Quality enhancement of cryopreserved spermatozoa of sutchi catfish (*Pangasianodon hypophthalmus*) with honey addition

N.A. Fanni*[1], M.B. Santanumurti[2], T.W. Suprayogi[1] and S.S. Bendryman[1]

[1]Fisheries and Marine Biotechnology, [2] Department of Fish Health Management and Aquaculture, Faculty of Fisheries and Marine, Universitas Airlangga, Surabaya, East Java, Indonesia, *Corresponding Author: Email: normaapriliafanni@gmail.com

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Abstract

Sutchi Catfish is one of the important fish commodities in Indonesia. Unfortunately, its seasonal spawning pattern causes limited supply. Cryopreservation is a solution to solve limited supply since it can store the spermatozoa in low temperature so that physiological, biological and morphological functions still remain. Improving the quality of cryopreservation is important to increase the success of Sutchi Catfish aquaculture. Adding honey in cryopreservation process is expected to increase the quality of spermatozoa since it contains with sugars as a source of spermatozoa's energy. This study tried to compare the effectivity of honey in cryopreservation process with no addition. The treatments used in this study were T1 (0% honey), T2 (0.2% of honey), T3 (0.4% of honey), T4 (0.6% of honey) and T5 (0.8% of honey). 30 days after stored, the spermatozoa were checked their motility, viability, abnormality, fertility and hatching rate. This study showed that honey addition could increase the motility significantly (P<0.01) to 23.14% better than control. The viability increased significantly (P<0.01) to 23.17% better than control. The abnormality test did not show significant difference between honey addition and control although the abnormality value in control was the highest (10.75%). The fertilization rate increased significantly (P<0.01) to 28.85% better than control. The hatching rate increased significantly (P<0.01) to 29.78% better than control. The success of all test indicated that the addition of honey in cryopreservation process of spermatozoa could be performed on Sutchi Catfish to increase its production even though the limited spawning pattern.

Keywords: Cryopreservation, Honey, Spermatozoa, Sutchi Catfish
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تحسين جودة الحيوانات المنوية المجمدة لسمك تشوسي السلور باضافة العسل

نورما ابريليا فاني[١]، محمد براويجويو سانتان مورت[٢]، تري واهيوسوبرايوغي[١] و وسريسوبيكتي بيندريمان[١]

تقنات الثروة السمكية والكائنات البحرية[١]، قسم ادراة وصحة الاسماك والكائنات البحرية[٢]، كلية الثروة السمكية والكائنات البحرية، جامعة إيرلانكا، سورابايا، جاوا الشرقية، اندنوسيا

الخلاصة

تشوسيالسلور هو إحدى السلع السمكية الهامة في اندونيسيا. ولكن لديه الأنماط الإنجابية الموسمية التي تؤدي إلى محدوديته. والحفظ بالتبريد هو الحل للتعامل مع محدودية امدادات ذلك السمك لأنه يحفظ الحيوانات المنوية في درجات حرارة منخفضةٌ جدًّا حتّى يعمل الاحتفاظ الفسيولوجية والبيولوجية والمورفولوجية. يعتبر تحسين نوعية الحيوانات المنوية أمرا ضروريا لتحسين الزراعة الناجحة لتشوسي السلور. ومن المتوقع أن تحسن إضافة العسل في عملية الحفظ بالتبريد نوعية الحيوانات المنوية لأن العسل يحتوي على السكر الذي يستخدم لطاقة الحيوانات المنوية. يحاول هذا البحث على مقارنة فعالية إضافة العسل مع عدم إضافة العسل على عملية الحفظ بالتبريد. تمت الإضافة في هذه الدراسة T1 (٠٪ العسل)، T2 (٠,٢٪ العسل)، T3 (٠,٤٪ العسل)، T4 (٠,٦٪ العسل)، T5 (٠,٨٪ العسل). بعد ثلاثين يوما من حفظ، تتم مراقبة الحيوانات المنوية على الحركة، والسلامة، والشذوذ، والتسميد ونسبة الفقس. وتشير هذه الدراسة إلى نتيجة إضافة العسل في تحسين القدرة على الحركة بشكل ملحوظ (ف <٠,٠١) ليصبح ٢٣,١٤% أفضل من مجموعة السيطرة. وتزيد

