic differences between C3H/He substrains.

In mouse ARTs, optimization of IVF conditions to produce normally fertilized ova with high consistency and efficiency allows for estimation of the number of animals required and the concomitant reduction in the number of animals used for zygote or embryo manipulation. Our study indicated that conditions such as high calcium levels during sperm:ova co-incubation are not recommended for IVF in C3H/He mice except for the J substrains. In the future, detailed genetic analysis and genotyping of single nucleotide polymorphisms (SNPs) to demonstrate genetic differences in C3H/He substrains need to be performed.

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Conflicts of interest. None.

Ethical standards. All animals were handled and treated according to the Recommendations for Handling of Laboratory Animals for Biomedical Research, compiled by the Institutional Animal Care and Use Committee for Laboratory Animal Experiments of the National Institute of Radiological Sciences.

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