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THE EFFECT OF STORAGE TIME ON THE QUALITY OF COMMON CARP SPERM (*Cyprinus carpio*) STRAIN PUNTEN IN NaCl SOLUTION AND COCONUT WATER (*Cocos nucifera* L.) EXTENDER

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ABSTRACT

Cyprinus carpio is a freshwater species with high reproduction and adaptability. These factors make C. carpio being the freshwater fish that majority distributed and has high commercial value in some countries, including Indonesia. The maturation phase of male and female fish gonads does not occur simultaneously. Thus, sperm preservation could be an alternative technique to maintain the breeding process throughout the year. This research aims to determine the effect of storage time on C. carpio sperm quality strain Punten in NaCl solution-coconut water (Cocos nucifera L.). This study used common carp (C. carpio) 8 months of age with 500 g.bw^{-1}. The C. carpio semen sample was divided into four treatment groups (0, 3, 6, and 9-hour storage time). The extender used in this research was composed of coconut water (C. nucifera L.) and NaCl solution with a ratio of 70% : 30%. The samples were stored in a refrigerator at 5^{o}C. Fresh sperm sample was analyzed macroscopically using various parameters, such as pH, volume, color, and consistency. Meanwhile, the post-preserved sperm quality sample was analyzed microscopically (motility, viability, concentration, and abnormality). Furthermore, we also obtained the fertilization rate and hatching rate. The result showed that storage time affects the reduction of C. carpio sperm quality significantly ($p<0.05$). The highest sperm viability was obtained in NaCl solution-Cocos nucifera with a 3-hour storage time (78.33%±5.49). Our study found that the storage time significantly affected the sperm quality of C. carpio that was given with C. nucifera extender. Adding C. nucifera with 3,6,9-hour storage time also increases the fertilization and hatching rate compared with the group without C. nucifera. It can be shown that the addition of C. nucifera as a natural extender has the essential role of maintaining C. carpio sperm quality after a short storage period.

Keywords: *Cocos nucifera, Cyprinus carpio, preservation, sperm quality*

INTRODUCTION

Common carp (*Cyprinus carpio*) is a freshwater species with high reproduction and adaptability. These factors make the *C. carpio*, the freshwater fish, which is mostly distributed and has high commercial value in some countries, including Indonesia. The common carp had a percentage of 70% of total aquaculture global production [1]. The percentage of *C. carpio* production reaches approximately 46% of freshwater cultivation [2]. Common carp are also known as species with high fecundity. Research about the storage and preservation of *C. carpio* spermatozoa is widely investigated, but the ideal natural extenders as antioxidants still need to be explored to maintain sperm quality after storage time. This is because it is associated with successful hatchery and maintaining the genetic diversity of this species [3].

Sperm quality is the essential factor that influences the productivity of *C. carpio*. The disturbance of fish sperm quality could directly affect fertility capability and the offspring [4]. Common carp sperm have a lifespan of 72 seconds inside the water [5]. In general, the lifespan of spermatozoa outside of the environment is only 1-2 minutes [6]. Thus, advanced methods to maintain the spermatozoa's lifespan outside were needed, including storage at a lower temperature, widely known as fish sperm preservation [7]. This technique has advantages for artificial spawning because of the difference between the maturation phase of male and female fish gonads. Short-term sperm storage usually occurs at 4-5°C [8, 9]. Spermatozoa storage is strongly associated with extender quality. Using an extender has an important role in increasing the quality and viability of spermatozoa when it is stored [5]. Fish sperm are very sensitive to peroxidative damage. Antioxidant sources were an essential component needed during the fish sperm preservation process. The natural antioxidants obtained from *Vitis vinifera* could protect spermatozoa from the peroxidative damage caused by Reactive Oxygen Species (ROS). Thus, fish sperm quality is still maintained [10].

