Applying bases for "double control" artificial breeding of giant panda in captivity

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THE giant panda is a kind of endangered rare animal, the number of which is less than one thousand at present, specially distributed in China. According to the protective scheme issued by The International Association of Nature Protection, stock population in captivity must be established in order to make artificial breeding when an animal species has decreased to less than 1 000 individuals[1]. It is an unshrinkable duty for Chinese researchers to make studies on the reproduction and breeding of giant panda in captivity.

Artificial insemination (AI) was successfully applied in the giant panda firstly at Beijing Zoo in 1963, and AI with frozen-thawed semen succeeded at Chengdu Zoo later. However, the artificial breeding situation in recent decades is not stable all over the country, with very slow increase of success rate and high empty pregancy rate. We believe that the most important reason is the failure of sperm-egg binding and fusion. Therefore, both the male and the female beasts should be paid attention to in the practice of AI. As long as the fertilization problem is solved, the empty pregancy rate will be lowered and the AI success rate will be increased. Having analyzed the research data about the pituitary gonadotrophic cells, testis, ovary, sperm, egg and hormone secretion in giant panda's reproductive axis, we have found that the basic reason for its low fertility is the reproductive endocrine disorder, mainly expressed in the insufficient secretion of gonadotrophins (FSH, LH), which in turn influences the development and maturation of gametes and lowers the sexual and reproductive ability, but not the high specification of its reproductive ability in giant panda[2]. In view of the situation mentioned above, we put forward an idea of "double (male and female) control", i.e. supplementing external gonadotrophins to females to stimulate follicle development, egg maturation, oestrus and ovulation; selecting the proper thawing solution, diluting solution and culture solution for cryopreserved semen pellets to improve sperm viability and elongate their living time *in vitro*.

1 Materials and methods

1.1 Induction of ovulation

FSH was supplemented to irregular or unwell oestrus fermale at 100 iu per day for 3 successive days, followed by the supplement of LH at 300 iu per day after the beginning of oestrus

during the breeding season.

1.2 Avoidance of empty pregnancy and pregnancy protection

To stimulate the development of corpus luteum and the secretion of progesterone, 300 iu LH was supplemented to an oestrous female individual at the time of AI or 30 min before AI, which could avoid empty pregnancy and protect pregnancy.

1.3 Improvement of sperm viability and extension of sperm living time

The giant panda semen was collected form healthy male individuals by electrical stimulation and cryopreserved semen pellets were made. The semen pellets used were provided by Chengdu Research Base of Giant Panda Breeding and Chengdu Zoo.

1.3.1 The influence of different thawing solutions on sperm viability. Four kinds of thawing solutions were tested, i.e. PSCM (NaCl: 5.5 mg/mL; KCl: 0.3 mg/mL; $MgCl_2$: 0.2 mg/mL; KH_2PO_4: 0.4 mg/mL, $KHCO_3$: 2.1 mg/mL; $CaCl_2 \cdot 2H_2O$: 0.3 mg/mL; glucose: 1 mg/mL; sodium pyruvate: 0.03 mg/mL; sodium lactate: 0.2% (v/v); penecillin: 0.05 mg/mL; streptomycin: 0.065 mg/mL; BSA: 0.4 mg/mL, pH 6.8) TCM-199, 199M2 (culture solution for hamster egg and sperm) and citrate-glucose solution (thawing solution for cryopreserved semen pellets of dairy cattle). After equal aliquots of semen at the same concentration were added to different solutions, the sperm viability and living time at the same pH, osmolarity, temperature and CO_2 concentration were compared.

1.3.2 The influence of pH on sperm viability. The best thawing solution was adjusted to different pH levels (6.8, 7.2, 7.4, 7.8) and equal aliquots of semen at the same concentration were added. The sperm viability and living time were compared.

1.3.3 The influence of temperature on sperm viability. The temperature range, in which AI was conducted, was simulated. The temperature of semen, thawed in PSCM and TCM-199 at 40℃, pH 6.8, was lowered to 37, 25, 18 and 15℃ respectively and the sperm viability and living time were observed. The thawing procedure was as follows: 1 mL of each thawing solution in a small test tube was heated to 40℃ and the cryopreserved semen pellets of the same lot were added to each of them. As soon as the cryopreserved pellets were thawed, the number of dead and immotile sperm in each sample was counted using a hemacytometer under a phase-contrast microscope. Then all the sperm in every sample were immobolized by heat or cold treatment and the total number was counted. Finally, viability rate was obtained.

