Biochemical and Biophysical Research Communications 463 (2015) 907–911

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# *In vitro* culture and *in vitro* fertilization techniques for prairie voles (*Microtus ochrogaster*)

Kengo Horie [a], Shizu Hidema [a], Takashi Hirayama [b], Katsuhiko Nishimori [a, *]

[a] *Laboratory of Molecular Biology, Department of Molecular and Cell Biology, Graduate School of Agricultural Science, Tohoku University, 1-1 Tsutsumidori-Amamiyamachi, Aoba-ku, Sendai, Miyagi 981-8555, Japan*
[b] *Department of Obstetrics and Gynecology, Juntendo University, 2-1-1, Hongo, Bunkyo-ku, Tokyo 113-8421, Japan*

ARTICLE INFO

*Article history:*
Received 9 May 2015
Accepted 4 June 2015
Available online 9 June 2015

*Keywords:*
Prairie vole
Assisted reproductive technology (ART)
*In vitro* culture
*In vitro* fertilization

ABSTRACT

Prairie vole (*Microtus ochrogaster*) is a highly social animal and is a commonly used animal model for neuropsychopharmacological and psychiatric studies. To date, only a few reports on the development of transgenic prairie voles which was primarily due to the suboptimal development of assisted reproductive technology (ART) in prairie voles. Limitations in ART further hinder the development of genetically modified prairie voles such as the application of conventional gene targeting technologies using embryonic stem (ES) or induced pluripotent stem (iPS) cells to generate chimeric prairie voles. Moreover, recent advancement in genome-editing tools such as transcription activator-like effector nuclease (TALEN) and clustered regulatory interspaced short palindromic repeat (CRISPR)/Cas technology provide an unprecedented opportunity to create gene targeting animal model and the development of ART in prairie voles is necessary for future development of novel transgenic prairie vole model. We have established efficient method for *in vitro* embryo culture and sperm cryopreservation with high fertilization rate. In G-1 PLUS and G-2 PLUS sequential culture condition, 81.0% (# of Blastocysts/total n) of one-cell embryos developed to blastocysts. In contrary, no embryos were developed to blastocyst stage in KSOM medium (0/total # of embryos in culture). *In vitro* fertilization rate using fresh and frozen-thawed sperm was 32.6% and 29.3%, respectively. This is the first report of IVF using cryopreserved prairie vole sperm. We employed mouse IVF methods in prairie voles and optimize culture conditions using human G-1/G-2 PLUS sequential culture method that resulted in high embryonic development rate. The development in vole reproductive technology will facilitate the generation of transgenic voles in the future.



## 1. Introduction

Animal model is important for studying psychiatric disorders and development of therapeutic methods for depression, schizophrenia, and other psychiatric diseases. In case of autistic spectrum disorders, there is increase interest in developing transgenic rodent model with deletion or genetic modification of a selected gene of interest that lead to deficit in social skills [1–3]. However, that fact that mouse social behavior is relatively limited [4], the development of an animal model with more robust social behavior is necessary for studying psychiatric disorders and the neurological physiology in humans.

Prairie vole, a native rodent species of North America, is known for its higher level of sociality, such as monogamous pair-bonding, minor-type mammalian social behavior, and prairie vole pairs nursing their offsprings together [5–7]. Additionally, mating within littermates is not observed. These social behavioral characteristics are different from those in mice and therefore, prairie voles are being considered a more suitable animal model for the study of human psychiatric disorders. However, very limited studies on these species have been reported. Of these, one of the first reports on genetically modified prairie voles suggested the potential of this unique animal model in studying social behavior. Prairie voles were infected with adeno-associated virus (AAV) expressing the arginine vasopressin receptor 1A (*Avpr1a*) gene in the ventral pallidum. This study showed that the expression of *Avpr1a* in ventral pallidum was critical for formation of pair-bonding in male voles [8]. Similarly, overexpression of oxytocin receptor (*Oxtr*) in the nucleus

* Corresponding author.
*E-mail address:* knishimori@m.tohoku.ac.jp (K. Nishimori).

http://dx.doi.org/10.1016/j.bbrc.2015.06.033
0006-291X/

accumbens (NAcc) of female prairie voles by AAV showed that the expression level of *Oxtr* in the NAcc was important for pair-bonding behavior in females [9]. These studies were achieved by focal AAV gene transfer in selected brain region of adult prairie voles and only a limited population of neural at the injection site were transfected, thus the systemic impact of the gene of interest such as *Oxtr* cannot be determined without the development of germline transgenic prairie voles. So far, there are only two reports on the creation of germline transgenic prairie voles using lentiviral vector [10,11]. To our knowledge, there are no reports to date on generation of gene knock-out (KO) or knock-in (KI) prairie voles.

