Effect of Sperm Preservation Solution of *Pseudobagrus hwanghoensis* in Artificial Reproduction Based on SCSA

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Abstract The quality of fish sperm is an important factor affecting artificial reproduction. Since the sexual maturation of male and female is not synchronized or for the fish that need to kill the male fish for sperm, sperm preservation solution is used to preserve the sperm temporarily during reproduction, in order to meet the needs of large-scale reproduction. Therefore, the preservation effect of sperm preservation solution directly affects the artificial reproduction. The quality of sperm in the preservation solution can be judged macroscopically by microscopic examination, but it is not accurate, while sperm chromatin structure analysis (SCSA) can quantitatively judge the quality of sperm in the preservation solution, thus providing guarantee for reproduction. The Zhaos sperm preservation solution of *Pseudobagrus hwanghoensis* has been verified by artificial breeding and SCSA analysis for many times, and it is better than Hanks sperm preservation solution. When the DNA fragmentation index is less than or equal to 0.15, the sperm is vigorous and the effect is obvious.

Keywords SCSA; Sperm preservation solution; DNA fragmentation index; Sperm motility

Pseudobagrus hwanghoensis, belonging to Siluriformes, lives in the bottom of the water and feeds on benthic animals, such as mayflies and hairy winged larvae. The young fish feeds on chironomid larvae. The fish is distributed in Yellow River System in Henan, and it is a rare species due to limited overall resources. The 3rd instar juvenile fish reaches sexual maturity, and the spawning period is from the middle of May to the end of July. The natural spawning amount is very small. Artificial breeding needs to kill the male fish for sperm, and large-scale breeding needs to store sperm with preservation solution in advance to improve efficiency and effect.

Sperm chromatin structure analysis (SCSA) is a method to detect the integrity of sperm DNA. Its principle is that the normal sperm DNA is double stranded, while the damaged sperm chromatin is loose, and the DNA is denatured into single stranded under the action of acid. According to the characteristics that acridine orange (AO) combines with double stranded DNA to emit green fluorescence, while it combines with single stranded DNA to emit red fluorescence, the Ao-staining sperm suspension can be analyzed by flow cytometry to determine the quality of sperm[1].

1 Materials and Methods

Reagents: acridine orange (AO) staining solution, hydrochloric acid, Hanks sperm preservation solution (commercially available), Zhaos sperm preservation solution (self prepared).

Sample: the sperms were ground [7], microscopically observed, and diluted in Zhaos and Hanks sperm preservation solutions for 8 min. According to the experimental design time period, the semens were diluted for 4, 96 and 144 h in advance, labeled, and then stored in the refrigerator at 4 ℃[2–4].

Accurately 30 μL of sperms stored in Zhaos and Hanks for 4, 96 and 144 h were taken respectively, successively added with 80 μL of acid aspirate (reaction for 30 s) and 100 μL of Ao staining solution (reaction for 3 min), and finally detected by cyto flex flow cytometer.

In artificial breeding, the mature parent fishes were injected with oxytocin[8–9]. The water temperature for oxytocin injection was above 20 ℃, with the optimum temperature of 22–24 ℃. Oxytocin was injected twice into the thoracic cavity, with a depth of about 1 cm. The first injection was carried out at 5:00–6:00 pm. The dose was LRH-A2 15–20 μg+ HCG 600 IU per kg of female parent fish, and the male fish was reduced by half. The needle distance was 12 h, and the second needle was injected at 5:00–6:00 am the next day. The dose was LRH-A2 15 μg + HCG 500 IU, and the male fish was reduced by half. When the water temperature was 20–24 ℃, the effect time was about 24 h. After the injection, the parent fish should be stimulated by flowing water.

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The pool was kept with slight flowing water at a flow rate of 10–30 m^3/h, so as to better promote the success of labor. During the water injection, attention shall be paid to prevent water from over flowing the pool. The dissolved oxygen in the tank should be more than 5 mg/L. The environment was kept quiet and away from light. Since the estrous behavior of pseudo parent fish was not obvious, artificial insemination was started after about 24 h of labor induction, and the extruded eggs (the eggs were orange, and the average diameter of the eggs was 2.0 mm) of mature female fish were artificially inseminated by semi dry method. When squeezing eggs, the pool water should not be brought into the basin to avoid affecting the fertilization rate. Proper amount of sperms stored in advance were sprinkled on the extruded eggs, and stirred continuously by hard chicken feathers so that the sperm and eggs can be better combined and fertilized[10–15].

The actual artificial insemination verification was to inseminate the sperm with different storage time and *P. hwanghoensis* eggs extruded after artificial induction[8]. The inseminated eggs were put into the incubation box according to the label, and incubated in the incubation tank for 5 h to calculate the fertilization rate[9].

