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by Cek Turnitin UHAMKA

Submission date: 07-Dec-2023 03:38PM (UTC+0700)
Submission ID: 2251149432
File name: Hatching_Rate_Embryo_of_Tor_Soro_after_48h_PostCold_Storage.pdf (795.17K)
Word count: 5283
Character count: 23524

第 48 卷 第 12 期
2021 年 12 月

湖南大学学报（自然科学版）
Journal of Hunan University（Natural Sciences）

Vol. 48 No. 12
December 2021

Open Access Article

The Use of Honey as Anti-Oxidative Agent: Hatching Rate Embryo of *Tor Soro* after 48h Post-Cold Storage

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Abstract: Honey as an anti-oxidative agent contains simple sugars (monosaccharides) such as fructose, glucose, and antioxidants that maintain motility, viability, and integrity of spermatozoa membranes in the preservation process. The use of honey as an antioxidant agent in sperm preservation varies between species due to differences in biophysical characteristics between cell types, and it is necessary to optimize the sperm preservation protocol for Mahseer (*Tor soro*) fish. Although the ability of honey as an antioxidant agent in the preservation of *T. soro* sperm has been proven through the analysis of abnormalities, viability, and sperm motility, the ability of post-preserved sperm fertility has rarely been reported. This study aims to evaluate sperm function after preservation by looking at the percentage of hatching rate of *T. soro* establishing a honey-optimized sperm preservation protocol on *T. soro*. Different concentrations of honey were investigated in postfertilization embryos by looking at the sperm quality of *T. soro* fish. For this purpose, the stripping technique is carried out by diluting the fish ringer solution and honey in a ratio of 1:10. After storage at 4°C for 48 hours, sperm is thawed at room temperature for 35 seconds and mixed with 100 eggs for fertilization. The percentage hatching rate of embryos was analyzed by ANOVA and Tukey tests. This study showed that honey affected the hatching rate of *T. soro* embryos after storage at 4°C ($p < 0.05$). The concentration of 1% honey resulted in the highest hatching rate of embryos with a total of 16.33 ± 1.35%.

Keywords: honey, anti-oxidative agent, sperm, cold storage, hatching rate.

蜂蜜作為抗氧化劑的用途：托索羅 48 小時後冷藏後的孵化率胚胎

摘要：作為抗氧化劑的蜂蜜含有單醣（單醣):如果糖、葡萄糖和抗氧化劑，可在保存過程中保持精子膜的運動性、活力和完整性。由於細胞類型之間生物物理特性的差異，蜂蜜作為抗氧化劑在精子保存中的使用因物種而異，因此有必要優化馬希爾魚的精子保存協議。雖然蜂蜜作為抗氧化劑在馬希爾魚精子保存中的能力已通過異常、活力和精子活力的分析得到證實，但保存後精子生育能力的報導很少。本研究旨在通過觀察馬希爾魚的孵化率百分比來評估保存後的精子功能，在馬希爾魚上建立蜂蜜優化精子保存方案。通過觀察馬希爾魚魚的精子質量，在受精後胚胎中研究了不同濃度的蜂蜜。為此，通過以 1:10 的比例稀釋魚林格溶液

Received: August 22, 2021 / Revised: October 17, 2021 / Accepted: November 13, 2021 / Published: December 30, 2021

Fund Project: 2nd National Innovative and Talent Program of Ministry of Education, Culture, Research, and Technology, The Republic of Indonesia

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和蜂蜜來進行剝離技術。在 4 攝氏下儲存 48 小時後，精子在室溫下解凍 35 秒，然後與 100 個卵子混合受精。通過方差分析和圖基檢驗分析胚胎的孵化率。該研究表明，蜂蜜會影響馬希爾魚胚胎在 4 攝氏下儲存後的孵化率 ($p < 0.05$)。1% 蜂蜜濃度導致胚胎孵化率最高，總計 16.33 ± 1.35%。

关键词：蜂蜜、抗氧化劑、精子、冷藏、孵化率。

1. Introduction

The most important ingredient of honey is carbohydrates present in monosaccharides, fructose, glucose, and disaccharides. It also contains oligosaccharides, including panose, and enzymes, including amylase, oxidase peroxide, catalase, and acid phosphorylase. Furthermore, honey contains amino acids, trace vitamin B, Vitamin B6, Vitamin C, niacin, folic acid, minerals, iron, zinc, and antioxidants. Honey is commonly used as an anti-inflammatory, antibacterial and antioxidant agent [1]. Antioxidants can play a negative role in the oxidation of molecules. Oxidation in cells can turn free radicals into chain reactions that can damage cells, tissues, and physiological functions [2].