2 Results and discussion

The crux in giant panda protection and salvage is to strengthen artificial breeding, and the core problem is to improve fertilization. Therefore, the control of oestrus and ovulation in female beasts and the guarantee of sperm vitality in male beasts directly determine the successive failure of artificial breeding. Part of the female individuals in captivity behave unoestrous, irregularly oestrous or unobviously oestrous, which severely influence their reproduction. Cooperating with Chengdu Zoo, we firstly explored the possibility of supplement of external gonadotrophin (FSH, LH) to stimulate oestrus and ovulation and at the same time, we also measured the hormone levels. Our experiment proved that FSH stimulates oestrus and LH stimulates ovulation. We obtained the first offspring named "Xingxing" after the treatment of female beast named "Qingqing" with external pituitary gonadotrophin in 1989[3] (fig. 1). Thereafter, the hormone-treated "Xingxing" bore three twins in 1990, 1991 and 1993. The above evidence sufficiently demonstrates that the supplement of external hormones not only has no side-effects on fe-

male beasts, but also does not influence the occurrence of normal oestrus, ovulation, pregnancy and farrowing next year[4]. In addition, the supplement of external gonadotrophin probably adjusts the endocrine condition, leading to the successive production of twin offspring.

LH can stimulate the normal development of corpusluteum and secretion of progesterone, which is beneficial to blastocyst implantation. Based on the above fact, we designed an experiment to protect pregnancy. 300 iu LH was injected into female beast named "Chengcheng" 30 min before the AI using the semen of male beast named "Yueyue" on April 14 and 15 respectively and offspring was produced on September 20, 1991. Experiments were repeated on female beasts named "Chengcheng" and "Bing-bing", and satisfactory results were obtained. The progesterone secretion was measured from oestrus to farrowing and it was found that the hormone secretion was consistent with fetal development and parturation. The experimental results demonstrate that LH can indeed induce ovulation and its function of pregnancy protection is realized by stimulation of progesterone secretion of corpus luteum cells.

Fig. 1. Offspring named "Xingxing" born after the treatment of female beast named "Qingqing" with FSH in 1989. X, Xingxing; Q, Qingqing.

Supplement of external gonadotrophin to female got satisfactory results. But sperm viability of male individuals is also an important factor influencing the reproduction, determining another 50% of success opportunity. The situation that the success rate of AI increased very slowly for a long time and no offspring were produced in three successive years before 1988 made us begin to pay attention to sperm viability. We found that the vitality of sperm of the frozen-thawed semen used in AI was very poor, and most of the sperm almost lost their moving ability, the probable reason of which was that the thawing solutions used previously in giant panda AI were used for or improved from the solutions for the AI of dairy cows. Although a few offspring were obtained, the success rate was very low. It seemed that the thawing solutions used for dairy semen were not fit for the thawing of cryopreserved giant panda semen, which would greatly influence the sperm vitality, meeting of the gametes and fertilization. In view of the above mentioned facts, we began to carry out research on the thawing solutions of cryopreserved semen pellets of giant panda.

For the convenient use, we systematically considered the thawing solution, dilution solution and culture system as an integral whole. Through ten times of experiments on the effect of thawing solution composition and pH on sperm vitality, we have proved that PMSC is the best solution, TCM-199 is also suitable for the thawing of giant panda semen and citrate-glucose solution is the poorest solution. After the thawing of cryopreserved semen pellets made from fresh semen with 90% sperm motile at the same temperature, and the culture of the sperm at the same osomolarity, pH, temperature and CO_2 concentration, the results of 5 replicates are shown as follows: (ⅰ) The recovery rates were 61%, 51%, 42% and 22%, respectively and the viability rates were 55%, 46%, 38% and 20%, respectively in PSCM, TCM1992, 1M2 and citrateglucose groups. (ⅱ) The living times were 17.5—21 h, 7.5—13 h, 5—7 h, 2 min—1.5 h (the majority died within 30 min and most lived for 10 min), respectively in PSCM, TCM-199, 199M2 and citrate-glucose groups. In order to test the influence of pH of the same kind of thawing solution (taurine and adrenalin were added to evaluate acrosome status) on sperm, we designed 4 experi-

