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Superovulatory Response, Oocyte Spontaneous Activation, and Embryo Development in WMN/Nrs Inbred Rats

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Abstract: WMN/Nrs inbred rats have been widely used in radiation biology for years. However, their reproductive profile has never been examined. We examined various reproductive characteristics of WMN/Nrs inbred rats such as superovulatory response, oocytes spontaneous activation (OSA), and embryo development *in vitro* and *in vivo*. Superovulation was induced in 3- to 9-week-old females by injection of 150 IU/kg PMSG and 150 IU/Kg hCG by 48 h apart. Only 8- and 9-week-old animals superovulated averaging 31.4 and 43.9 oocytes, respectively, and superovulation did not depend on estrous cycle. Animals 3–7 weeks of age did not superovulate. Because Wistar strains have been known to show a high incidence of OSA, factors expected to affect OSA in WMN/Nrs, including the time interval of various steps from euthanasia to oocyte recovery, incubation media, estrous cycle, and anesthetic treatments, were examined. The time from animal euthanasia to oviduct excision was the only factor shown to affect OSA. We also compared *in vitro* and *in vivo* embryo developmental competence between embryos obtained by natural ovulation and superovulation. Although percent *in vitro* development of 2-cell embryos to blastocysts was similar for embryos obtained by natural ovulation (63.7%) and superovulation (69.7%), fetus development after oviductal transfer of 2-cell embryos was significantly lower in embryos obtained by superovulation than in those obtained by natural ovulation (60.2% vs. 87.5%, P=0.02). Our results provide important normative data regarding future applications of rat assisted reproductive technologies (ARTs) such as *in vitro* fertilization and cryopreservation in WMN/Nrs strain and may be applicable to other strains of laboratory rats.

Key words: embryo development, inbred WMN/Nrs rats, oocyte spontaneous activation, superovulation

Introduction

In vitro manipulation of gametes and embryos is a fundamental technique practiced in assisted reproductive technologies (ARTs) and various basic researches in mammalian development. For these purposes, the successful collection of oocytes or embryos with full developmental competence is essential. In mice and hamsters, superovulation followed by *in vitro* fertilization is well established [3, 7]. Although the number of superovu-

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lated oocytes varies depending on strain among inbred mice, sufficient numbers of oocytes and embryos for practical and research use can be easily obtained. However, researchers often encounter difficulty obtaining sufficient numbers of oocytes and embryos from laboratory rats due to their limited responses to superovulatory treatment.

Various methods, such as the PMSG-hCG injection regimen used in mice [4, 15, 24–26, 33, 42, 44], continuous infusion of the gonadotropin [1], and anti-inhibin antiserum treatment [12] have been shown to induce superovulation in rats. Among these methods, the PMSG-hCG injection regimen seems to be the most practical and economical method of inducing superovulation and may be applied to a wide range of ages in Wistar rats [15, 21, 26, 30, 33]. However, quantitative data on the actual use of this technique in rats is limited to studies of juvenile rats and a few adult strains. Another issue with superovulation is the impaired quality of superovulated oocytes or embryos compared to naturally ovulated ones. Data reported for mice and hamsters are controversial, with some studies showing significant differences in quality and other showing no difference [5, 6, 22, 23, 32, 34, 37]. However, in rats the quality of superovulated oocytes or embryos, as indicated by developmental ability to the fetus, has not been directly compared with that obtained by natural ovulation [10, 30, 33, 40], and such information is essential for estimating the efficiency of animal production and the normality of embryos. Thus, obtaining normative information concerning superovulation and the quality of superovulated oocytes or embryos on a strain-by-strain basis, especially for inbred rats, would be quite useful for ARTs of laboratory rats.

One problem in handling rat oocytes is that they often activate spontaneously and release the second polar body when excised from oviductal environments without forming the pronucleus [17, 45, 46]. This abortive phenomenon is specific to rat oocytes and has been called 'oocyte spontaneous activation' (OSA) [17]. In spontaneously activated oocytes, the chromosomes are scattered around the cytoplasm or form the metaphase III spindle [17, 46]. Thus, a method to overcome OSA has been important issue in the application of ARTs, such as *in vitro* fertilization and nuclear transfer for the production of cloned animals. The mechanisms underlying OSA are likely to be involved in reduction of the activity of cytostatic factor, and the use of calcium-free medium or addition of reagents to stabilize cytostatic factor, such as MG132, is effective to reduce the incidence of OSA [11, 10, 14, 47]. However, the influence of these conditions on oocyte quality, especially fertilization and developmental competence, has been detrimental thus far [10, 11]. Another factor known to reduce OSA is the time interval from animal sacrifice to oocyte collection [17]. Keefer and Schuetz reported that when oocytes were collected within 5 min of animal sacrifice, the incidence of OSA was significantly reduced [17]. However, it is not clear which steps in the procedure of oocyte collection (e.g., oviduct excision, oocyte retrieval from the oviduct, or the removal of cumulus cells) should be performed within what time interval. Further information on how variations in techniques of oocyte collection influence quantity and quality of oocytes and embryos obtained from laboratory rats is needed.