The temperature was the important factor that affected sperm quality. The lower temperature during the preservation process would affect the

fertility capability of fish sperm [11]. Seminal plasma components have the role of protecting the spermatozoa from oxidative stress. While the preservation process, the reduction of seminal plasma could affect the sensitivity of spermatozoa to oxidative stress that resulted in spermatozoa DNA damage [12]. Several factors affect the success of sperm preservation, such as storage medium, dilution factor (sperm:extender), and extender type. The ideal extender contains antioxidants, antibiotics, nutrition, and buffer [13]. The extender that is often used for fish sperm preparation is physiological NaCl. The role of physiological NaCl is to buffer and maintain the optimization of sperm pH [14]. The sperm storage with physiological NaCl as the extender usually only gives the sperm a lifespan of 60 minutes because of the low energy source spermatozoa need. Furthermore, the other components that have a role in energy bioavailability to elongate the lifespan of spermatozoa are still required [15].

Coconut water (*Cocos nucifera* L.) is a natural extender with several components, such as glucose and fructose, used as an energy source for spermatozoa [16, 17]. The research about sperm preservation of *Clarias batrachus* at 4°C for six days with *C. nucifera* as an extender has shown increased motility, viability, and fertilization rate [18]. The osmolality of the extender is one factor that affects sperm motility after storage time. This osmolality factor is associated with ion concentration inside the extender. Na^+ ion is the primary electrolyte that has a role in maintaining the osmolality of semen plasma. Thus, sperm viability and motility are still preserved. Sodium also has a crucial role in stabilizing the pH [19]. Coconut water also has high antioxidants, sugar, vitamins, electrolytes, and amino acids. Antioxidant compounds found in coconut water include vitamin C and flavonoids, which protect sperm from lipid peroxidation [17]. Vitamin C is a ROS scavenger with high polarity caused by the hydroxyl chain inside; thus, it is easily soluble in water.

Furthermore, vitamin C will directly react with ROS, such as hydroxyl radicals and hydrogen superoxide [20, 21]. Glucose and fructose inside coconut water are energy sources for spermatozoa strongly associated with sperm motility [22, 23]. This research aims to determine the effect of various storage times 3, 6, and 9 hours or short-term periods on common carp (*C. carpio*) sperm quality added with NaCl solution-*C. nucifera* extender to determine the ideal extender composition to improve the expected carp sperm quality after storage time.

METHODS

Study design. This research consists of four groups with six replications, including P0 or control group (a group with 0-hour storage time), P1 (a group with 3-hour storage time), P2 (a group with 6-hour storage time), and P3 (a group with 9-hour storage time). The whole group fresh semen was stored at 5°C. Fresh semen of *C. carpio* was added with coconut water as a natural extender inside of NaCl solution 0.9%. Semen was diluted with NaCl solution-coconut water with a ratio 1 : 9 (semen:extender, v/v). Each tube contained 0.3 mL semen and 2.7 mL extender, thus resulting in a total suspension volume of 3 mL (Table 1).

Table 1. Semen dilution ratio

Dilution ratio		Total
NaCl solution 30% (0.81 mL)	*Cocos nucifera* 70% (1.89 mL)	2.7 mL
Semen 1 (0.3 mL)	Extender 9 (2.7 mL)	Semen+Extender 3 mL

Common carp (*C. carpio*) selection and sperm collection. This study used common carp 8 months of age with 500 $g.bw^{-1}$. The common carp were selected based on SNI 8296.1:2016 standard and obtained from Punten Freshwater Fisheries and Aquaculture Installation Laboratory (IPBAT), Batu City, East Java. The male *C. carpio*, whose gonads have matured, has characteristics in the form of slightly red and protruding genitals. Fresh sperm collection used the stripping technique with a slow press from the abdominal area to the urogenital area [23, 24].

Extender preparation and sperm preservation. The extender used in this research is composed of physiological NaCl and coconut water. The comparison of coconut water and physiological NaCl was 70% : 30% with the extender ratio and sperm was 1 : 9 (0.3 mL sperm : 2.7 mL extender).