The development of zinc-finger nuclease and TALEN technologies have accelerated genome-editing in fertilized eggs for the production of gene targeted animals [12–14]. Recent advancement of CRISPR/Cas technology has greatly accelerated the creation of gene targeted animals because of its high efficiency and simplicity. Since the first report of the creation of gene KO mouse using CRISPR/Cas [13], widespread applications in different animal species such as rats and monkeys have been increasing [15–19]. We expect that gene targeted prairie voles can be produced by using CRISPR/Cas technology.

One of the major challenges in the creation of gene targeted prairie voles by using genome-editing tools such as CRISPR/Cas technology is the suboptimal development of ARTs in prairie voles. This study was aimed to establish ARTs in prairie by adopting methods that are commonly used in mice, which include IVF and *in vitro* culture [20]. The success of *in vitro* culture of mice embryos from one-cell to blastocyst stage was first reported by Witten [21], and soon after, the generation of newborn mice was achieved by implantation of *in vitro* produced embryos in the uterus [22], laying the foundation of today mouse molecular genetic technologies.

In this study, we reported the development of ovulation procedures, IVF procedure, and *in vitro* embryo culture until blastocyst stage in prairie voles. Our results were comparable to mice and expected to facilitate future gene editing in prairie voles.

## 2. Materials and methods

### 2.1. Animals

The protocols for care and use of prairie voles in this study were approved by the Institutional Animal Care and Use Committees of Tohoku University. All animals were maintained on a 12 h light: 12 h dark cycle and were provided with *ad libitum* food and water access.

For breeding, 1 male, between 2 months and 1 year old, was housed with 1 female of a similar age. Pups were weaned 3 weeks after birth and fed together, with 3–4 animals per cage.

### 2.2. Stud males

Male prairie voles, > 2 months old, were used as studs. Up to 4 animals were housed in each cage and were separated just before breeding.

### 2.3. Vasectomized males

Male prairie voles, > 2 months old, were selected to prepare vas voles (vasectomized male prairie vole). Voles were anesthetized with 3% isoflurane (Wako Pure Chemical Inc. Ltd, Osaka, Japan), laparotomized, seminiferous tubules were closed by heated forceps, and the incisions were closed. Vas voles were allowed to heal for at least a week before use.

### 2.4. Superovulation

To induce ovulation, 8- to 10-weeks-old female prairie voles were abdominally injected with 30, 60, or 100 IU/100 μl pregnant mare serum gonadotropin (PMSG; Peamex 1000 IU, Novartis Animal Health Co. Ltd.) 2 h before the beginning of the dark cycle. Each female was placed in a cage with a stud male, separated by a transparent and perforated PLC divider. After 48 h of separated cohabituation, female prairie voles were abdominally injected with 30, or 60 IU/100 μl human chorionic gonadotropin (hCG; Puberogen 1500 IU, Novartis Animal Health Co. Ltd.), and the divider was removed to induce copulation. After 15 h of removal of the divider, female voles with confirmed mating were sacrificed using $CO_2$, and their oviducts were dissected.

### 2.5. Harvesting embryos

Using a 26-gauge needle, oviducts were cut in M16 medium (M7292, Sigma Co Ltd., St Louis, MS, USA) under a microscope, to harvest fertilized eggs. An egg was confirmed to be fertilized by observing presence of sperm. Eggs were stored in the same medium under mineral oil (2613785, Nakarai Tesque Co Ltd., Kyoto, Japan), in a humidified 5% $CO_2$ chamber at 37 °C. For extended culture until blastocyst stage, fertilized eggs were moved to different media as follows: G-1 PLUS (Vitrolife, 10128) followed by G-2 PLUS (Vitrolife, 10131), Global (Life Global, LGGG-100), or KSOM (Arc resource) medium, and incubated further at 37 °C with 5% $CO_2$. G-1 PLUS medium were exchanged to G-2 PLUS medium at 8 cell stage (2.5–3.5 day *in vitro* (DIV)). Five to ten embryos were co-cultured per a well in 4 well plate (Thermo Fisher Scientific Inc.,).