In this study, we obtained normative information on the reproductive profile of an inbred strain of Wistar rats, called WMN/Nrs (National BioResource Project Rat No: 0439), formerly known as WM/MsNrs or Wistar/MsNrs rats. This strain of rats is commonly used in radiobiological research to evaluate the effects of various sources of radiation on whole body animals [8, 27, 28].

Materials and Methods

Animals

Animals used in this study were kindly provided by Drs. K. Anzai and M. Ueno of the National Institute of Radiological Sciences who purchased them from SLC Inc. (Shizuoka, Japan). Animals were maintained under conventional conditions at 22 ± 2°C and 55 ± 10% humidity under a lighting regimen of 12 light: 12 dark (lights on from 07:00 to 19:00) with free access to food (MB-1, Funabashi Farm Co., Ltd., Chiba, Japan) and water. Animal treatment and experimental procedures were approved by the Institutional Animal Care and Use Committee of the National Institute of Radiological Sciences, Japan. Under the conditions employed in this study, most mature WMN/Nrs females showed 4-day

estrous cycles. An average of 12.2 embryos (range 6–17 embryos) and an average litter size of 10.2 (n=31, range 5–14) were observed during natural ovulation and mating (Ueno M., personal communication). Body weights of WMN/Nrs females were 30.2 ± 0.5 g at 3 weeks of age, 50.7 ± 0.8 g at 4 weeks, 76.2 ± 1.2 g at 5 weeks, 97.9 ± 1.1 g at 6 weeks, 117.0 ± 1.4 g at 7 weeks, 134.5 ± 1.6 g at 8 weeks and 147.0 ± 2.0 g at 9 weeks of age (n=15) (Yamada Y., personal communication).

Hormone treatment

Female rats at 3–9 weeks of age were injected intraperitoneally with 150 IU/kg of pregnant mare serum gonadotropin (PMSG; Serotropin; ASKA Pharmaceutical Co., Ltd., Tokyo, Japan), and 150 IU/kg of human chorionic gonadotropin (hCG, Gonatropin; ASKA Pharmaceutical Co., Ltd.) 48–50 h later at 17:00–19:00. Theses amounts of gonadotropins were determined in a preliminary study in which the ovulatory response after injection of the above amounts (8/8, 100%) to 8-week-old females was better than that induced by 150 IU/Kg PMSG and 75 IU/Kg hCG (6/8, 75%), which used in previous studies [26, 33]. When necessary, the estrous cycle was examined by examining vaginal smears between 11:00–12:00 am.

Counting ovulated oocytes

Oviducts were excised from the animals and immersed in mineral oil (M8410, Sigma-Aldrich St. Louis, MO, USA) warmed at 37°C after cervical dislocation at 17–18 h post hCG. The cumulus and oocytes complexes (COCs) were collected from oviducts by puncturing the ampullae with a 26G syringe needle. The cumulus cells were removed with 1 mg/ml bovine testis hyaluronidase (type I-S, H-3506, Sigma-Aldrich) containing 0.01 mg/ml soybean trypsin inhibitor (202-09221, Wako Pure Chemical Industries, Osaka, Japan) in HEPES-buffered modified Krebs-Ringers bicarbonate solution (mKRB) [29], in which 20 mM $NaHCO_3$ was replaced with 20 mM HEPES. Then, they were rinsed 3 times prior to counting the number of ovulated oocytes with normal morphology.