2 Results and Analysis

2.1 Results The results of the on-line test of sperms stored in Zhaos solution are shown in Fig.1.

The on-line test results of sperms stored by Hanks solution are shown in Fig.2.

2.2 Analysis

2.2.1 Fracture index analysis. The sperm cells were circled on the FSC/SSC diagram and the gate was set to the FL1 (FITC)/FL3 (ECD) scatter diagram for analysis. Sperms with good DNA integrity mainly emitted green fluorescence (P2 gate), and those with poor DNA integrity mainly emitted red fluorescence (P4 gate). The sperm DNA quality was evaluated by calculating the DNA fragmentation index by the formula, at=red/(red+green).

The following results were obtained from the experimental graphs and data.

Zhaos:

at_1=6.37/(39.03+6.37)=0.14

at_2=11.28/(40.65+11.28)=0.21

at_3=9.48/(23.23+9.48)=0.29

Hanks:

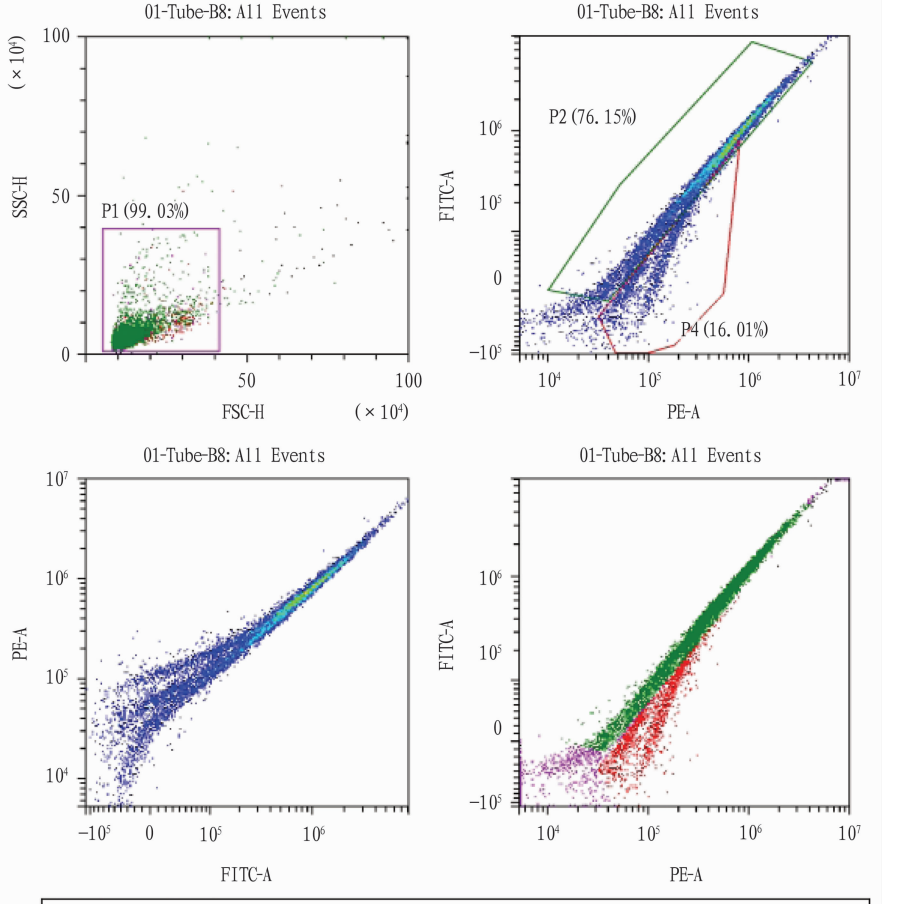
at_1=9.44/(32.10+9.44)=0.23

at_2=8.29/(17.18+8.29)=0.32

at_3=18.85/(38.54+18.85)=0.32

From the above breaking index results, the breaking index of the sperms stored in the Zhaos sperm preservation solution was smaller than that in the purchased Hanks sperm preservation solution: Zhaos at_1 < Hanks at_1; Zhaos at_2 < Hanks at_2; Zhaos at_3 < Hanks at_3. The results showed that Zhaos sperm preservation solution was superior to Hanks sperm preservation solution.