Sperm quality analysis. The sperm quality analysis was carried out on fresh sperm macroscopically, while the post-preserved were microscopically. The macroscopic analysis included pH, volume, color, and consistency [25]. The pH was measured with litmus paper, and consistency was visually analyzed (white, milky white, or cream) [13]. The microscopic analysis included motility, viability, concentration, and abnormalities. The 10 µL sperm suspension was taken to this analysis with a microscope with 100 – 400X magnification [25]. The sperm viability and abnormalities were calculated with the following formula below:

$$Viability = \frac{Total\ of\ live\ sperm}{Total\ of\ sperm} \times 100\% \ldots\ldots (1)$$

$$Abnormalities = \frac{Total\ of\ abnormal\ sperm}{Total\ of\ sperm} \times 100\% \ldots\ldots (2)$$

The sperm viability and abnormalities were evaluated based on eosin-nigrosin staining. The live sperm will look transparent, and the dead sperm will be stained with eosin-nigrosin [26]. The sperm concentration was calculated with the 10 µL taken and dropped inside of the hemocytometer in the microscope with 400X magnification [27].

Fertilization and hatching rate. This research used the female common carp with 3 years of age and 4 kg.bw^{-1} based on the SNI 8296.1:2016 standard. The ovum collection was done with the stripping technique by slowly pressing the abdominal area to the genital pore. The ovum obtained will homogenize with semen with chicken feathers to initiate fertilization. [28]. The homogenate was dropped into the pool surface that was given by aeration [29]. Fertilization rate was observed at 10-12 hours after fertilization [29, 30]. The successful fertilized ovum will appear transparent and clear inside. Meanwhile, the unfertilized ovum will look pale white [30]. The fertilization rate was calculated using the following formula (3):

$$Fertilization\ Rate\ (FR) = \frac{Fertilized\ ovum}{Total\ of\ ovum} \times 100 \ldots\ldots (3)$$

The time it takes for a fertilized ovum to hatch is approximately 2–3 days. The hatch ovum will form larvae, while the unhatched ovum will look pale white, indicating the egg was dead [31]. The hatching rate was calculated with the following formula (4).

$$Hatching\ Rate\ (HR) = \frac{Hatched\ egg}{Hatched\ fertilized} \times 100 \ldots\ldots (4)$$

Data analysis. The sperm quality data, including pH, volume, color, consistency, and sperm abnormalities, were descriptively analyzed. Meanwhile, the sperm motility, viability, concentration, fertilization and hatching rate were statistically analyzed with Statistical Product and Service Solution (SPSS) software. The statistical analysis used is one-way ANOVA ($p<0.05$) followed by the Tukey HSD test.

RESULTS AND DISCUSSION

***Cyprinus carpio* sperm quality.** The quality of fresh *C. carpio* semen was analyzed macroscopically and microscopically. Macroscopic parameters include color, pH, volume and consistency. The color of fresh semen from *C. carpio* visually shows a milky white color, indicating that fresh semen is classified as normal. Normal fresh fish semen is milky white or slightly pale cream. Abnormal colors of fresh semen, such as greenish, indicated faecal contamination. The yellowish-green color indicates the presence of *Pseudomonas aeruginosa* bacterial contamination, while a pink or purplish color indicates contamination in the form of blood on the external genitals. The brown color is caused by the presence of blood in the upper genitals [32]. Fresh semen of *Cyprinus carpio* has an average volume of 7 mL and is categorized as normal because the average volume of fresh common carp semen is approximately 2.5 – 7 mL [33, 34]. The consistency of fresh semen is considered thick and categorized as normal because the consistency of good quality sperm is like thick milk, representing the number of spermatozoa. The degree of acidity (pH) of fresh semen *C. carpio* as a result of this study is in the range 6 – 7 (Table 2). The pH of good common carp fresh semen is in the range of 6.8 – 7.6 [35]. The optimal pH for common carp and catfish semen is 7, which correlates with increased motility and viability of spermatozoa. Semen with a pH that is too high or too low causes a decrease in metabolism, thereby reducing the motility and viability of spermatozoa [4].

Sperm motility. The study found that the addition of coconut water in NaCl solution with a storage time of 3, 6, and 9 hours resulted in an increase in the percentage of sperm motility compared to the group treated with NaCl solution only. The percentage of sperm motility at P1, P2, and P3 were 68.33% ± 5.16, 62.50% ± 2.73, and 37.50% ± 5.2, respectively (Table 3). The storage time significantly affected sperm motility ($p<0.05$), where the longer the storage time, the lower the sperm motility due to time dependence.