Factors affecting OSA

Oocytes used in this series of experiments were obtained from superovulated animals. In the first set of experiments, the time interval of each step from animal euthanasia to oocyte collection was varied as indicated in Fig. 2a. Female rats at 8–9 weeks of age were administered PMSG and hCG, and oocytes were obtained 17–18 h post hCG injection. In Treatment 1, the time interval from animal euthanasia to oviduct excision, in Treatment 2 from oviduct excision to COC collection, and in Treatment 3 from COC collection to incubation in medium was varied: 0, 1, 2, and 3 min. After 3 h incubation in 100 μl mKRB [29] under 5% CO_2 in air at 37°C, COCs had their cumulus cells removed by hyaluronidase as described above. Denuded oocytes were fixed in 2% formaldehyde and stained with Hoechst 33342 for examination of OSA by observation of chromosome distribution and extrusion of the second polar body. In the second set of experiments, the effects of incubation media, estrous cycle and anesthetic treatments on OSA were studied. Based on the results of the first set of experiments, COCs were collected from the oviduct immediately after animal euthanasia. To study the effects of the incubation media, COCs were divided into 3 pieces of the approximately the same mass and incubated in 100 μl of mKRB [29], TYH [38], or mHTF [19] for 3 h under 5% CO_2 in air at 37°C. These media were selected because they are possible candidate media for *in vitro* fertilization. To study the effects of the estrous cycle, females were examined by vaginal smear, then treated with gonadotropins for superovulation. To study the effect of anesthesia, oviducts were excised from superovulated female rats under anesthesia after 10 min of induction with Nembutal (Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan) at 50 mg/Kg BW, avertin (tribromoethanol) at 250 mg/Kg BW, or isoflurane (Escain®, Mylan Seiyaku, Tokyo, Japan) (2% at 0.2 l/min for induction and 1.5% at 0.15 l/min for maintenance), and COCs were incubated for 3 h in 100 μl mKRB under 5% CO_2 in air at 37°C. After 3 h incubation, COCs had their cumulus cells removed, and denuded oocytes were fixed and stained as in the first experimental series.

Collection, culture, and transfer of 2-cell embryos

To obtain embryos after natural ovulation, 8–9 week-old females at proestrus were caged with mature adult males (>12-week-old). To collect embryos from su-

Table 1. Ovulation of 3- to 9-week-old WMN/Nrs rats by superovulatory treatment[a)]

Age (weeks)	No. animals treated	No. ovulated animals (%)	No. ovulated oocytes with normal morphology (mean ± MSE)
3	6	2 (33.3)	16.0[b)] ± 5.0
4	3	3 (100)	12.3[b)] ± 5.6
5	6	4 (66.6)	11.0[b)] ± 4.5
6	6	2 (33.3)	13.0[b)] ± 1.0
7	5	2 (40)	14.0[b)] ± 0.0
8	16	15 (93.8)	31.4[c)] ± 2.6
9	16	13 (81.3)	43.9[d)] ± 3.0
9 (Natural ovulation)	12	N/A	12.2[b)] ± 0.6

[a)]Animals were injected 150 IU/Kg PMSG and hCG 48–50 h apart and ovulation was observed 17–18 h post hCG injection. [b, c, d)]Percentages with different superscripts are significantly different in the same column (P<0.05).

perovulated animals, females were injected with PMSG at 17:00 of metestrus, and 48 h later hCG was administered to only females at proestrus and caged with mature males. In the morning of the following day (day 1), mating was confirmed by observation of sperm in vaginal smears. At 15:00–16:00 of day 2, 2-cell embryos were collected by flushing the oviducts with modified Rat Embryo Culture Medium (mR1ECM) [29] equilibrated for 3 h at 5% CO_2 in air, as described previously [20]. To examine *in vitro* embryo development, groups of 8–16 embryos were cultured for 96 h in 50 μl mR1ECM supplemented with 3 mg/ml bovine serum albumin (A3311, Sigma-Aldrich) under 5% CO_2, 5% O_2, and 90% N_2 at 37°C. The embryo culture experiments were replicated 6 and 12 times for natural ovulation and superovulation, respectively. After scoring development, blastocysts were fixed in 2% formaldehyde and stained with Hoechst 33342 for nuclear counting as described previously [20]. To compare *in vivo* developmental competence of embryos obtained by natural ovulation and superovulation, 2-cell embryos were transferred to the oviducts of pseudopregnant recipients. To induce pseudopregnancy, mature female rats at proestrous were caged with a vasectomized male overnight, and only females with a vaginal plug on the following day (day 1) were used as recipients. Two-cell embryos were transferred to the oviducts on day 1 of pseudopregnancy under anesthesia by isoflurane (2% at 0.2 l/min for induction and 1.5% at 0.15 l/min for maintenance). Each recipient received 10–16 embryos because this is within the range of natural ovulation (6–17 oocytes/female). On day 20, recipient rats were sacrificed for observation of implantation and fetus development.

Statistical analysis

The number of ovulated oocytes and nuclear counting of blastocysts were calculated using raw data. For the experiments on OSA and development, the percentage data were transformed by the Tukey-Freeman arcsin transformation to control for unequal variances [43]. Analysis of variance (ANOVA) was performed using the SAS software (SAS Institute Inc., Cary, NC); least significant difference tests were used for multiple comparisons. A probability of P<0.05 was considered to be statistically significant.