2.2.2 Verification analysis. The results of artificial insemination showed that the fertilization rate of sperms preserved in Zhaos solution was 81.4% at 144 h, which was kept at a high level and was consistent with the lower level of DNA fragmentation index Zhaos at_3=0.29 measured by flow cytometry (Tab.1). Similarly, the 144 h



Tube name: 04-Tube-B8
Sample ID:

Group	Particle number	% Parent group	Mean FITC-A	Median FITC-A	Mean PE-A	Median PE-A
A11 Events	10 000	100. 00%	606 386. 5	345 408. 8	532 885. 9	330 489. 3
P1	9 903	99. 03%	572 015. 3	339 914. 2	500 383. 4	326 515. 3
P4	1 601	16. 01%	78 474. 3	31 598. 4	160 210. 4	119 594. 8
P2	7 615	76. 15%	731 553. 9	547 137. 8	617 882. 8	491 705. 9

Fig.1 On-line test of sperms stored in Zhaos solution

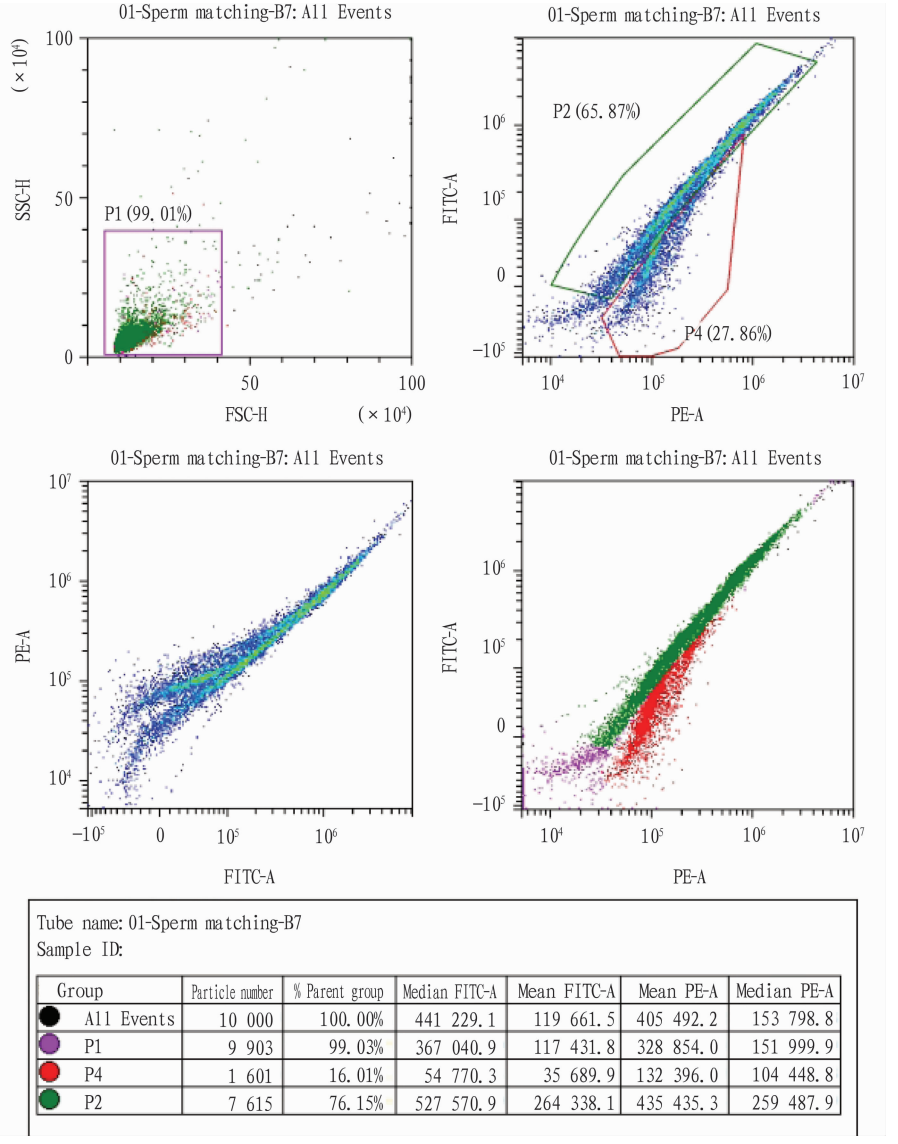


Fig.2 On-line test of sperms stored in Hanks solution

fertilization rate of sperms preserved in Hanks solution was 11.5%, which reached a very low level and can not be used for actual artificial reproduction, consistent with the results of higher level of DNA fragmentation index Hanks at_3=0.32 measured by flow cytometry.

2.2.3 Microscopic observation. Microscopic observation[5-6] showed that the sperms stored in Zhaos preservation solution were still vigorous on the 6th day, began to weaken on the 7th day, and died on the 9th day. The spermatozoa stored in Hanks preservation solution had stronger vitality on the 1st and 2nd day, weaker vitality on the 4th day and died on the 5th day.