السلامة بشكل ملحوظ (ف <٠,٠١) ليصبح ٢٣,١٧ ٪ أفضل من مجموعة السيطرة. ولم يظهر اختبار الشذوذ فرقا كبيرا بين إضافة العسل ومجموعة السيطرة بالرغم من قيمة الشذوذ في عنصر التحكم هي الأعلى (١٠,٧٥٪). يزيد التسميد بشكل كبير (ف <٠,٠١) ليصبح ٢٨,٨٥ ٪ أفضل من مجموعة السيطرة. وتزيد نسبة القفس بشكل ملحوظ (ف <٠,٠١) ليصبح ٢٩,٧٨ ٪ أفضل من مجموعة السيطرة. ويشير نجاح جميع الاختبارات إلى أن إضافة العسل على عملية الحفظ بالتبريد يمكن تطبيقها في الحيوانات المنوية ليزيد إنتاج سمك تشوسي السلور رغم وجود أنماط التكاثر المحدودة.

Introduction

Sutchi Catfish is one of the important freshwater fish commodities in Indonesia. In 2011, Sutchi Catfish production reached 229,267 tons or about 16.1% of total world production (1). Sutchi Catfish is well-known since its high price, fast growth rate, high fecundity, cultured easily and tasty (2). However, Sutchi Catfish's seasonal spawning pattern which is once a year (October to April) causes limited supply to fulfill the demand (3). Therefore cryopreservation is a solution for limited supply in Sutchi Catfish's breeding.

Cryopreservation is a technique that can provide good quality spermatozoa for artificial insemination (4). Cryopreservation is used in fish biotechnology in order to solve the cost, risk in fish transport, limited broodstock, gamete storage and also spermatozoa banking (5,6). Thus, cryopreservation can be used in Sutchi Catfish to provide spermatozoa from male broodstock for breeding activity continuously.

The main factor that affects the success of cryopreservation is an extender. The extender is important for dilution of fish spermatozoa prior to cryopreservation. The most important function of the extender is to keep the spermatozoa in an immotile state until it is used (7). The diluents are required in cryopreservation to inhibit the energy used by spermatozoa and prolong the life of spermatozoa by reducing their activity (8). The energy needed by spermatozoa is obtained from simple sugars (monosaccharides) such as fructose and glucose which are found in honey. Honey contains 41% fructose and 35% glucose that can be used as a source of energy of spermatozoa (9). In addition, honey also contains salt ions for utilizing energy sources by spermatozoa, defending themselves and helping the fertilization of the egg after the cryopreservation process (10).

This study aimed to improve cryopreservation quality of Sutchi Catfish's spermatozoa by adding honey into diluent. The quality of spermatozoa after cryopreservation process would be tested through motility, viability, abnormality, and fertilization rate (11-14). The final result was expected to increase the quantity of Sutchi Catfish culture in Indonesia.

Material and methods

Material

Materials used were spermatozoa from Sutchi Catfish (IBAT Mojokerto, East Java), honey (Lawang Bee Forest, Malang), NaCl, glycerol, distilled water, eosin-nigrosin (Bred Life Science), 0.25 ml mini straw and liquid nitrogen. This research was conducted in July to November 2017. The maintenance of broodstock was conducted at "Laboratorium Reproduksi dan Pemuliaan Ikan", Brawijaya University, Malang. The cryopreservation process and the examination were conducted at Teaching Farm, Universitas Airlangga, Surabaya and LSIH, Brawijaya University, Malang. Fertilization process was conducted at "Sheva Fish", Boyolali, Central Java.