Oxidative stress is one of the effects of the preservation process [3]. The reproductive technologies used for the conservation of sperm, short-term storage, and cryopreservation are two common practices in aquaculture used for routine management in artificial reproduction and management of gene banks, respectively. However, due to the poor initial quality or the intrinsic fragility of sperm in some species, cryopreservation is difficult to carry out, so cold storage is the most used tool as it is a viable, inexpensive, and simple procedure to perform [4]. Short-term storage is a prerequisite for different breeding programs such as cryopreservation, artificial fertilization, and hybridization. Sperm short-term storage techniques have been developed for several known fish species of commercial interest. The development of short-term storage protocols for fish sperm would allow solving the problem of asynchrony between sexually mature males and females, eliminating the need to keep broodstock available for artificial fertilization and thus maintain a continuous supply of gametes and have a constant stock of sperm and fingerlings throughout the reproductive period [5]. Additionally, its application would allow for further genetic studies and preservation of the genetic heritage of threatened or endangered species [6].

However, changes during the cold-storage process result in decreased sperm motility, plasma membrane function, acrosome integrity, and decreased sperm fertility [4]. Oxidative stress also causes an imbalance between free radicals and antioxidant protective activity. Honey contains phenolic compounds that have the potential as strong anti-oxidative agents [7]. Strong oxidative ability can reduce oxidative stress on sperm preservation. Therefore, as a natural ingredient that is easily found, honey has the potential to ward off oxidative stress.

Honey is a natural ingredient used in the freezing technique. Based on its composition, honey can function to maintain the motility, viability, and integrity of the spermatozoa membrane after dilution [3]. The main components of honey are sugars, proteins, minerals, phenolic compounds, and other minor compounds [8]. Fructose and glucose are the most commonly used in preservation. Fructose and glucose can provide the required energy and support the vitality of spermatozoa after dilution [9]. In addition, the availability of honey in nature is easy to obtain and can be purchased at an affordable price. Muhammad [10] revealed that brown sugar containing glucose at a concentration of 15% had a maximum percentage of T. soro spermatozoa viability which was 83.75%. Honey has a significant effect on the level of motility (80.48 ± 7.18%) [11]. In addition, cryopreservation of T. soro spermatozoa with a 5% concentration of honey cryoprotectant gave a maximum motility percentage of up to 85.97% [12].

Tor soro is one freshwater fish from the family *Cyprinidae* found in Indonesia. The presence of *T. soro* in nature has begun to decline because it has high economic value [13], which causes overfishing [9], habitat destruction, and the entry of foreign species [14]. Efforts to cultivate *T. soro* have been carried out, but the availability of seeds is still an obstacle [15] due to the mismatch of gonad maturity between males and females, so that it is necessary to do restocking and conservation. For that reason, spermatozoa preservation needs to be done with limited seed conditions, different gonad maturity times, and a limited cultivation area. Although the ability of honey as a natural anti-oxidative agent in sperm preservation of *T. soro* has been proven through the analysis of sperm abnormalities, viability, and motility, the ability of post-cryopreservation sperm fertility is rarely reported. The study focused on the changes during the research and evaluated the concentration of honey as a natural oxidative agent in the further development of *soro* embryos. In addition, the research contributes to the knowledge of various types of damage and their

consequences on successful fertilization using *T. soro* sperm after storage.

This study aims to evaluate sperm function after preservation by looking at the percentage of hatching rate of *T. soro* establishing a honey-optimized sperm preservation protocol on *T. soro*. For that reason, we use honey with various concentrations as one of the ingredients that have potential oxidative agents.

2. Methods

2.1. Preparation Extender

The fish Ringer's solution and honey were used as an extender. The stock of fish Ringer's solution was prepared by dissolving 3.25 g of NaCl; 0.125 g of KCl; 0.175 g of $CaCl_2.2H_2O$; and 0.1 g of $NaHCO_3$ in distilled water up to 500 mL, and then the solution was kept at 4°C temperature following [16]. Honey was purchased from the local market and six different concentrations of honey solution were tested: 0%, 0.5%, 1%, 1.5%, and 2%.

2.2. Sperm Collection and Dilution

Sperm was collected according to natural egg-laying time at the Freshwater Fisheries Germplasm Research Installation, Cijeruk, West Java, from August to September. Sperm retrieval using a stripping technique with the help of a syringe was then collected in a microtube containing a stock solution. Then, it was put in a cryotube using a micropipette.

