NPPC/NPR2 signaling is essential for oocyte meiotic arrest and cumulus oophorus formation during follicular development in the mouse ovary

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Abstract

Natriuretic peptide type C (NPPC) and its high affinity receptor, natriuretic peptide receptor 2 (NPR2), have been assumed to be involved in female reproduction and have recently been shown to play an essential role in maintaining meiotic arrest of oocytes. However, the overall role of NPPC/NPR2 signaling in female reproduction and ovarian function is still less clear. Here we report the defects observed in oocytes and follicles of mice homozygous for \textit{Nppc}^{lbab} or \textit{Npr2}^{cn}, mutant alleles of \textit{Nppc} or \textit{Npr2} respectively to clarify the exact consequences of lack of NPPC/NPR2 signaling in female reproductive systems. We found that: i) \textit{Npr2}^{cn}/\textit{Npr2}^{cn} female mice ovulated a comparable number of oocytes as normal mice but never produced a litter; ii) all ovulated oocytes of \textit{Npr2}^{cn}/\textit{Npr2}^{cn} and \textit{Nppc}^{lbab}/\textit{Nppc}^{lbab} mice exhibited abnormalities, such as fragmented or degenerated ooplasm and never developed to the two-cell stage after fertilization; iii) histological examination of the ovaries of \textit{Npr2}^{cn}/\textit{Npr2}^{cn} and \textit{Nppc}^{lbab}/\textit{Nppc}^{lbab} mice showed that oocytes in antral follicles prematurely resumed meiosis and that immediately before ovulation, oocytes showed disorganized chromosomes or fragmented ooplasm; and iv) ovulated oocytes and oocytes in the periovulatory follicles of the mutant mice were devoid of cumulus cells. These findings demonstrate that NPPC/NPR2 signaling is essential for oocyte meiotic arrest and cumulus oophorus formation, which affects female fertility through the production of oocytes with developmental capacity.


Introduction

Natriuretic peptide type C (NPPC) is a member of the natriuretic peptide family, which also includes natriuretic peptide type A (NPPA) and natriuretic peptide type B (NPPB) in mammals (Potter \textit{et al.} 2006). NPPC exerts its biological action as a local factor by binding to a high affinity receptor, natriuretic peptide receptor 2 (NPR2), followed by the production of intracellular cGMP via the guanylyl cyclase catalytic domains of NPR2 (Suga \textit{et al.} 1992). NPPC has been shown to be associated with various physiological functions in mammals (Schulz 2005). In particular, its physiological role in regulating longitudinal bone growth has been revealed using genetically altered mice (Chusho \textit{et al.} 2001, Tamura \textit{et al.} 2004).

Expression of the \textit{Nppc} and \textit{Npr2} is higher in the ovary and uterus than in other tissues (Stepan \textit{et al.} 2000). These genes are also detected in granulosa cells and are modulated by the estrous cycle in rats (Jankowski \textit{et al.} 1997). In addition, cGMP is involved in the regulation of oocyte meiotic arrest (Tönnell \textit{et al.} 1990) and ovarian follicle survival (McGee \textit{et al.} 1997). These observations suggest that NPPC/NPR2 signaling may play an important role in female reproduction. Recently, Zhang \textit{et al.} (2010) reported that NPPC and NPR2 play an essential role in maintaining meiotic arrest of oocytes and that a significant number of oocytes in \textit{Nppc} or \textit{Npr2} mutant mice exhibit premature resumption at the late antral stage. However, the overall role of NPPC/NPR2 signaling in female reproduction, including follicle development, oocyte maturation, and ovulation, remains to be elucidated.

Achondroplasia (CN) and long bone abnormality (LBAB) are spontaneous mutant mouse strains characterized by disproportionate dwarfism with short limbs and tails (Lane & Dickie 1968, Jiao \textit{et al.} 2007). Missense mutations have been identified as the causative genetic alterations in the \textit{Npr2} and \textit{Nppc} genes of the CN and LBAB mice respectively (Tsuji & Kunieda 2005, Jiao \textit{et al.} 2007). These mutations, represented as \textit{Npr2}^{cn} and \textit{Nppc}^{lbab}, have been confirmed to impair function of the NPPC/NPR2/cGMP system (Tsuji & Kunieda 2005, Tsuji \textit{et al.} 2008). In this study, we investigated detailed phenotypes in the oocytes and ovaries of \textit{Npr2}^{cn}/\textit{Npr2}^{cn} and \textit{Nppc}^{lbab}/\textit{Nppc}^{lbab} mice to elucidate the overall role of NPPC/NPR2 signaling in female reproduction.
Table 1 Mating test of Npr2<sup>cn/cn</sup>/Npr2<sup>cn/cn</sup> mice.