Spermatozoa collecting

Preparing a matured Sutchi Catfish with 2 kg weight. Injected the ovaprim at a dose of 0.4 ml/kg for intramuscular induction of hormone stimulation (15). Stripping was conducted 8-10 hours after injection then accommodated in a dry, sterile container. Sperm quality was evaluated by checking its color, morphology, volume, smell, motility, viability, progressive motility and concentration. The concentration of spermatozoa obtained was 4.7×10^9 cell/ml.

Preparation before freezing

Preparing the diluent solution was divided into two parts: diluent A and B. Diluent A was 10 ml each consist of NaCl and honey. The treatments were T1 (0% honey as control), T2 (0.2% of honey), T3 (0.4% of honey), T4 (0.6% of honey) and T5 (0.8% of honey). Diluent B was 10 ml each consisted of glycerol, honey, and NaCl. Honey dissolved in NaCl appropriate treatment concentration as before (T1, T2, T3, T4 and T5) made in 9 ml then added 10% each glycerol.

The spermatozoa were inserted in diluent A in each treatment, then stored in the cool room together with diluent B until the temperature reaches 5^{o}C. Then it mixed the diluent B with the diluent A gradually through the tube wall according to the treatment. Equilibration was performed for one hour. Filled and sealed the spermatozoa into the mini straw and continued into cryopreservation process (11).

Spermatozoa cryopreservation

Mini straw was placed 1-2 cm above the surface of liquid nitrogen for 9 minutes, then put into liquid nitrogen (deep freezing). After 15 minutes, the mini straw was quickly transferred into the stored tank with temperature of -196^{o}C for 30 days (11).

Thawing

After stored in 30 days, thawing process was conducted. Put the mini straw into the water bath with temperature of 30^{o}C for 30 seconds (7). Then dripped the spermatozoa from mini straw on glass object then examined the motility, viability, abnormality, fertilization and hatching rates.

Motility test

The characteristic of live spermatozoa was moving quickly, slowly or the head/tail movement, while the dead spermatozoa did not show any movement at all from the head or tail (16).

Viability test

Observation of spermatozoa viability was done by using a eosin-nigrosin staining. Dead spermatozoa were shown in red color while the living spermatozoa were shown in the white color (transparent) (12).

Abnormality test

Spermatozoa abnormalities could be known by eosin-nigrosin staining (Bred Life Science) (13).

Fertilization rate test

Sutchi Catfish's eggs were obtained from Ovaprim-induced broodstock. Females were given injections two times with an interval of 8 hours. The first injection was given a dose of 0.6 ml/kg and the second injection of 0.3 ml/kg (17). The eggs were taken 8-10 hours after the second injection, then stored in a dry, sterile container for use of fertilization check (8).

Fertilized eggs were mixed with post-thawing spermatozoa using the ratio of 1: 300,000 (18). Fertilization was done by putting post-thawing spermatozoa in a petri dish and then mixed with eggs. Afterward, 25°C of distilled water was added to activate spermatozoa and stirred slowly using feather for 5 minutes (19). Then the catfish eggs were dispersed in a tank which was aerated and incubated at 30-32°C.

Fertilization occured when the nucleus of spermatozoa cell was able to fertilize the core of egg in the cytoplasm to form a zygote (14).The fertilization was checked 6 hours after spermatozoa and egg were mixed.

Hatching rate test

The egg's hatching rate observation was performed 24 hours after fertilization. Hatching rate was determined based on the percentage of eggs hatched compared with total fertilized eggs (16).

Statistical analysis

The data obtained were tested using analysis of variance (Anova) according to the design used. If there was a significant difference then proceed with Duncan Multiple Range Test with the level of 0.05. SPSS (16.0) was used in this study.

Results

Spermatozoa motility

This study showed that the percentage of spermatozoa motility with honey addition was significantly (P<0.01) higher than control after stored for 30 days (Table 1). The highest spermatozoa motility resulted in the treatment T4 (honey 0.6%) with 45.72% while the lowest was 22.58% in T1 (control). This study showed that honey addition could increase the motility of spermatozoa about 23.14% better than control.