Results

The superovulatory responses are shown in Table 1. Except for 4-week-old females, the number of females responding to hormonal treatment was lower among premature females (3–7 weeks of age, 33.3–66.6%) than among 8- and 9-week-old females (93.8 and 81.3%, respectively). In 3- to 7-week-old females, the average numbers of ovulated oocytes (11–16 oocyte/ovulated female) were similar to that obtained with natural ovulation (12.2 oocytes/female) and significantly lower than that seen in 8- and 9-week-old females (31.4 and 43.9 oocytes/female, respectively. P<0.05). In 8- and 9-week-old females, superovulation was induced independent of estrous cycle (Table 2). However, females never mated with males unless superovulation was induced synchro-

Table 2. Mean numbers (± MSE) of ovulated oocytes throughout estrous cycle of 8- and 9 week-old WMN/Nrs rats[a)]

Estrous cycle at PMSG injection	Age (weeks)	
	8 (n)	9 (n)
Proestrus	34.3 ± 6.6 (3)	45.7 ± 3.4 (3)
Estrus	30.5 ± 8.0 (4)	40.3 ± 7.4 (4)
Metestrus	27.8 ± 1.3 (4)	42.8 ± 2.3 (3)
Diestrus	33.9 ± 1.3 (4)	47.3 ± 11.9 (3)

[a)]Numbers of oocytes collected from 8- and 9-week-old females (Table 1) were further categorized based on the estrous cycle.

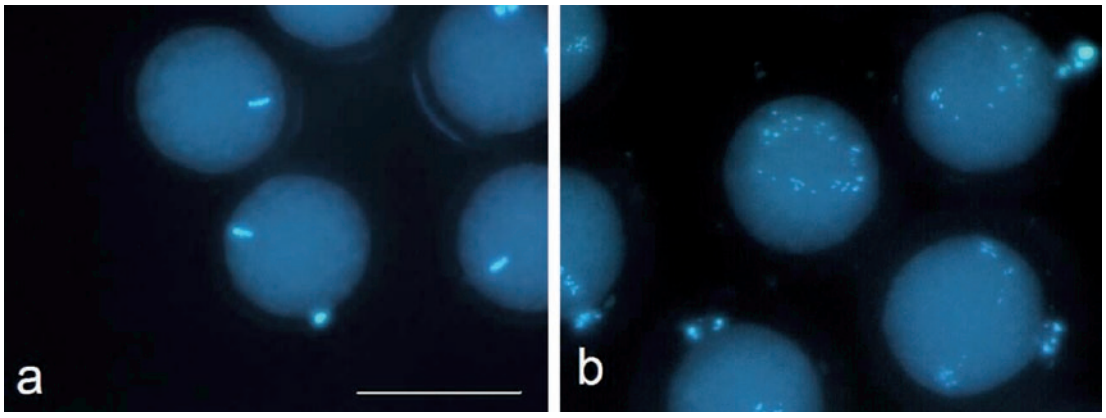

Fig. 1. Representative photos of spontaneously activated oocytes of WMN/Nrs rats stained by Hoechst 33342. (a) Metaphase II oocyte right after oocyte collection. (b) Spontaneously activated oocyte after 3 h incubation. Chromosomes are scattered in the cytoplasm. Bar in (a) indicates approximately 100 μm.

nously with the estrous cycle: i.e., PMSG injection on the day of metestrus and hCG injection and mating on the day of proestrus (Kito, personal observation).

In order to clarify which experimental procedures affect OSA in WMN/Nrs, the effects of the time interval of each step of oocyte collection, media, estrous cycle and anesthetic treatments were examined. Representative pictures of metaphase II oocytes and spontaneously activated oocytes with scattered chromosomes in WNM/Nrs rats are shown in Fig. 1. In the first set of experiments, in which we studied the effect of the time interval from animal euthanasia to oocyte collection, 0–3 min delays were inserted into all three steps: 1) from animal euthanasia to oviduct excision, 2) oviduct excision to COC-retrieval from the oviducts, and 3) COC-retrieval to transfer into the incubation medium (Fig. 2a). The results are shown in Figs. 2b–d. A significant increase in the incidence of OSA was observed only when the time interval from animal sacrifice to oviduct excision was longer than 1 min ($P<0.05$, Fig. 2b). In the other steps of oocyte collection, i.e., from oviduct excision to COC collection (Fig. 2c) and from the COC collection to incubation (Fig. 2d), the time interval had no effect on the incidence of OSA. In the second set of experiments, effects of incubation media, estrous cycle, and anesthetic treatment were examined. The results are presented in Figs. 3a–c. No significant increase in OSA was observed for any treatment (all $P>0.05$). It should be noted that incubation of COCs in mHTF which had a higher Ca^{2+} concentration (5.1 mM) than the other media (1.7 mM) had no significant effect on the incidence of OSA.