### 2.6. Collection of 2-cell embryos

After 45 h post removal of the cage divider, 2-cell embryos were collected from the ampulla of oviducts from post-ovulation female voles, via a similar procedure as that used for preparation of fertilized eggs described above, and embryos were cultured in M16 medium.

### 2.7. In vitro fertilization (IVF)

Male voles, 10 weeks old, were euthanized using $CO_2$. Sperm were collected from the epididymides and incubated in Fertiup medium (Cosmo Bio Co. Ltd, Japan) at 37 °C with 5% $CO_2$ for 30 min and subsequently added to human tubal fluid (HTF) medium (Cosmo Bio Co. Ltd, Japan) containing unfertilized eggs which had no first polar body. After incubation with the sperm for 5 h, eggs were washed 3 times in G-1 PLUS medium. The next day, efficiency of fertilization was evaluated by the rate of development to 2-cell stage.

### 2.8. Sperm viability

Male voles, 17 weeks old, were euthanized using $CO_2$. Sperm were collected from epididymies and placed into 120 μl Fertiup medium. The sperm suspension was diluted 1:1000, and active and inactive sperm were counted under a stereomicroscope using a hemocytometer.

### 2.9. Sperm cryopreservation

Sperm were collected through a procedure similar to that used for sperm viability described above, 10 μl Fertiup medium with sperm was placed into each vial and frozen-thawed according to

procedures previously described for cryopreservation and thawing of mice sperm [23].

### 2.10. Embryo transfer

A vasectomized male prairie vole and a 10-week-old female vole were housed together in the same cage but separated by a transparent and perforated divider. After co-habitation for 3 days, the divider was removed and mating was allowed to proceed. The next day, pseudopregnant female voles were anesthetized using 3% isoflurane and 4 to 15, 2-cell embryos were transferred into oviducts of each animal.

### 2.11. Statistics

The results are expressed as mean ± standard error (SE). Student's *t* test was used for statistical analysis of the results and a P value of <0.05 was considered as statistically significant.

## 3. Results

### 3.1. Fertilized eggs from superovulated, female prairie voles

We used superovulation procedures in prairie voles as previously described [11] and compared the conditions established in this study. Administration of PMSG/hCG at 3 different dose combinations produced the following result: (i) 100 IU/30 IU dose: an average of 2.6 eggs per individual vole regardless of fertilization, (ii) 60 IU/60IU dose: an average of 5.6 eggs, and (iii) 30 IU/30IU dose: an average of 3.2 eggs, respectively. However, these were not significantly different, where (i) v.s. (ii) F = 0.51, P = 0.22; (ii) v.s. (iii)F = 0.28, P = 0.37 (Fig. 1). Recalculation of their value with avoidance of data from infertile voles showed 11.2 ± 1.1 for 60 IU/60 IU dose level, which was similar to that previously reported by Keebaugh et al. [11].

### 3.2. In vitro culture of fertilized eggs

We performed *in vitro* culture of fertilized eggs until blastocysts stage using KSOM medium, however, no one-cell eggs developed into 2-cell or subsequent stages (Fig. 2). We then tested Global medium (or the combination of G-1 PLUS medium followed by G-2 PLUS medium for *in vitro* vole embryo culture.

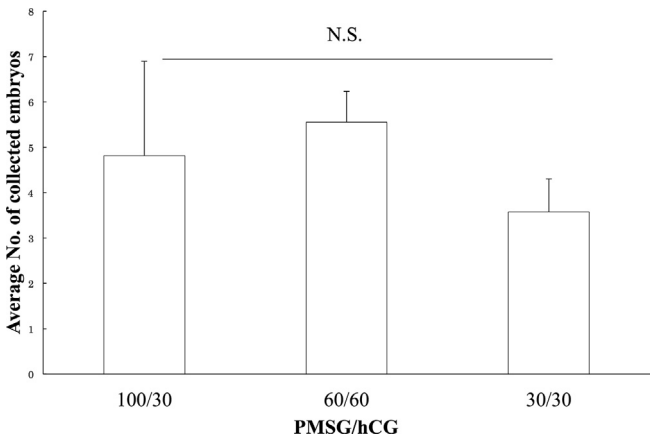


**Fig. 1.** Comparison of hormonal regimen for the production of onc-cell embryos. PMSG was administered at 48 h before the administration of hCG. The number of embryos collected from superovulated females was not significant differences between each hormone regimen.