Table 1: Spermatozoa motility after cryopreservation

Treatment	Motility (%) ± SD
T1	22.58 ± 1.46 ^{a}
T2	29.63 ± 3.62^{b}
T3	35.75 ± 1.25^{c}
T4	45.72 ± 2.55^{d}
T5	43.07 ± 1.90^{d}

Different superscripts showed significant difference (P<0.01).

Spermatozoa viability

This study (Table 2) showed that the percentage of spermatozoa viability with honey addition was significantly (P<0.01) higher than control after stored for 30 days. The highest spermatozoa viability was 48.85% in the treatment T4 while the lowest was 25.68% in T1 (control). This study showed that honey addition could increase the viability of spermatozoa about 23.17% better than control.

Table 2: Spermatozoa viability after cryopreservation

Treatment	Viability (%) ± SD
T1	25.68 ± 2.43^{a}
T2	32.28 ± 2.72^{b}
T3	37.4 ± 2.91^{c}
T4	48.85 ± 3.58 ^{d}
T5	47.43 ± 1.64^{d}

Different superscripts showed significant difference (P<0.01).

Spermatozoa abnormality

This study (Table 3) showed that the percentage of spermatozoa abnormality with honey addition significantly decreased than control after stored for 30 days. The highest spermatozoa abnormality was 10.75% in T1 (control) while the lowest spermatozoa abnormality was 8.25% in T4. This value indicated that the physical quality of spermatozoa after freezing on honey addition treatment was better than control. The abnormalities form found in the study were a curved tail and tail break (Figure 1).

Table 3: Spermatozoa abnormality after cryopreservation

Treatment	Abnormality (%) ± SD
T1	10.75 ± 1.50 [a]
T2	9.25 ± 0.96 [a]
T3	8.25 ± 1.71 [a]
T4	8.25 ± 0.96 [a]
T5	8.75 ± 1.26 [a]

Same superscripts showed no significant difference (P>0.05).

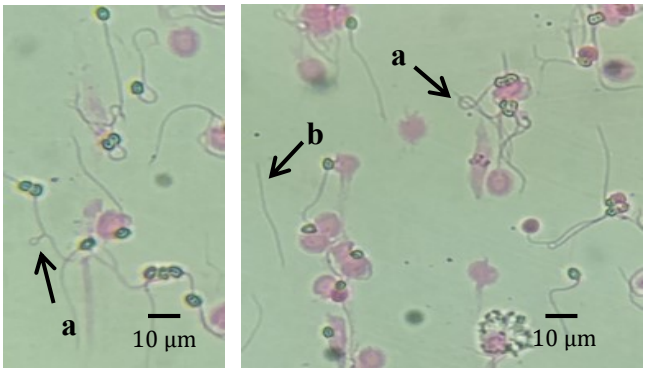

Figure 1: Abnormality test of spermatozoa, (a) Curved Tail, (b) Tail break.

Fertilization rate

This study (Table 4) showed that the percentage of fertilization rate of spermatozoa with honey addition was significantly (P<0.01) better than control after stored for 30 days. The highest fertilization rate was 30.23% in T4 while the lowest fertilization rate was 1.38% in T1 (control). This study showed that honey addition could increase the fertilization rate of Sutchi Catfish about 28.85% better than control.

Hatching rate

This study (Table 5) showed that the percentage of hatching rate with honey addition after stored for 30 days was significantly (P<0.01) better than control. The highest hatching rate was 29.78% in T4 while the lowest hatching rate was 0% in T1 (control). This study showed that honey addition could increase the hatching rate of Sutchi Catfish about 29.78% better than control.

Table 4: Fertilization Rate After Cryopreservation

Treatment	Fertilization Rate (%) ± SD
T1	1.38 ± 0.59 [a]
T2	20.23 ± 1.64 [b]
T3	24.23 ± 1.55 [c]
T4	30.23 ± 1.44 [d]
T5	28.48 ± 1.02 [d]

Different superscripts showed significant difference (P<0.01).