The results of *in vitro* development and fetus development after embryo transfer are shown in Tables 3 and 4, respectively. *In vitro* development of 2-cell embryos to the blastocysts stage and the expanded blastocyst stage

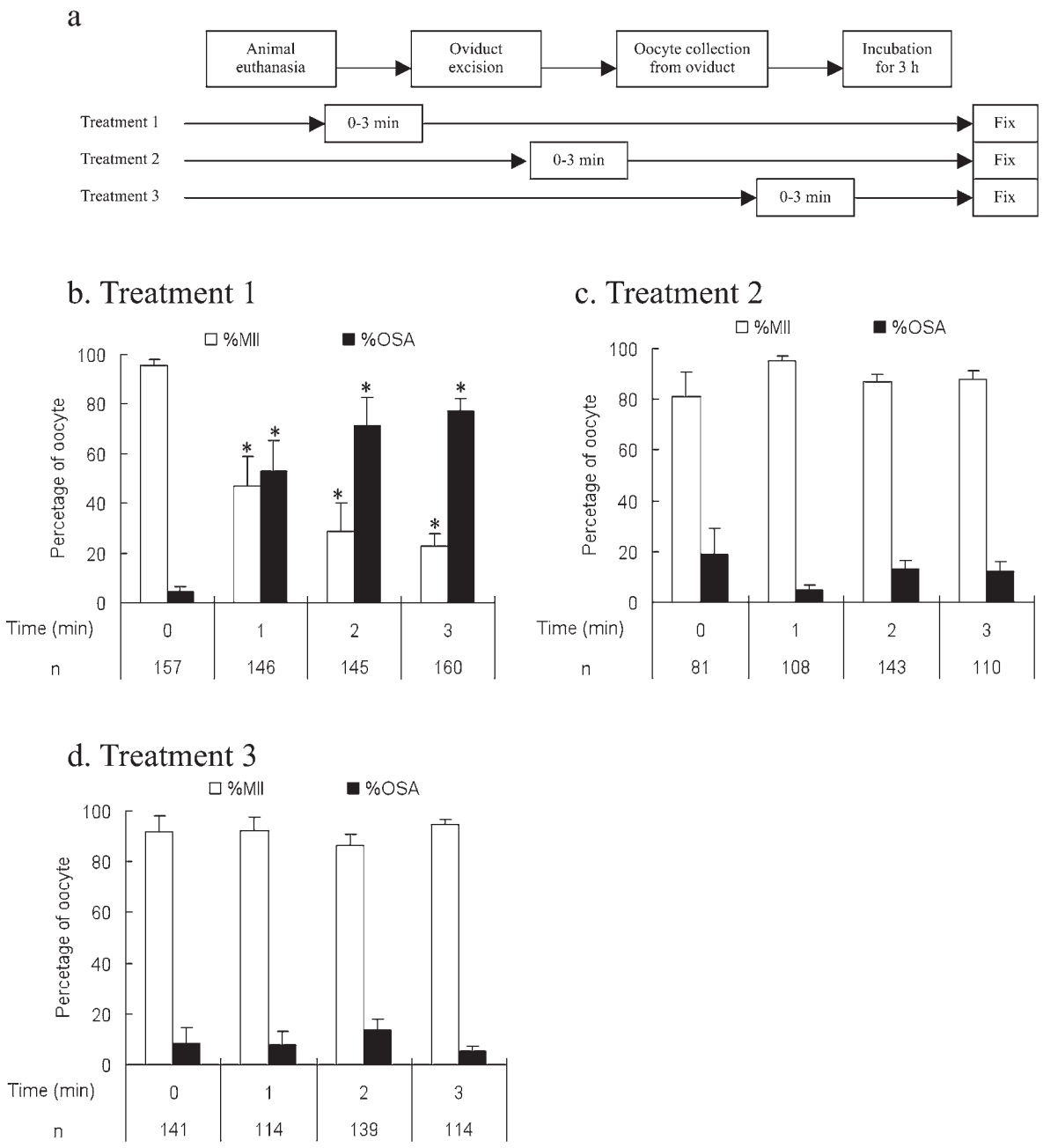

Fig. 2. Effects of time interval from animal euthanasia to COC collection on OSA of WMN/Nrs rats. (a) Protocol to study the effects of the time interval of each procedure on OSA. (b)–(d) Incidence of OSA after Treatments 1–3, respectively. Open bars indicate percentages of oocytes maintained at MII stage after 3 h incubation and filled bars indicate oocytes activated spontaneously. Error bars indicate mean standard errors (MSEs). Asterisks (*) in (b) indicate significant difference from 0 min group ($P<0.05$). No statistical significances among experimental groups were observed in (c) and (d). Four females were used in each experimental group. n: total number of oocytes.

as well as nuclear numbers of blastocysts were not significantly different between embryos obtained by natural ovulation and superovulation (Table 3). However, percent fetus development, but not implantation, of embryos obtained by superovulation (60.2%) was significantly lower than when embryos were obtained by natural ovulation (87.5%, $P=0.02$, Table 4).