<table>
<thead>
<tr>
<th>Genotype of female mouse</th>
<th>Number of successful mating</th>
<th>Litter size (mean ± S.D.)</th>
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<tbody>
<tr>
<td>+/?(n=6)</td>
<td>6</td>
<td>6.6±2.7</td>
</tr>
<tr>
<td>Npr2&lt;sup&gt;cn/cn&lt;/sup&gt;/(n=5)</td>
<td>5</td>
<td>0</td>
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Results

Fertility of Npr2<sup>cn/cn</sup> female mice

To confirm that the lack of NPPC/NPR2 signaling results in defective reproductive ability in mice, the fertility of Npr2<sup>cn/cn</sup>/Npr2<sup>cn/cn</sup> and normal (+/?) female mice was examined by a mating test with normal male mice. Successful mating, as judged by the presence of a vaginal plug, was confirmed in five Npr2<sup>cn/cn</sup>/Npr2<sup>cn/cn</sup> female mice and six normal female mice. In addition, we observed all stages of the estrus cycle, namely, proestrus, estrus, metestrus, and diestrus stages, in the Npr2<sup>cn/cn</sup>/Npr2<sup>cn/cn</sup> mice based on the cytology of vaginal smears; however, the cycle of these stages was not as clear as in the normal (+/?) mice (data not shown). No signs of pregnancy or delivery of pups were observed in Npr2<sup>cn/cn</sup>/Npr2<sup>cn/cn</sup> mice, whereas the normal mice gave birth to an average of 6.6 pups (Table 1). These results indicate that although female mice lacking NPPC/NPR2 signaling have estrus cycle and mating ability, they are infertile.

Morphology of ovulated oocytes in Npr2<sup>cn/cn</sup>/Npr2<sup>cn/cn</sup> female mice

We assessed the oocytes ovulated from Npr2<sup>cn/cn</sup>/Npr2<sup>cn/cn</sup> mice to investigate the etiology of infertility. First, the number of ovulated oocytes was counted in both naturally ovulated and superovulated Npr2<sup>cn/cn</sup>/Npr2<sup>cn/cn</sup> female mice at 10–12 weeks of age. As shown in Fig. 1, although the number of oocytes was significantly lesser in naturally ovulated Npr2<sup>cn/cn</sup>/Npr2<sup>cn/cn</sup> mice than in normal (+/?) mice, ovulation of oocytes was confirmed in Npr2<sup>cn/cn</sup>/Npr2<sup>cn/cn</sup> mice. Furthermore, no significant difference was found in the number of oocytes between the superovulated Npr2<sup>cn/cn</sup>/Npr2<sup>cn/cn</sup> and normal (+/?) mice. These findings indicated that the follicular development and ovulation processes appear to be unaffected in Npr2<sup>cn/cn</sup>/Npr2<sup>cn/cn</sup> mice and that the infertility of Npr2<sup>cn/cn</sup>/Npr2<sup>cn/cn</sup> female mice is not caused by lack of ovulated oocytes.

However, no cumulus cells were observed around the ovulated oocytes of Npr2<sup>cn/cn</sup>/Npr2<sup>cn/cn</sup> mice, whereas oocytes were ovulated as oocyte–cumulus cell complexes in normal mice, implying aberrant development of cumulus cells in the ovaries of Npr2<sup>cn/cn</sup>/Npr2<sup>cn/cn</sup> mice (data not shown). Furthermore, under stereomicroscopic observation, a significant number of the ovulated oocytes of Npr2<sup>cn/cn</sup>/Npr2<sup>cn/cn</sup> mice appeared to be fragmented. Immunocytochemical staining using anti-α-tubulin antibody revealed that the ovulated oocytes of the normal mice were arrested at MII stage, as demonstrated by appropriate meiotic spindle structure and chromosome alignment and a clearly evident excreted polar body (Fig. 2A). In contrast, all oocytes collected from the superovulated Npr2<sup>cn/cn</sup>/Npr2<sup>cn/cn</sup> mice showed condensed chromatin with or without fragmented cytoplasm, and no meiotic spindle or chromosome configurations were observed (Fig. 2B). At 1.5 days after coitus, the fertilized eggs collected from the normal female mice had reached the two-cell stage (Fig. 2C), whereas those collected from the Npr2<sup>cn/cn</sup>/Npr2<sup>cn/cn</sup> mice exhibited morphologically abnormal with fragmented or degenerated cytoplasm (Fig. 2D). These results indicate that abnormal oocytes with no developmental capacity were ovulated in the Npr2<sup>cn/cn</sup>/Npr2<sup>cn/cn</sup> mice.