Table 5: Hatching Rate After Cryopreservation

Treatment	Hatching Rate (%) ± SD
T1	0 ± 0.00 [a]
T2	14.15 ± 1.58 [b]
T3	20.45 ± 0.60 [c]
T4	29.78 ± 2.19 [d]
T5	27.80 ± 1.56 [d]

Different superscripts showed significant difference (P<0.01).

Discussion

Motility or movement of spermatozoa is the most significant functional parameters influenced by the morphology and structure of spermatozoa (20). The motility of spermatozoa is useful for estimating the survival of spermatozoa (15). Spermatozoa with higher speed can fertilize more eggs (20).

In this study, the control treatment was the lowest value of motility. This result was due to the movement of individual spermatozoa was strongly influenced by the availability of energy supply produced in the metabolism of ATP (21). The contractions of the spermatozoa's fibrils would halt and stop moving without energy supply. It was necessary for spermatozoa to move through respiration or metabolism from ATP (22). This study suggested that honey addition in the diluent for cryoprotectant process had been shown to be effective in long-term spermatozoa storage as honey was contained in sugar.

Viability is one of the indicators to determine the quality of spermatozoa. The test purpose is to ascertain live or unviable spermatozoa by the percentage of viability based on the absorption of the eosin-nigrosin (23). The addition of honey showed higher viability than the control. This was because honey contains high fructose and glucose and can be used as a source of energy for spermatozoa survival (24).

The abnormality is one of the indicators in determining the quality of spermatozoa since abnormal spermatozoa cell structure can cause disruption in fertilization (25). Abnormal spermatozoa can reduce the occurrence of fertilization such as failure to reach the place of fertilization, inability to fertilize the egg or cannot support the development of the embryo (26). In this study, the addition of honey in the diluent showed no significant difference. The acceptable of spermatozoa morphological abnormalities was 20-30% (27). However, the honey addition was better when compared with the control. The abnormalities form found in the study were a curved tail and tail break and caused by differences in osmosis concentration during dilution, cold shock at the cooling time and sample preparation process (28).

Fertilization is the ability of the spermatozoa to fertilize the egg which later evolves into an embryo (15). This study showed that fertilization in control treatment was the lowest value of 1.38%. The hatching rate in control treatment was also the lowest value of 0%. The presence of honey could protect the spermatozoa in the low temperature. Antioxidant and anti-cold shock contained in honey could work optimally to protect spermatozoa, by breaking the lipid peroxidation chain reaction on the plasma membrane and prevent or reduce the damage that occurs in the plasma membrane during frozen stage (24).

Thus the number of spermatozoa that live is enough for fertilization and hatching process. The larger of the number of active spermatozoa will give a greater chance of a number of eggs to fertilize as the active spermatozoa to enter into the open egg microfil. Fertilization can occur when there are active spermatozoa entering the open egg microfil (31).

Honey is unique since its viscosity would increase when the temperatures became colder (32). Honey contains 41% fructose and 35% glucose that can be used as a source of energy of spermatozoa (9). In addition, honey also contains salt ions and carbohydrates for utilizing energy sources by spermatozoa, defending themselves and fertilize the egg after the cryopreservation process (10,32). Honey had also been used to improve the cryopreservation process in humans and mammals so that it can be used also in Channel Catfish (32-34).

The treatments used in this study were T1 (0% honey), T2 (0.2% of honey), T3 (0.4% of honey), T4 (0.6% of honey) and T5 (0.8% of honey). The study showed that higher addition of honey concentration would increase the value of motility, viability, fertilization rate and hatching rate. However, the highest value of motility, viability, fertilization rate and hatching rate was presented by T4. The value of all parameter was decreased in T5. Using honey of 0.8% could give toxic effect on spermatozoa. The toxicity in the form of changes in osmotic pressure in the diluent thus causing damage to plasma membrane spermatozoa cells (35).

It could be concluded that addition honey in cryopreservation process could improve spermatozoa quality of Sutchi Catfish. However, adding much honey could decrease the quality of Sutchi Catfish's spermatozoa in cryopreservation process. 0.6% of honey was the best result in cryopreservation process of Sutchi Catfish.

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