Fresh sperm was suspended in the iluents mixtures containing fish Ringer's solution and the respective honey. The composition of the solution was modified after [16]. The dilution ratio of the fresh sperm and eluent solution was 1:10 based on [13].

2.3. Preservation, Storage, and Post-Preservation

The total stock solution made was 250 mL. The stock solution consisted of honey (concentrations of honey used were 0%, 0.5%, 1%, 1.5%, 2%): fish ringer (NaCl 3.25 g, KCl 0.125 g, $CaCl_2.2H_2O$ 0.175 g, and $NaHCO_3$ 0.1 g in 250 mL of aqua dest) [11]. The ratio of semen to a stock solution is 1:10. According to its concentration, the semen is diluted with a stock solution containing honey. The semen was refrigerated at 4°C for 48 hours. After 48 hours, the cement was stored at room temperature for 35 seconds for analysis.

The observation was carried out on the fertilization of fresh and post-preserved sperm of *T. soro*. Each post-preserved spermatozoa treated with honey at a concentration of 0%, 0.5%, 1%, 1.5%, and 2% was used for egg fertilization. The eggs were collected from the mature female fish by gentle abdominal pressure, and the eggs were put in the plastic basin and kept at 5°C. One hundred eggs were taken randomly then fertilized with the treated sperm. The fertilized eggs were incubated in different plastic basins. A total of 0.2 mL of eggs were mixed with 0.6 mL of thawed sperm (1:3 v/v) and two drops of tap water and then mixed with a soft feather. Furthermore, egg and spermatozoa cells were mixed and incubated in a container filled with water.

2.4. Statistical Analysis

The sperm viability and hatching rate data were analyzed using one-way ANOVA, followed by the Tuckey's test to determine the best treatment. The analysis was conducted using SPSS 23 (SPSS, Chicago, IL, USA). The qualitative data such as semen color, volume, and pH were analyzed descriptively.

2.5. Ethical Approval

Health Research Ethics Committee, Faculty of Medicine, Universitas Indonesia, Cipto Mangunkusumo Hospital approved the study. Ethical approval number: KET919/UN2.F1/ETIK/PPM.00.02/2019.

3. Result and Discussion

The results showed that the highest percentage of hatching rate was found at 1% honey concentration with 16.33 ± 1.35 ($p < 0.05$).

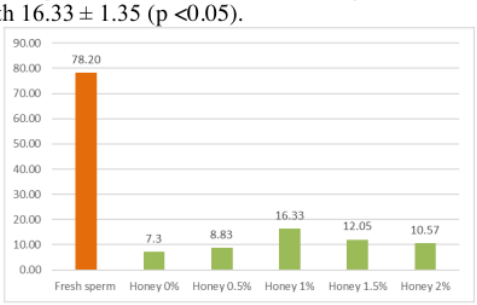

Fig. 1 Hatching rate of *T. soro* embryo after 48 hours

Based on Fig. 1, we first investigated the effectiveness of honey as an antioxidant agent, which contained 0.5%, 1%, 1.5% and 2% honey, and 0% honey, respectively, as a control group. The hatching rate of embryos was maintained at the same level with concentrations of 1.5% (12.05%) and 2% (10.57) used. However, a further increase in honey concentration up to 2% significantly reduced the hatching rate to 10.57% ($P<0.05$). In contrast, the use of 1% concentration resulted in a significant increase in hatchability to 16.33% compared to honey concentrations of 0%, 0.5%, 1.5%, and 2%.

Suci *et al*. [13] reported that a 10% concentration of honey was a natural compound that produced the highest percentage of embryo viability at 1080 minutes after fertilization with a total of 94 ± 2.79 ($p<0.05$). In addition, Berliana *et al*. [12] showed that honey affected ($P<0.05$) the percentage of motility after cryopreservation with an optimum concentration of 5%, namely 85.97±1.91%. The use of honey as an anti-oxidative agent tends to be different in each treatment; a concentration of 1% after fertilization using

preserved sperm *T. soro* has the highest average percentage of hatching rates. The study [17] reported that honey contains trehalose, glucose, and fructose joined by glycosidic bonds. This disaccharide is very useful because it has low insulinemic and glycemic indices. Trehalulose is also known to be cariogenic and a very active antioxidant.