Histology of ovaries of Npr2<sup>cn/cn</sup>/Npr2<sup>cn/cn</sup> female mice

The ovaries of Npr2<sup>cn/cn</sup>/Npr2<sup>cn/cn</sup> mice were histologically examined to determine the nature of abnormalities occurring during oogenesis. In the ovaries of 12-week-old Npr2<sup>cn/cn</sup>/Npr2<sup>cn/cn</sup> mice, primary, preantral, and antral stage follicles were observed, as in normal mice, but the gross appearance of the ovaries in these mice was smaller than those of normal mice. In normal mice, oocytes in the follicles at these stages are arrested at prophase of the first meiotic division until the induction of maturation by the preovulatory LH surge, which is characterized by the presence of a visible germinal vesicle (GV) as shown in Fig. 3A. However, in antral follicles of the Npr2<sup>cn/cn</sup>/Npr2<sup>cn/cn</sup> mice, almost all of the oocytes had no clear GV and showed apparent morphological abnormalities such as the presence of condensed chromatin (Fig. 3B) and fragmented ooplasm (Fig. 3C), indicating precocious resumption of meiosis.
followed by degeneration. The proportion of oocytes with condensed chromatin or fragmented ooplasm in Npr2cn/Npr2cn mouse was 70.5 or 29.5% (n = 62; the number of oocytes examined in three ovaries) respectively. In normal mice, all oocytes examined (n = 103) were at GV stage.

To further confirm the precocious resumption and degeneration of the oocytes in the Npr2cn/Npr2cn, Nppc1bab/Nppc1bab and normal (+/+) mice. Ovulated oocytes collected at MI stage from normal (A and E; littermates of B and F respectively), Npr2cn/Npr2cn (B), and Nppc1bab/Nppc1bab (F) mice. Fertilized eggs at the two-cell stage collected from normal (C) and Npr2cn/Npr2cn (D) mice.

along with the surrounding cumulus cells as a cumulus-oocyte complex. However, despite no significant difference in the granulosa cell layers of the preantral follicles, the cumulus oophorus surrounding the oocytes in the antral follicles of 12-week-old Npr2cn/Npr2cn mice were markedly thinner than those of normal mice (Fig. 3B and C). Furthermore, 50% of oocytes (n = 54) were devoid of cumulus cells in the periovulatory follicles of Npr2cn/Npr2cn mice (Fig. 3E and F), which accounts for the lack of cumulus cells surrounding the ovulated oocytes of mutant mice.