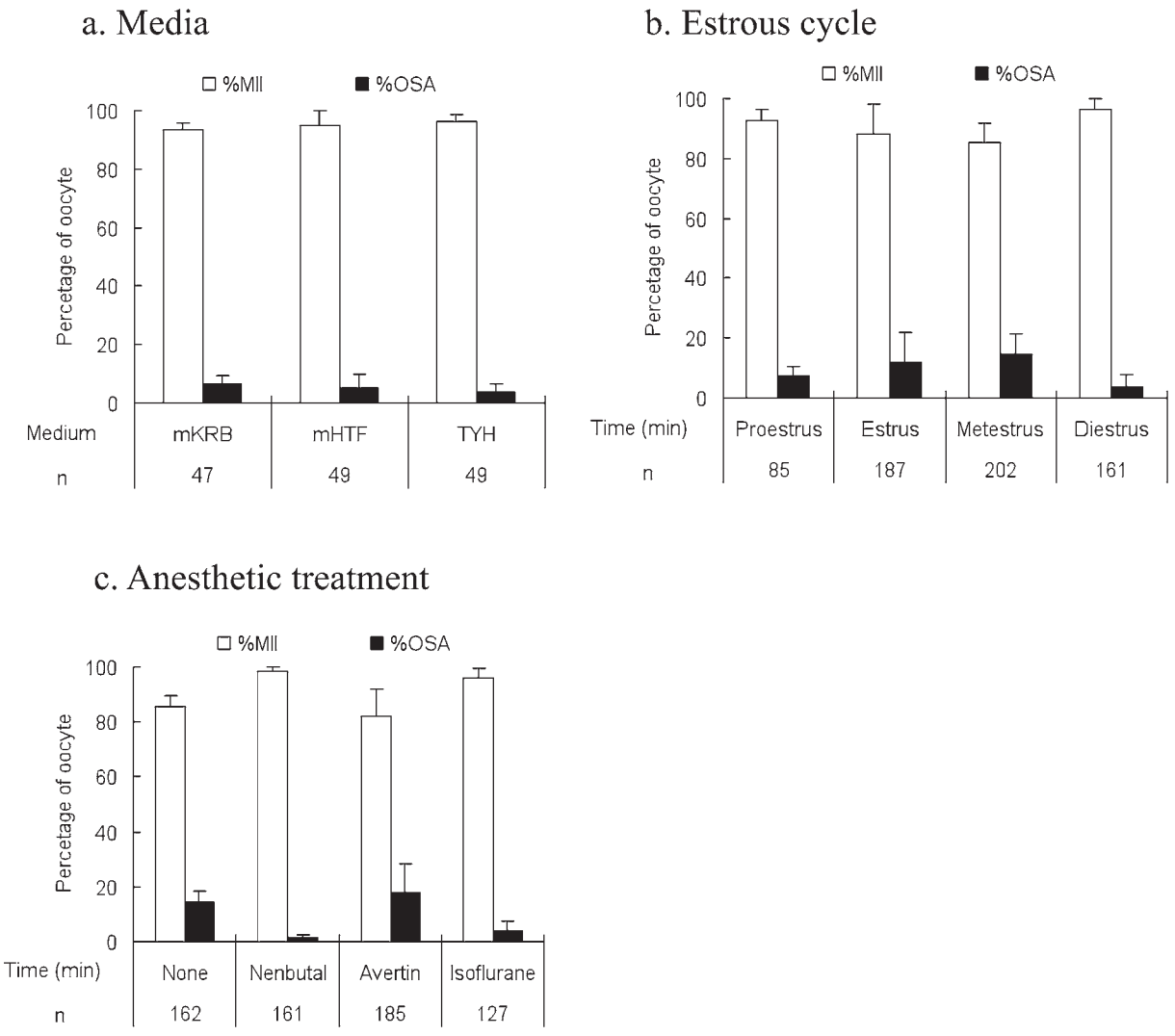

Fig. 3. Effects of media (a), estrous cycle (b), and anesthetic treatment (c) on the incidence OSA of oocyte collected from WMN/Nrs rats. Open bars indicate percentages of oocytes maintained at MII stage after 3 h incubation and filled bars indicate oocytes activated spontaneously. Error bars indicate MSEs. No statistical significances among experimental groups were observed by any treatment. Four females were used in each experimental group. n: total number of oocytes.