2.3 Actual verification of artificial propagation When all the larvae were hatched, the newly hatched larvae were gathered in the 4 corners of the incubation frame, with a total length of only about 6.0 mm. They looked like tadpoles, and the yolk sac was large and nutritious. They were weak and tender and can not swim freely. After 5 –7 d of temporary feeding, the total length of the larvae reached 1.0 cm, and the yolk sac was greatly reduced. When the larvae started to eat, the fry can be cultured. However, during temporary feeding, the water quality was required to be clean, the dissolved oxygen should be greater than 5 mg/L, and the direct sunlight was also required[15].

The number of fries obtained in 5 batches of propagation were 300, 5 000, 10 000, 50 000 and 300 000 tails, respectively. Judging from the number of fries obtained in 5 batches, the propagation technology had been constantly improving and maturing.

Tab.1 Two practical effects of sperm preservation solution

Fertilization rate//%	Time//h		
	4	96	144
Zhaos solution	98.2	87.6	81.4
Hanks solution	83.0	13.3	11.5

3 Conclusions

(1) SCSA is a fast and accurate method to determine the quality of sperm, and can be used for quantitative analysis. It is an important means to detect the vitality of artificial preserved sperm before use.

(2) Through SCSA analysis and practical application test, Zhaos, a sperm preservation solution of *P. hwanghoensis*, can be artificially stored for one week in a 4 ℃ refrigerator and keep sperms vigorous, which is the guarantee for large-scale reproduction.

(3) For the artificially preserved spermatozoa of the Yellow River, according to the analysis of reference data[1] and test results, it is believed that if the DNA fragmentation index is less than or equal to 0.15, the spermatozoa are vigorous, and the preserved spermatozoa can be used as the source of artificial insemination. If the DNA fragmentation index is larger than or equal to 0.3, the sperms tend to be exhausted and are no longer used as the semen source of artificial insemination.

(4) The artificial reproduction of *P. hwanghoensis* is to prepare "artificial semen" with self-developed sperm preservation solution before insemination. The repro-

duction effect was very good, the fertilization rate was as high as 98.2%, with an average of 93%, and the hatching rate was 75%. After 5 batches of experiments, the reproduction technology has been gradually maturing, and the reproduction can be customized according to the number of users. In conclusion, a special sperm preservation solution has been developed independently, and an evaluation system based on SCSA sperm preservation solution has been established through DNA fragmentation index analysis.

References

[1] APEDAILE AE, GARRETT C, LIU DY, *et al*. Flow cytometry and microscopic acridine orange test: relationship with standard semen analysis[J]. Reprod Biomed Online, 2004, 8(4): 398–407.

[2] CHEN SL. Theory and technology of fish sperm and embryo cryopreservation [M]. Beijing: China Agricultural Press, 2007.

[3] CHEN SL, LIU XT, LU DC, *et al*. Study on cryopreservation of semen of silver carp, carp, Bluntnose bream and grass carp [J]. Acta Zoologica Sinica, 1992, 38(4).

[4] YU HT, ZHANG XM, CHEN C. Research prospect of fish semen cryopreservation [J]. Bulletin of Oceanology and Limnology, 2004(2): 66–72.

[5] LUO XZ, ZOU GW, PAN GB. Studies on the physiological characteristics of largemouth catfish sperm [J]. Freshwater fisheries, 2002, 32(2): 51–53.

[6] LU DC, FU CJ, LIU XT, *et al*. Biological characteristics of semen of main freshwater cultured fishes in China [J]. Freshwater Fisheries, 1989(2): 34–37.

[7] YANG JY. Study on the development and sperm biology of pelteobagrus fulvidraco [J]. Journal of Southwest Normal University: Natural Science Edition, 2005, 30(4): 719–724.

[8] WANG MH, CAI SX, CHEN XH, *et al*. Schilbagrid artificial propagation technology [C]. Jiangsu Institute of Freshwater and Fisheries, 2009.

[9] GAO ZZ, CUI J, XU GH, *et al*. Ussuri bagrid artificial propagation method [M].Changchun: Jilin Agricultural University Press, 2010.

[10] XIE YH. Freshwater fishes in Northeast China [M]. Liaoning Science and Technology Press, 2007.

[11] XIA YJ, YANG FY. Practical technology of fish farming [M]. China Agriculture Press, 1999.

[12] WANG W. Science of fish breeding [M]. China Agriculture Press, 2006.

[13] The Wangji Bridge. Fish Breeding Science [M]. Dalian University of Technology Press, 2000.

[14] Chinese Academy of Fishery Sciences. Practical Book of Freshwater Aquaculture [M]. China Agriculture Press, 2004.

[15] ZHAO DQ, LI XM, XIE GQ, *et al*. Summary of artificial propagation techniques of Ussuri Bagrid [J]. Henan Fisheries, 2019(1): 5–8.