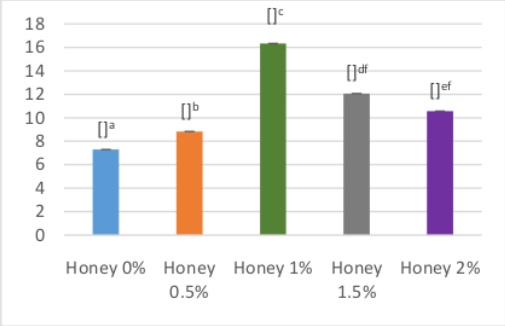

Fig. 2 Comparison of hatching rate percentage in fresh sperm with preserved sperm

Based on Fig. 2, the percentage value of hatching rate on fresh sperm and sperm that has been treated has an average ratio of 78.20±2.17% and 6.33±1.35%, respectively. These results indicate that honey is one of the natural components that can be used as a natural protective preservative precursor in preserving *T. soro*. However, further research is still needed on the amount of honey concentration because the hatchability value of sperm that has been treated has not been able to reach the portion of the hatchability value of fresh sperm.

According to Junita [18], External factors that affect the hatchability of fish eggs are temperature, dissolved oxygen, pH, salinity, and light intensity. The hatching process generally takes place faster at higher temperatures because, at high temperatures, the metabolic process runs faster so that embryo development will be faster, resulting in more intensive embryo movement in the shell. The fecundity of *T. soro* fish is 1320-2700 eggs with a fertilization rate of 91–96%, while the ideal temperature for hatching *T. soro* fish eggs is 19-22°C [15]. In addition, sperm DNA quality and gene expression are critical for early embryonic development. Abnormal genomic processes can cause permanent damage to totipotent cells, thereby altering the differentiation capacity of cells [19].

DNA is packaged in the form of chromatin. Chromatin integrity in spermatozoa has been shown to play an important role during embryonic development, and a close relationship has been established between the stability of the structural organization of chromosomes and the fertilization capacity of spermatozoa. Moreover, sperm chromatin plays a more important role in early embryonic development than during fertilization [20].

Chromatin packaged in one of three distinct schemes: (a) DNA linked to histones by the nucleosomal organization (HDNA): representing up to 15% of chromatin; (b) DNA linked to protamine (PDNA) adopts the typical toroid shape of the sperm nucleus, and (c) a small piece of DNA located between the nucleosome and the toroid and bound to the nucleus of the sperm matrix [21]. This difference in paternal gene compartmentalization leads to different accessibility associated with early or late transcription during embryonic development, according to studies by Figueroa et al. conducted in 2020. The packaging of the sperm genome in teleost fish is highly variable; some species use only histones, while others use protamine or protamine-like proteins in DNA compaction. These variations have been studied to understand their sensitivity to environmental effects, including exposure to toxins or radiation, aging, cold storage, cryopreservation, or changes in the thermal regime during spermiogenesis [22].

However, honey has chemical components that can be used as an anti-oxidative agent, which is indicated by the success of post-cryopreserved sperm to fertilize with eggs. Based on Berliana *et al*. [12] that the cryopreservation of *T. soro* spermatozoa with a 5% concentration of honey cryoprotectant gave the maximum motility percentage up to 85.97%. According to, honey contains antioxidants that are important for reproduction, antioxidants contained in honey significantly increase the concentration of SOD (Superoxide Dismutase) in semen. SOD plays a role in counteracting the increase in ROS (Reactive Oxygen Species) that occurs during the cryopreservation process. Increased ROS [23] causes oxidative stress conditions that result in decreased sperm quality after cryopreservation due to DNA damage. ROS attacks purine and pyrimidine bases, causing DNA damage, and in addition, it initiates apoptosis in sperm, causing cotton enzymes to become active [22].

4. Conclusion

Based on these studies, it was concluded that honey solution affects the hatching rate of *T soro* after storage at 4°C. In addition, 1% of the honey solution showed the highest hatching rate of the embryo, with a total of 16.33 ± 1.35%. Further research is needed on the basic components of honey which have great potential to be used as anti-oxidative agents, especially in the preservation of *T. soro* sperm. The molecular analysis will also be conducted to determine DNA damage and other external factors that can reduce the hatching rate of *T. soro's* embryos.

Acknowledgment

This research was supported by the Directorate of Research and Community Service, University of Indonesia (HIBAH PIT 9 2019: NKB-

0015/UN.R3.1/HKP.05.00/2019) on behalf of Dr. Abinawanto. Research and Development Institute, University of Muhammadiyah Prof. DR. Hamka (Doctoral Dissertation Research: 855/F.11/07/2021) and 2nd National Innovative and Talent Program of Ministry of Education, Culture, Research, and Technology, Republic of Indonesia, and also to the Installations for Freshwater Fish Genetic Resources, Ministry of Marine Affairs and Fisheries, Cijeruk, West Java.

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