In the culture using Global medium, 47.5% one-cell eggs developed to blastocyst stage. Sequential culture by using G-1 PLUS medium and G-2 PLUS medium yielded the best the development of one-cell embryos to blastocyst stage (81.0%) as well as of the development of *in vivo* produced 2-cell embryos to blastocyst stage (88.9%). Thus, we overcame the challenge of the 2-cell block in the development of prairie vole embryos by adopting the G-1/G-2 PLUS medium system.

### 3.3. IVF using fresh sperm

IVF using unfertilized eggs, that were confirmed that they had no first polar body, from female prairie voles and freshly-obtained vole sperm resulted in 32.6% (SEM ± 7.30) fertilization rate, confirmed by cell division to 2-cell embryos (Fig. 3).

### 3.4. IVF using frozen-thawed sperm

Although viability of frozen-thawed sperm was lower than that of fresh sperm, the efficacy of IVF with frozen-thawed sperm (29.3% SEM ± 12.3) was similar to that with fresh sperm (F = 0.42, P = 0.86) (Fig. 3).

### 3.5. Oviduct transfer of 2-cell stage embryos

We implanted 4 embryos at 2-cell stage into the oviduct of pseudopregnant female prairie voles and we obtained 2 newborn animals.

## 4. Discussion

We were successful in obtaining an adequate number of fertilized, prairie vole eggs and confirmed previously-described conditions used for superovulation [11]. We evaluated 3 different dose combinations of PMSG/hCG and obtained a similar yield.

In this study, we developed modified procedures to facilitate the development of fertilized prairie vole eggs from single-cell to blastocyst stage, with higher efficiency. We anticipate the need for advanced technology to produce genetically-modified prairie vole lines, including precise insertions of certain genes with specific modifications, use of vole ES cells, etc. The procedures developed in this study will significantly facilitate this kind of work in the future.

To our knowledge, this is the first report of *in vitro* fertilization (IVF) of prairie vole oocytes using freshly and frozen-thawed prairie vole sperm at fertilization rate at 32.6% and 29.3% respectively. Cryopreservation of vole sperm was also successfully achieved. Although the viability of frozen-thawed vole sperm was lower than that of freshly prepared sperm, the fertilization rate was not different between fresh or frozen-thawed sperm using commercially available medium developed for cryopreservation of mice sperm. Success in these procedures could provide a means to not only preserve and distribute newly-generated vole lines, but also generate pathogen-free SPF vole lines.

A previous report described the possibility of *in vitro* culture of prairie vole fertilized eggs until blastocyst stage [24]. However, details of media, culture conditions, and developmental rate were not provided. Here we developed optimal vole embryo culture conditions with high blastocyst yield from one-cell embryos using G-1 PLUS and G-2 PLUS sequential media, originally developed for human fertilized eggs.

Prairie vole blastocysts are essential to obtain vole ES cells and to generate chimeric embryos using ES or iPS cells. *In vitro* culture of blastocysts from one-cell embryos is advantageous over *in vivo* produced blastocysts due to higher recovery of blastocysts from

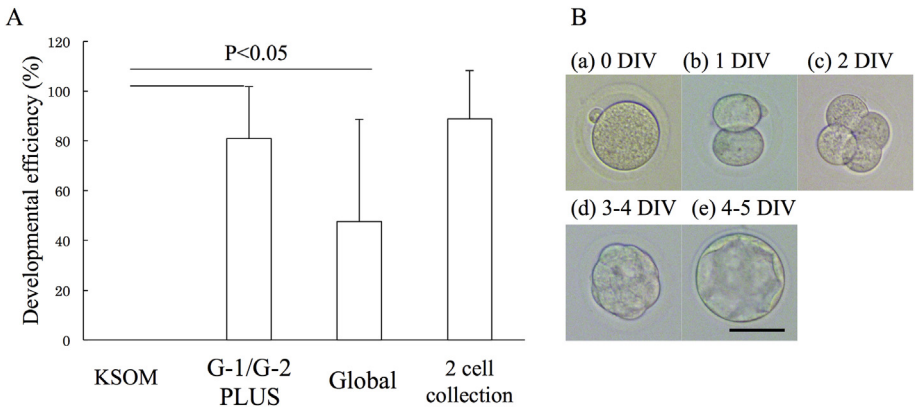


**Fig. 2.** Comparison of *in vitro* embryo culture media. A. Embryos collected from female prairie voles were cultured in different medium (KSOM, G-1 PLUS/G-2 PLUS, Global). B. Pictures of prairie vole embryos cultured *in vitro*; (a), 1 cell (0 DIV); (b), 2 cells (1 DIV); (c), 4 cells(2 DIV); (d), morula(3-4 DIV); (e), blastocyst(4-5 DIV). Scale bar = 50 μm.