mental groups, i.e. pH 6.8, pH 7.2, pH 7.4 and pH 7.8. The experimental results showed that there was no evident influence of pH on sperm viability immediately after thawing, the vitality rates were 55%, 55%, 54% and 50%, respectively in the four groups. However, pH influenced the beginning time of acrosome reaction (pH 6.8, 8—9 h; pH 7.2, 10—12 h; pH 7.4, 10—11 h; pH 7.8, 12—13 h), the beginning time of acrosome losing (hyperactivation, pH 6.8, 10 h; pH 7.2, 13 h; pH 7.4, 12—13 h; pH 7.8, 14.5 h) and the sperm living time (pH 6.8, 17.5—21 h; pH 7.2, 18.5—23 h; pH 7.4, 18—25 h; pH 7.8, 21—23 h). In the research of the effects of temperature on sperm viability, the cryopreserved semen pellets were thawed in PSCM (pH 6.8) and TCM-199 (pH 6.8) at 40℃ and cultured at 37, 25, 18 and 15℃ respectively. The temperature of thawing (dilution, culture) solution had no evident influence on sperm viability and living time. The above results demonstrate that PSCM and TCM-199 at pH 6.8 are probably most suitable for the maintenance of sperm viability and enable the sperm to be hyperactivated earlier, which is beneficial to sperm-egg binding and fertilization. The technical personnels of Chengdu Zoo repeated the experiment on the selection of thawing solutions. Of the solutions (Eagle's MEM, TCM-199, BMOC-3, skim milk and citrate-glucose solution) tested, TCM-199 was the most suitable one for the thawing of cryopreserved giant panda semen[5].

The thawing of cryopreserved semen pellets is one of the key links in AI. The selected solutions are not only used for the thawing and dilution of semen, but also used for the maintaining sperm viability and elongating sperm living time, which half determined the pregnancy of female beasts.

The authors firstly used PSCM solution to thaw the cryopreserved giant panda (No. 9) semen pellets in 1990. After the oestrous female individuals named "Qingqing" was artificially inseminated with this semen, baby twin were produced and they were the first twin raised successfully by both the dam and the nursing personnels[6] (fig. 2). However, controversial views lasted for a long time as to the birth of the twin, the focusing point being whether they came from natural copulation or from AI. The "riddle" was uncovered by DNA fingerprinting test conducted by Feng Shengguo *et al*. at the Institute of Molecular Biology, Sichuan Normal University 4 a later. They proved that the father of the twin named "Yaya" and "Xiangxiang" born by female beast named "Qingqing" in 1990 was male beast No. 9[7], whose cryopreserved semen pellets were thawed and diluted with PSCM for AI. Thereafter, using PSCM or TCM-199 as thawing solutions, four twins were born in 3 successive years after AI. A total of 7 twins were born from 1990 to 1995. Having not got offspring for many years, the Fuzhou Giant Panda Research

Fig. 2. Twin offspring named "Yaya" and "Xiang xiang" born after the female beast named "Qingqing" was artificially inseminated with the frozen-thawed semen of male beast No. 9 in 1990. Y, Yaya; Q, Qingqing.

Fig. 3. Offspring born after LH was used to stimulate ovulation and protect pregnancy of female beast named "Chengcheng" in 1992. C, Chengcheng.

Center used PSCM as thawing solution and got one infant in 1994, which further proved the effectiveness of this thawing solution. The above mentioned are breakthrough achievements in giant panda breeding (figure 3).

In conclusion, the empty pregnancy rate has been evidently lowered and farrowing rate has been enhanced at Chengdu Research Base of Giant Panda Breeding and Chengdu Zoo since 1989. A striking event is that twins are often born. The good situation sufficiently demonstrates that the supplement of gonadotrophin and the use of newly selected thawing solutions are closely related with the breeding. The experimental results show that the supplement of external gonadotrophins not only has effect in the present year, but also has the function of endocrine adjustment, which will be beneficial to the reproduction later. The measure of "double control" will lead to the application of theories in endocrinology and fertilization biology to the practice of giant panda breeding and contribute a lot to the conservation of giant panda, which we believe will bring invaluable social and economic benefits. We deeply believe that the application of the measure will not only improve and amplify the breeding of giant panda, but also guide the breeding of other rare animals, economic animals and even domestic animals.

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