**Morphology of ovulated oocytes and histology of ovaries of Nppc1bab/Nppc1bab female mice**

To further demonstrate that NPPC signaling through NPR2 is crucial for oogenesis and follicle development, we investigated the ovulated oocytes and ovaries of mutant mice (Nppc1bab/Nppc1bab) carrying a mutation in the Nppc gene. Since the Nppc1bab mice of the original LBAB strain established by the Jackson Laboratory (Bar Harbor, ME, USA) exhibit early postnatal mortality, we were not able to obtain adult homozygous mouse of this strain. Therefore, we substituted the genetic background of the mutant strain with that of the CN strain (Npr2cn/Npr2cn) to obtain adult homozygous mice for morphological examination of oocytes and ovaries. The Nppc1bab/ + mice were backcrossed for more than eight generations to wild-type (+/+). Ovulated oocytes of mutant mice were collected 9 h after human chorionic gonadotropin (hCG) injection and examined to observe the progression of abnormalities in oocytes immediately before ovulation. In normal mice, oocytes of periovulatory follicles showed a metaphase spindle and underwent typical meiosis with hCG stimulation. However, in the Npr2cn/Npr2cn, Nppc1bab/Nppc1bab mice were collected 9 h after human chorionic gonadotropin (hCG) injection and observed the progression of abnormalities in oocytes immediately before ovulation.
homzygous female mice were obtained by sib-mating of these Nppc\(^{\text{lbab}}\)/Nppc\(^{\text{lbab}}\) mice with the CN genetic background. No signs of pregnancy or delivery of pups in Nppc\(^{\text{lbab}}\)/Nppc\(^{\text{lbab}}\) female mice after continuous housing with male mice were confirmed. MII-stage oocytes of heterozygous (Nppc\(^{\text{lbab}}\)/Nppc\(^{\text{lbab}}\)) mice showed normal meiotic spindle structures and chromosome alignments (Fig. 2E), whereas oocytes ovulated from the resultant Nppc\(^{\text{lbab}}\)/Nppc\(^{\text{lbab}}\) female mice showed condensed chromosomes (Fig. 2F), similar to those of the Npr2\(^{cn}\)/Npr2\(^{cn}\) mice. In the ovaries of the 12-week-old mice, oocytes in the antral follicles of normal mice were maintained at the GV stage (97.5%, \(n = 81\); Fig. 4A), whereas those of Nppc\(^{\text{lbab}}\)/Nppc\(^{\text{lbab}}\) mice resumed meiosis and developed a metaphase spindle (59.3%, \(n = 59\); Fig. 4B) or fragmented ooplasm (39.0%; Fig. 4C). In contrast to a typical cumulus expansion of normal mice that were treated with PMSG and hCG (Fig. 4D), oocytes immediately before ovulation exhibited a dispersed configuration of chromosomes (18.4%, \(n = 49\)) or fragmented ooplasm (44.9%) in the ovaries of Nppc\(^{\text{lbab}}\)/Nppc\(^{\text{lbab}}\) mice (Fig. 4E and F). Furthermore, the cumulus oophorus in the antral follicles of the Nppc\(^{\text{lbab}}\)/Nppc\(^{\text{lbab}}\) mice was markedly thinner (Fig. 4B and C), and 53% of oocytes (\(n = 49\)) in the periovulatory follicles were devoid of cumulus cells (Fig. 4E and F), as in the Npr2\(^{cn}\)/Npr2\(^{cn}\) mice. These results indicate that the abnormalities observed in the ovaries of Npr2\(^{cn}\)/Npr2\(^{cn}\) and Nppc\(^{\text{lbab}}\)/Nppc\(^{\text{lbab}}\) mice were apparently identical, confirming that the NPPC signaling through NPR2 is crucial for oocyte meiotic arrest and cumulus oophorus formation in mice.

**Discussion**

In this study, we revealed that: i) Npr2\(^{cn}\)/Npr2\(^{cn}\) female mice are infertile despite successful mating and ovulation; ii) the oocytes of mice homozygous for Npr2\(^{cn}\) or Nppc\(^{\text{lbab}}\) prematurely resume meiosis in antral follicles and exhibit abnormal morphology, such as condensed chromatin or fragmented ooplasm, with no cumulus oophorus in periovulatory follicles; and iii) ovulated oocytes of mice homozygous for Npr2\(^{cn}\) or Nppc\(^{\text{lbab}}\) have no developmental capacity.