Table 2. Common carp (*Cyprinus carpio*) semen pH

Group	Semen pH	
	NaCl solution only	NaCl solution-*Cocos nucifera*
P0	7	7
P1	6	6
P2	6	6
P3	7	7

Table 3. The effects of storage time on *C. carpio* sperm quality

Group	NaCl solution only			NaCl solution-*Cocos nucifera*		
	SM (%)	SV (%)	SC (x 10^9 cell/ml)	SM%	SV (%)	SC (x 10^9 cell/ml)
P0	73.33±2.58^c	84.14±6.69^c	5.57±43.96^b	70.00±4.47^c	77.27±5.72^b	4.67±57.51^a
P1	65.83±3.76^b	67.72±4.47^b	4.99±43.37^a	68.33±5.16^{bc}	78.33±5.49^b	4.58±64.22^a
P2	60.00±5.47^b	64.17±3.43^b	4.92±21.41^a	62.50±2.73^b	74.30±4.58^b	4.51±32.54^a
P3	35.83±3.76^a	50.73±7.48^a	4.81±19.98^a	37.50±5.24^a	55.83±3.77^a	4.46±38.19^a

Note: different superscript letters in the same column indicate significant differences between groups ($p<0.05$). (P0) 0 hours storage time, (P1) 3 hours storage time, (P2) 6 hours storage time, (P3) 9 hours storage time

SM (%) : percentage of sperm motility
SV (%) : percentage of sperm viability
SC (x 10^9 cell/ml) : sperm concentration

The decrease in sperm motility caused by time-dependent during the storage process was due to a significant decrease in ATP levels. Low temperatures during the preservation process affect the functionality of spermatozoa, such as sperm motility, membrane integrity, DNA integrity, and fertilization ability [36]. Long storage time also causes alterations in the structural components of flagella microtubules and cytoskeleton proteins. The cytoskeleton plays a crucial role in maintaining the normality of spermatozoa structure and their movement. A previous study demonstrated that long storage time could affect the disorganization of the spermatozoa flagella axoneme structure, which represents kinetic alteration post-storage time [37].

Sperm motility is an essential parameter in sperm quality, primarily due to its direct correlation with the spermatozoa's ability to exhibit movement towards the female gametes [5, 38]. When spermatozoa are outside the testicles, spermatozoa require nutritional sustenance for energy to maintain their longevity. Depletion of ATP within spermatozoa leads to the cessation of spermatozoa fibril contraction, resulting in immotility [39]. This is caused by alterations in proteins associated with the tricarboxylic acid cycle (TCA). Most proteins that play a role in the TCA cycle are found in the midpiece of spermatozoa. This pathway is essential because it is related to the ATP formation in spermatozoa [40]. Preservation of *C. carpio* sperm has been shown to elevate the production of Reactive Oxygen Species (ROS) within spermatozoa due to protein carbonylation. Carbonylated proteins exert notable effects on sperm motility, axoneme-bound proteins, and proteins involved in energy metabolism. The increased ROS production adversely impacts the flagellar structure and energy supply in spermatozoa, potentially resulting in abnormalities [37].

Coconut water is composed of organic and inorganic molecules. Components found in coconut water include carbohydrates, protein, mineral salts, amino acids, and sugars (sucrose, glucose, fructose and sorbitol). The availability of nutritional sources such as glucose and fructose in coconut water is a source of energy for spermatozoa. This component has the capability to maintain sperm motility post-preservation process [41]. This study demonstrated that sperm motility percentage value of 68.33% ± 5.16 at 3 hours storage time, which tends to be close to the motility percentage of fresh sperm. Coconut water also contains vitamin C, a flavonoid which plays an important role as a ROS scavenger, thereby preventing lipid peroxidation and stimulating endogenous antioxidant activity, such as Superoxide Dismutase (SOD) and catalase (CAT) [42].