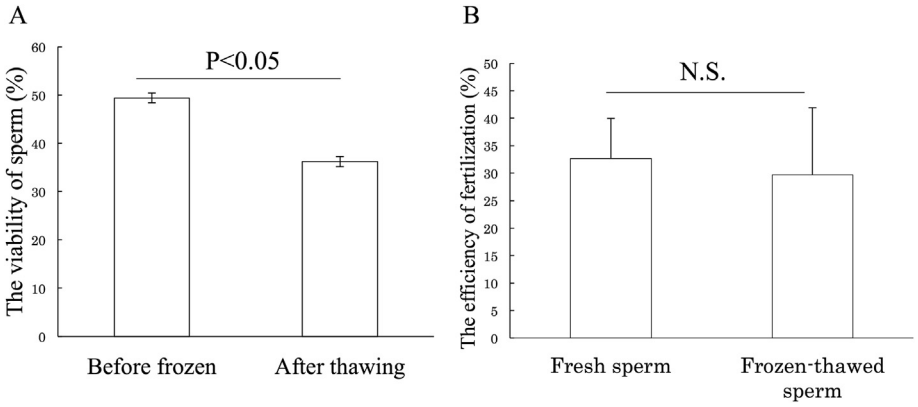


**Fig. 3.** Cryopreservation of prairie vole sperm and IVF. A. The viability of frozen-thawed sperm and freshly prepared sperm. B. Fresh and frozen-thawed sperm showed similar *in vitro* fertilization rate.

single-cell embryos. We also successfully produced newborn vole pups, after oviduct transfer of 2-cell embryos.

We believe that the generation of transgenic prairie voles will be facilitated by the techniques developed in this study.

However, further optimization on the administration of gonadotropin derived from other animal species, dosage, duration of administration, age of animals for hormonal treatment are necessary to improve the yield of fertilized vole eggs.

## Acknowledgments

This work was supported in part by a Grant-in-Aid for Exploratory Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (25660074). We thank Dr.Larry J Young (Yerkes National Primate Research Center, Dept. of Psychiatry, Emory University) for his providing prairie vole, Dr. Anthony Chan (Yerkes National Primate Research Center, Dept. of Human Genetics, Emory University) for his critical reviewing of this manuscript, Dr. Yuichi Hiraoka (Dept. of Agricultural Science, Tohoku University) for constructive discussions.

## Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.06.033.

## References

[1] J.N. Ferguson, L.J. Young, E.F. Hearn, M.M. Matzuk, T.R. Insel, J.T. Winslow, Social amnesia in mice lacking the oxytocin gene, Nat. Genet. 25 (2000) 284–288.

[2] Y. Takayanagi, M. Yoshida, I.F. Bielsky, H.E. Ross, M. Kawamata, T. Onaka, T. Yanagisawa, T. Kimura, M.M. Matzuk, L.J. Young, K. Nishimori, Pervasive social deficits, but normal parturition, in oxytocin receptor-deficient mice, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 16096–16101.

[3] D. Jin, H.X. Liu, H. Hirai, T. Torashima, T. Nagai, O. Lopatina, N.A. Shnayder, K. Yamada, M. Noda, T. Seike, K. Fujita, S. Takasawa, S. Yokoyama, K. Koizumi, Y. Shiraishi, S. Tanaka, M. Hashii, T. Yoshihara, K. Higashida, M.S. Islam, N. Yamada, K. Hayashi, N. Noguchi, I. Kato, H. Okamoto, A. Matsushima, A. Salmina, T. Munesue, N. Shimizu, S. Mochida, M. Asano, H. Higashida, CD38 is critical for social behaviour by regulating oxytocin secretion, Nature 446 (2007) 41–45.

[4] M.E. Modi, L.J. Young, The oxytocin system in drug discovery for autism: animal models and novel therapeutic strategies, Horm. Behav. 61 (2012) 340–350.

[5] C.S. Carter, A.C. DeVries, L.L. Getz, Physiological substrates of mammalian monogamy: the prairie vole model, Neurosci. Biobehav. Rev. 19 (1995) 303–314.

[6] B.J. Aragona, Z. Wang, The prairie vole (*Microtus ochrogaster*): an animal model for behavioral neuroendocrine research on pair bonding, ILAR journal/National Research Council, Inst. Lab. Anim. Resour. 45 (2004) 35–45.