Premature resumption of meiosis caused by defective NPPC/NPR2 signaling has been reported by Zhang et al. (2010) using Npr2\(^{cn-2j}\)/Npr2\(^{cn-2j}\) and Nppc\(^{\text{lbab}}\)/Nppc\(^{\text{lbab}}\) mice. However, it is notable that the premature resumption in these mutant mice was not fully proven to be caused by defective NPPC/NPR2 signaling. For example, the ovaries of the Nppc\(^{\text{lbab}}\)/Nppc\(^{\text{lbab}}\) mice were not collected under natural conditions. The authors transplanted the ovaries of Nppc\(^{\text{lbab}}\)/Nppc\(^{\text{lbab}}\) mice into the kidney capsules of normal mice to reveal the importance of NPPC produced in the ovaries for oocyte meiotic arrest. Furthermore, it has not yet been confirmed whether Npr2\(^{cn-2j}\) has disruption of the Npr2 gene. A mutation of Npr2 gene in Npr2\(^{cn-2j}\) was assumed from an allelism test using mice with the Npr2\(^{cn}\) allele, but the causative mutation of Npr2\(^{cn-2j}\) has not yet been identified. In contrast, we used adult mice homozygous for the Nppc\(^{\text{lbab}}\) mutation by introducing the genetic background of the CN mutant mice to overcome the early death of the mutant mice; we also used Npr2\(^{cn}\)/Npr2\(^{cn}\) mice, in which loss-of-function mutation of the Npr2 gene has been identified (Tsuji & Kunieda 2005). Zhang et al. (2010) reported that only 80 and 50% of oocytes in late antral follicles of Npr2\(^{cn-2j}\)/Npr2\(^{cn-2j}\) and Nppc\(^{\text{lbab}}\)/Nppc\(^{\text{lbab}}\) mice respectively had resumed meiosis. We further revealed that following the premature resumption of meiosis, most of the oocytes showed abnormal morphology with condensed chromatin or fragmented ooplasm immediately before ovulation. These results provide concrete evidence for the etiology of female mouse infertility caused by defective NPPC/NPR2 signaling.

Our data also provide novel findings regarding the role of NPPC/NPR2 signaling in the formation of the cumulus oophorus. We found that cumulus cells surrounding oocytes of Npr2\(^{cn}\)/Npr2\(^{cn}\) and Nppc\(^{\text{lbab}}\)/Nppc\(^{\text{lbab}}\) mice were significantly decreased in the antral follicles and were absent in periovulatory follicles. During follicular development, oocyte-secreted factors, such as growth differentiation factor-9 and bone morphogenetic protein-15, play crucial roles in controlling proliferation,
The presence of all follicular stages and ovulation of oocytes in mutant mice. In the present study, we confirmed the morphological differences in ovarian histology in the other hand, Zhang found in the ovaries of NPR2-deficient mice. On the that only primordial through secondary follicles were used in the present study and those used by Tamura et al. between the NPPC/NPR2-deficient mice used in follicular development of these mutant mice will cumulus cells directly or indirectly, and further study of implicated in the proliferation and/or maintenance of cumulus cells of antral follicles (Zhang et al. 2010). Therefore, the NPPC signal may exert its effect directly on cumulus cells through NPR2 and would thus be implicated in the proliferation and/or maintenance of cumulus cells. The effects of NPPC signal could affect the cumulus cells directly or indirectly, and further study of the follicular development of these mutant mice will provide novel findings regarding the interaction between oocytes and cumulus cells.

There are significant differences in follicular development between the NPPC/NPR2-deficient mice used in the present study and those used by Tamura et al. (2004) and Zhang et al. (2010). Tamura et al. (2004) reported that only primordial through secondary follicles were found in the ovaries of NPR2-deficient mice. On the other hand, Zhang et al. (2010) reported no major morphological differences in ovarian histology in the mutant mice. In the present study, we confirmed the presence of all follicular stages and ovulation of oocytes in both Npr2cn+/Npr2cn− and NppcIbab/NppcIbab mice, but found significant decreases in the number of cumulus cells in the antral follicles of both mutants. The reason for these differences in ovarian phenotypes is unknown, but a possible explanation is the different levels of residual NPPC/NPR2 activity caused by different mutations in the Npr2 or Nppc genes of these mutant mice. Exons 3 to 7 of the Npr2 gene were removed in the knockout mouse used by Tamura et al. (2004), resulting in null mutation of the gene. Missense mutations have been found in the Npr2cn− and NppcIbab used in the present study, which may raise the possibility for residual NPPC/NPR2 signaling in these mice. Actually, we have revealed that NPPC derived from the NppcIbab allele retained a slight ability to induce cGMP production through its receptor NPR2 (Tsuji et al. 2008). As mentioned earlier, no detailed information on Npr2cn−, including the type of mutation and the residual activity of NPR2, is currently available. Ovaries transplanted in normal mice were used to assess the ovaries of the NppcIbab/NppcIbab mice; therefore, considerable effects of the host mouse, including circulating NPPC, could not be excluded. Considering these differences, the different levels of residual NPPC/NPR2 activity in these mutant mice may be attributed to the different phenotypes of follicular development.