Sperm viability. The addition of coconut water in NaCl solution with a storage time of 3, 6, and 9 hours resulted in an increase in the percentage of spermatozoa viability compared to the NaCl solution-only group. The highest spermatozoa viability percentage was found in group P1 with a storage time of 3 hours with 78.33% ± 5.49 respectively (Table 3). Based on Tukey's test, it found that the longer of storage time will directly affect the decrease of sperm viability or time-dependent (P3) ($p<0.05$). It is caused by the cold temperatures that trigger the cold shock mechanism in spermatozoa. Low temperatures during the spermatozoa storage process cause increased ROS production [5]. High ROS production can cause damage to the plasma membrane and mitochondrial structure in spermatozoa. The spermatozoa membrane contains a lot of polyunsaturated fatty acids (PUFA), which are ROS targets. ROS can stimulate lipid peroxidation which causes loss of spermatozoa membrane integrity, resulting in sperm cell death either by apoptosis or necrosis [43].

Coconut water has natural ingredients that play a role in increasing the viability of spermatozoa after the storage process. Previous studies stated

that the minimum percentage of fish spermatozoa during storage was 70% [44]. These results correlate with this research because the NaCl solution-*Cocos nucifera* treatment group had a percentage of 78.33% ± 5.49 with a storage time of 3 hours and 74.30% ± 4.58 with a storage time of 6 hours. The phenolic and flavonoid compounds in *C. nucifera* act as a protective agent against ROS reactivity. Thereby, the membrane integrity can be maintained [41]. Spermatozoa viability is also associated with energy availability. The NaCl solution-*Cocos nucifera* extender produced higher spermatozoa viability than spermatozoa without adding coconut water. The sucrose and fructose content in coconut water provides an energy source that maintains the lifespan of spermatozoa during the storage period [33].

Sperm concentration. The storage times of 3, 6, and 9 hours resulted in spermatozoa concentrations that tended to decrease or were time-dependent but not significant (Table 3). The concentration of spermatozoa showed a decrease with the length of storage time. Based on this study, the lowest spermatozoa concentration was found in the treatment with a storage time of 9 hours (4.46 x 10^9 ± 38.19 cells/mL). The concentration of spermatozoa in *C. carpio* fish decreased due to the length of storage time at low temperatures because the spermatozoa lead to agglutination [37]. A previous study showed that the number of spermatozoa in *C. carpio* decreased with a storage period of 8 days [3].

Sperm abnormalities. Our research has found the existence of primary and secondary spermatozoa abnormalities. Primary abnormalities occur during the spermatogenesis process in the seminiferous tubules [45]. This study found a primary abnormality in the form of a double head (Figure 1a). Secondary abnormalities are spermatozoa damage that occurs during the maturation process in the epididymis. Several secondary abnormalities were found in this research, such as head without tail, tail only, and folded tail, Figures 1b; 1c; 1d [46, 47]. Spermatozoa abnormalities can also be due to cold storage at 5°C mechanism. The alteration of temperature affects the spermatozoa membrane permeability, thus becoming abnormal. Several other factors can cause spermatozoa abnormalities, including alterations in osmotic pressure due to the release of ions and dehydration of spermatozoa during the storage period, which causes damage to the spermatozoa membrane [48].

Fertilization rate. Adding coconut water to the NaCl solution as an extender significantly increased the fertilization rate in all treatment groups. The highest fertilization rate value was in the control group with the addition of coconut water (52.62% ± 5.71). The fertilization rate value decreased along with the length of storage time. The lowest fertilization rate value was found in the group without *C. nucifera* at 6.65% ± 2.25 with a storage time of 9 hours (Table 4). The fertilization rate is a parameter associated with spermatozoa motility and viability. This study showed that adding coconut water with a storage time of 3 hours produces a high percentage of motility, spermatozoa viability, and fertilization rate. Thus, these results show the potential of coconut water as a natural extender, which can improve the quality of *C. carpio* sperm with a storage time of 3 hours.