Discussion

Unlike mice, the practical application of ARTs including superovulatory techniques, *in vitro* fertilization, embryo culture, and cryopreservation in laboratory rats is still problematic. Researchers often encounter obstacles, such as OSA and poor fetus development of *in vitro* fertilized and developed embryos [16, 17, 20, 36, 45]. In addition, data regarding the application of these techniques to different strains is still limited [4, 31]. Thus, in this study, our objective was to provide data on the basic reproductive profile of WNM/Nrs inbred rats, which are often used to study the effects of radiation on whole animals [8, 27, 28]. The WNM/Nrs rats, formerly called Wistar/MsNrs, have been bred by sibmating for more than 30 generations since their introduction into the National Institute of Radiological Sciences, Japan, as a closed colony in 1960, from Hokkaido University, Japan, via the National Institute of Genetics, Japan, in 1951. Since 1992, the WMN/Nrs colony has been maintained as an inbred strain by SLC Inc.

Obtaining many eggs is one of the most important

Table 3. In vitro development of WMN/Nrs 2-cell embryos after 96 h culture[a)]

Ovulation	No. embryos cultured[b)]	No. of blastocysts (% ± MSE)	No. of expanded blastocysts	Nuclear no. of blastocysts
Natural ovulation	59	38 (63.7 ± 3.0)	24 (38.7 ± 6.9)	51.4 ± 2.6
Superovulation	154	101 (67.9 ± 4.9)	64 (45.1 ± 5.9)	49.8 ± 1.8

[a)]Two-cell embryos were cultured in R1ECM supplemented with BSA under 5% CO_2 and 5% O_2 at 37°C. Embryos derived from natural ovulation were cultured 8–13 embryos/50 μl medium, and embryos derived from superovulation were cultured 11–16 embryos/50 μl medium. [b)]Total of 6 and 12 replicates experiments for natural ovulation and superovulation, respectively.

Table 4. Development to fetus after transfer of WMN/Nrs 2-cell embryos into recipient females[a)]

Origin of embryos	No. recipients[b)]	No. pregnant recipients	No. of embryos transferred	No. implantation (% ± MSE)	No. fetus (% ± MSE)
Natural ovulation	4	4	40	36 (90.0 ± 5.8)	35 (87.5 ± 7.5)
Superovulation	5	5	78	63 (80.7 ± 4.6)	47 (60.2* ± 4.3)

[a)]Two-cell embryos were transferred into the oviducts of recipient females on day 1 of pseudopregnancy. [b)]Each recipient received 10 or 15–16 embryos obtained by natural ovulation and superovulation, respectively. *Significantly different at P=0.02 in the same column.

issues in the application of ARTs. In mice, superovulatory techniques are well established: e.g., intraperitoneal injection of PMSG and hCG. Although various superovulatory methods for rats have been described, such as continuous injection of gonadotropin [1] and immunization against inhibin [12], gonadotropin injection used successfully in mice, has also worked well in Wistar rats [21, 26, 33]. In most outbred and inbred rats, superovulation can be successfully induced using immature females [4, 30]. However, some Wistar rats such as Wistar-Imamichi and BrlHan:WIST@Jcl(GALAS) respond to superovulatory treatments independent of age and the estrous cycle [15, 21, 26, 33]. Our study indicates that the superovulatory response in WMN/Nrs is quite different from that reported in other strains of rats. In WMN/Nrs, only mature females (>8 weeks of age), not immature females (3–7 weeks of age), responded to superovulatory treatment (Table 1). Although superovulation in WMN/Nrs was found to be independent of the estrous cycle, gonadotropin injection did not influence the estrous cycle based on the vaginal smear, and copulation was observed only when gonadotropin injection was synchronized with the estrous cycle: i.e., PMSG injection on the day of metestrus and hCG on proestrus (Kito, personal observation). Thus, it is possible that only the ovaries responded to the exogenous gonadotropins, not the whole body, resulting in rejection of mating by females. These results indicate that the characteristics of the superovulatory response vary among rat strains, even among Wistar strains. Further information regarding superovulatory responses in other rat strains would be helpful, so as to more systematically organize the characteristics of the superovulatory response for rat research.

Ovulated rat oocytes are spontaneously activated without any stimuli when they are transferred into *in vitro* conditions [17, 45, 46]. In our study, this abortive activation (i.e., OSA), was inhibited by properly treating animals and oocytes. Keefer and Schuetz [17] found that collecting rat oocytes within 3 min after animal euthanasia significantly reduced OSA and that remaining oocytes in excised oviducts or leaving oocytes at room temperature only partly inhibited OSA [17]. Our detailed analysis using WMN/Nrs further clarified that it is the time from animal euthanasia to oviduct excision that exclusively affects the incidence of OSA. Even an 1 min delay of oviduct excision from an animal body significantly increased OSA from 4 to 53% (Fig. 2b). However, unlike the results of Keefer and Schuetz, neither leaving oocytes in the excised oviducts covered with mineral oil at 37°C, nor leaving the COCs under mineral oil at 37°C significantly increased OSA in our study.