Both Nppc and Npr2 are expressed in gonadotroph cells of the anterior pituitaries (McArdle et al. 1993) as well as in GNRH-secreting cells of the hypothalamus (Olesc et al. 1994, Herman et al. 1996, Middendorf et al. 1997). In addition, the promoter activity of Nppc has shown to be stimulated by GNRH stimulation (Thompson et al. 2009). In the present study, the number of oocytes at natural ovulation was significantly decreased in the Npr2cn+/Npr2cn− mice, but no difference was found in the number of ovulated oocytes in the superovulated Npr2cn+/Npr2cn− mice. In addition, the Npr2cn+/Npr2cn− mice showed no clear estrus cycles, although all stages of the estrus cycle were observed. Thus, NPPC/NPR2 signaling may contribute to the regulation of FSH and/or LH levels via GNRH-secreting cells or GNRH-responsive cells. Further investigations using NPPC/NPR2-deficient mice would help to clarify whether NPPC/NPR2 signaling is actually associated with the regulation of gonadotropin level in the hypothalamic–pituitary axis.

In conclusion, our data demonstrate that defective NPPC/NPR2 signaling in mice results in female infertility due to developmentally impaired oocytes. Since our study suggested that NPPC/NPR2 signaling is involved in oocyte meiotic resumption, ovulation rate, and the formation of the cumulus oophorus, further evidence of the roles of NPPC/NPR2 signaling in these events will be informative for therapeutic treatment of human infertility and for improving the reproductive ability of animals.

Materials and Methods

Animals

Heterozygous Npr2cn+/− and NppcIbab+ mice were obtained from the Jackson Laboratory. Mice homozygous for Npr2cn− and their normal littermates (+/? ) were obtained by sib-mating heterozygotes (Npr2cn+−). Mice homozygous for NppcIbab and their normal littermates (+/? ) were obtained by sib-mating of heterozygous (NppcIbab−) mice that were backcrossed for more than eight generations to wild-type (+/+ ) mice of the CN mouse strain. The genotypes of the Npr2 and Nppc genes in these mice were determined by PCR–restriction fragment length polymorphism, as described previously (Tsuji & Kunieda 2005, Tsuji et al. 2008). All animal experiments were approved by the Animal Committee of Okayama University and were conducted in accordance with the Guidelines for Animal Experiments at Okayama University.
Collection of ovulated oocytes and embryos

For the collection of ovulated oocytes and embryos, female mice were euthanized and their oviducts were removed from the ovaries. The oviducts were placed between two glass microscope slides and the number of ovulated oocytes was then counted by observing the ampullae of the oviducts under a stereoscopic microscope. For natural ovulation, the number of oocytes was counted in untreated female mice on the day when vaginal plugs were observed after mating with male mice. Superovulation was achieved by an i.p. injection of 5 IU PMSG followed by 5 IU hCG 48 h later. The number of oocytes from these superovulated mice was counted 14–16 h after the second injection. Data are expressed as mean±S.D. and statistical significance was determined using Student’s t-test. Two-cell stage embryos were obtained from the oviducts of the female mice mated with normal male mice at 32–36 h after the hCG injection on the day following confirmation of the presence of a vaginal plug.

Oocyte immunocytochemistry

Ovulated oocytes were collected from the oviducts of the mice 14–16 h after the hCG injection. For immunocytochemical analysis, the oocytes were briefly incubated with 0.3 mg/ml hyaluronidase in PBS and fixed for 1 h in 2% formaldehyde. The oocytes were then treated with 1% Triton X-100 for 20 min and incubated with anti-z-tubulin antibody (Sigma) for 1 h at 20°C to visualize tubulin. After washing with PBS containing 0.1% Triton X-100, the oocytes were reacted with FITC-conjugated goat anti-mouse IgG antibody for 1 h at 20°C. The oocytes were washed and finally mounted in Vectashield (Vector Labs, Burlingame, CA, USA) supplemented with DAPI.

Histological evaluation of ovarian follicles

Ovaries used for histological analysis were collected from female mice at 3 and 12 weeks of age. The 3-week-old mice were treated with 5 IU PMSG and their ovaries were subsequently excised 9 h after the hCG injection to observe oocytes immediately before ovulation. The ovaries were fixed overnight in Bouin’s solution, dehydrated in ethanol, and embedded in paraffin. Specimens were sectioned at 5-μm thickness and stained with hematoxylin and eosin.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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