[7] L.A. McGraw, L.J. Young, The prairie vole: an emerging model organism for understanding the social brain, Trends Neurosci. 33 (2010) 103–109.

[8] L.J. Pitkow, C.A. Sharer, X. Ren, T.R. Insel, E.F. Terwilliger, L.J. Young, Facilitation of affiliation and pair-bond formation by vasopressin receptor gene transfer into the ventral forebrain of a monogamous vole, J. Neurosci. Off. J. Soc. Neurosci. 21 (2001) 7392–7396.

[9] H.E. Ross, S.M. Freeman, L.L. Spiegel, X. Ren, E.F. Terwilliger, L.J. Young, Variation in oxytocin receptor density in the nucleus accumbens has differential effects on affiliative behaviors in monogamous and polygamous voles, J. Neurosci. Off. J. Soc. Neurosci. 29 (2009) 1312–1318.

[10] Z.R. Donaldson, S.H. Yang, A.W. Chan, L.J. Young, Production of germline transgenic prairie voles (*Microtus ochrogaster*) using lentiviral vectors, Biol. Reprod. 81 (2009) 1189–1195.
[11] A.C. Keebaugh, M.E. Modi, C.E. Barrett, C. Jin, L.J. Young, Identification of variables contributing to superovulation efficiency for production of transgenic prairie voles (*Microtus ochrogaster*), Reprod. Biol. Endocrinol. RB&E 10 (2012) 54.
[12] T. Gaj, C.A. Gersbach, C.F. Barbas 3rd, ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering, Trends Biotechnol. 31 (2013) 397–405.
[13] H. Wang, H. Yang, C.S. Shivalila, M.M. Dawlaty, A.W. Cheng, F. Zhang, R. Jaenisch, One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering, Cell 153 (2013) 910–918.
[14] M. Jinek, K. Chylinski, I. Fonfara, M. Hauer, J.A. Doudna, E. Charpentier, A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity, Science 337 (2012) 816–821.
[15] H. Wan, C. Feng, F. Teng, S. Yang, B. Hu, Y. Niu, A.P. Xiang, W. Fang, W. Ji, W. Li, X. Zhao, Q. Zhou, One-step generation of p53 gene biallelic mutant Cynomolgus monkey via the CRISPR/Cas system, Cell Res. 25 (2015) 258–261.
[16] D. Li, Z. Qiu, Y. Shao, Y. Chen, Y. Guan, M. Liu, Y. Li, N. Gao, L. Wang, X. Lu, Y. Zhao, M. Liu, Heritable gene targeting in the mouse and rat using a CRISPR-Cas system, Nat. Biotechnol. 31 (2013) 681–683.
[17] X. Ren, J. Sun, B.E. Housden, Y. Hu, C. Roesel, S. Lin, L.P. Liu, Z. Yang, D. Mao, L. Sun, Q. Wu, J.Y. Ji, J. Xi, S.E. Mohr, J. Xu, N. Perrimon, J.Q. Ni, Optimized gene editing technology for Drosophila melanogaster using germ line-specific Cas9, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) 19012–19017.
[18] W. Li, F. Teng, T. Li, Q. Zhou, Simultaneous generation and germline transmission of multiple gene mutations in rat using CRISPR-Cas systems, Nat. Biotechnol. 31 (2013) 684–686.
[19] K. Belhaj, A. Chaparro-Garcia, S. Kamoun, V. Nekrasov, Plant genome editing made easy: targeted mutagenesis in model and crop plants using the CRISPR/Cas system, Plant Methods 9 (2013) 39.
[20] J. Hammond Jr., Recovery and culture of tubal mouse ova, Nature 163 (1949) 28.
[21] W.K. Whitten, Culture of tubal mouse ova, Nature 177 (1956) 96.
[22] L.A. Mc, J.D. Biggers, Successful development and birth of mice cultivated *in vitro* as early as early embryos, Nature 182 (1958) 877–878.
[23] N. Nakagata, Cryopreservation of mouse spermatozoa, Mamm. Genome Off. J. Int. Mamm. Genome Soc. 11 (2000) 572–576.
[24] R.L. Roberts, K.N. Wolf, M.E. Sprangel, W.F. Rall, D.E. Wildt, Prolonged mating in prairie voles (*Microtus ochrogaster*) increases likelihood of ovulation and embryo number, Biol. Reprod. 60 (1999) 756–762.