The differences in results may have arisen because Keefer and Schuetz [17] left the oviducts and oocytes at room temperature, whereas we maintained the oocytes and the oviducts at 37°C during oocyte collection. This suggests that maintaining the oviducts and oocytes at 37°C, as was done in our study, may be beneficial for inhibiting OSA.

Ito *et al*. [13, 14] showed that ovulated rat oocytes, especially those from the Wistar strain, cannot be arrested at metaphase II due to the loss of activity of $p34^{cdc2}$ kinase maintained by high activity of Mos/MEK/MAPK pathways which in turn are negatively regulated by Ca^{2+}-dependent proteasome. Therefore, incubation of rat oocytes in Ca^{2+}-free conditions or addition of BAPTA-AM, a Ca^{2+} chelator, to the incubation medium effectively inhibited OSA [10, 11, 14]. However, the practical application of these approaches may be difficult as the fertilization and subsequent developmental competence of oocytes treated like this has is yet to be examined, and rat oocytes incubated under Ca^{2+}-free conditions have been reported to degenerate [10, 11]. In our study, the effects of various media on OSA were examined (Fig. 3a). We found that as long as the oviducts were excised soon after animal euthanasia, no medium effects were observed, even with mHTF which has a higher Ca^{2+} concentration (5.1 mM) than the other media (1.7 mM). mHTF has been reported to enhance the *in vitro* fertilization of various inbred mouse strains [18]. Our preliminary studies have indicated that mHTF also enhanced *in vitro* fertilization in Wistar rats (Kito *et al*. unpublished observation). Although there are strain differences in the incidence of OSA [31, 36], our results indicate that OSA can be inhibited if oocytes are carefully collected and transferred to *in vitro* conditions for further treatments such as *in vitro* fertilization or nuclear transfer.

We also showed that OSA was not influenced by anesthetic treatments and the estrous cycle (Figs. 3b and 3c). Anesthetic treatment by ether has been reported to induce parthenogenic activation in rodents [2]. We observed no significant effects of anesthetic treatment by Nenbutal, avertin and isoflurane on OSA (Fig. 3c). The collection of oocytes under anesthesia might allow the collection of oocytes without inducing OSA.

Superovulatory treatment has been known to increase the number of abnormal oocytes in rodents [5, 6, 23, 32]. The quality of superovulated oocytes with normal morphology is controversial. Some studies have shown that superovulatory treatment resulted in abnormal litter size and that oocyte quality is poorer than that of oocytes obtained by natural ovulation [5, 6]. Others have reported that superovulation resulted in failure of implantation and reduction of litter size due to environmental defects inside the oviducts and uterus induced by gonadotropins [35, 39, 41]. In mice, it has been reported that offspring obtained by superovulation have increased chromosomal abnormalities [23]. Because exogenous gonadotropin treatment may result in abnormal endogenous hormonal secretion [24], the quality of superovulated oocytes should be examined by embryo transfer into the gonads of normal untreated females. In our study, although no significant difference in *in vitro* development was observed after 96 h culture (Table 3), when transferred into recipient females, the embryos obtained by superovulation had lower developmental ability to 20-day fetus (60.2%) than those obtained by natural ovulation (87.5%, Table 4). It can be claimed that the differences in the numbers of embryos transferred to individual recipients between embryos derived from natural ovulation (10 embryos/recipient) and superovulation (15–16 embryos/recipient) may have contributed to the differences in fetus development, but, it is unlikely because the numbers of embryos transferred were within the range of natural ovulation (6–17 embryos/female). In addition, the percentages of fetus development in our study are comparable to those reported by Han *et al*. [9] and Kon *et al*. [21], who used embryos obtained by natural ovulation and superovulation, respectively. These results indicate that the quality of embryos is impaired by superovulatory treatment and that preimplantation development to blastocysts does not necessarily reflect the developmental competence of embryos. For practical purposes, use of superovulation may be helpful when the number of superovulated embryos is large enough to overcome reduced quality of the embryos, and superovulatory treatment of WMN/Nrs rats may possibly be applied to further studies of ARTs, such as *in vitro* fertilization, cryopreservation, and embryo culture.

In conclusion, we have reported various basic data of

WMN/Nrs inbred rats. In this strain, superovulation can only be induced in mature females and the superovulatory response is independent of the estrous cycle. OSA may be inhibited if the oviduct is excised from the animals immediately or under anesthesia, and OSA in this strain is unlikely to be affected by the medium composition, especially the Ca^{2+} concentration of the medium. Our data suggest that the use of superovulated oocyte/embryos has practical applications for *in vitro* fertilization and cryopreservation of the WNM/Nrs rat.

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