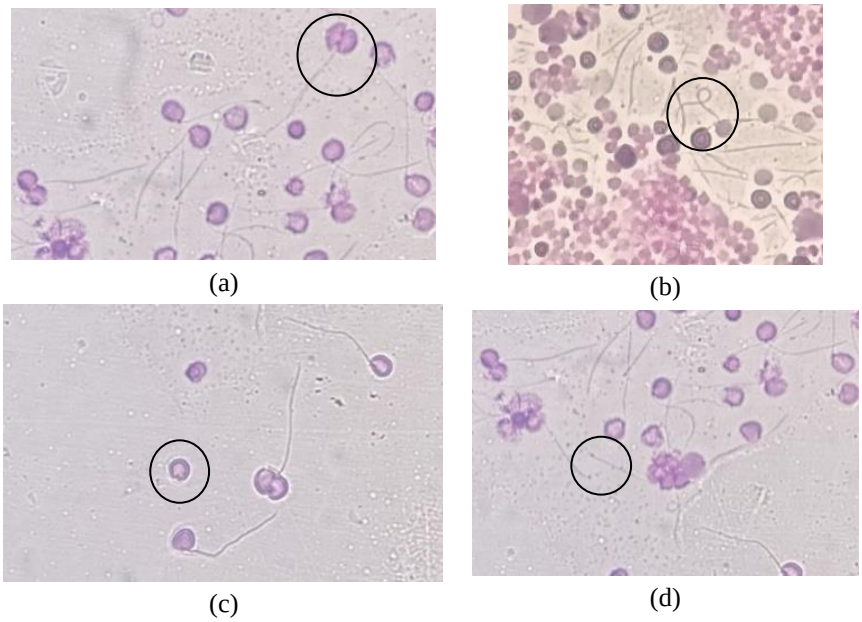

Figure 1. Sperm abnormalities (x400). Double head; (b) folded tail; (c) head without tail; (d) tail only

Table 4. Fertilization rate of *C. carpio*

Group	NaCl solution only	NaCl solution-*Cocos nucifera*
	Fertilization Rate	Fertilization Rate
P0	42.75±6.79^{d}	52.66±5.71^{c}
P1	31.40±7.84^{c}	38.64±5.15^{c}
P2	13.30±4.34^{b}	15.17±2.11^{b}
P3	6.65±2.25^{a}	10.09±2.09^{a}

Note: different superscript letters in the same column indicate significant differences between groups ($p<0.05$). (P0) 0 hours storage time, (P1) 3 hours storage time, (P2) 6 hours storage time, (P3) 9 hours storage time

The fertilization rate is the percentage value of eggs successfully fertilized from the total eggs released during spawning [49]. The fertilization rate is related to sperm quality. A good sperm quality of *C. carpio* leads to a high probability of gaining a higher fertilization rate. High spermatozoa motility can provide optimal fertilization rates [50]. Spermatozoa that move progressively tend to have the capability to fertilize egg cells at a higher rate. The progressive spermatozoa have long motility. The quantity of spermatozoa also affects the fertilization rate [51]. High spermatozoa viability represents the capability of spermatozoa to penetrate the microphyll holes in the egg cell. Thus, the opportunity for fertilization to occur is higher [52]. Our research positively correlated with previous research that proves the addition of coconut water in NaCl solution as an extender stimulates and prolongs the activity time of spermatozoa in fertilizing egg cells [29]. A study by Pangaribuan et al. showed that adding 70% coconut water in 30% NaCl resulted in a fertilization rate of 89% after a storage period of 12 hours [53]. The sucrose and fructose compounds in coconut water act as bioenergy sources for spermatozoa. In contrast, the vitamin C and flavonoid components act as protective agents that protect spermatozoa while stored at low temperatures [41].

Hatching rate. This research showed that the addition of coconut water had a higher hatching rate compared to samples without the addition of coconut water. The highest hatching rate value was found in the control treatment with the addition of coconut water (20.03 ± 8.38%) and the control without coconut water (16.82% ± 3.01). The hatching rate value decreased with storage time (Table 5).

Table 5. Hatching rate of *C. carpio*

Group	NaCl solution only	NaCl solution-*Cocos nucifera*
	Hatching Rate	Hatching Rate
P0	16.82±3.01^{b}	20.03±8.38^{c}
P1	11.96±3.19^{ab}	15.02±6.83^{bc}
P2	5.75±2.77^{ab}	7.62±2.42^{ab}
P3	2.91±1.67^{a}	4.41±1.95^{a}

This result was caused by the hatching rate positively correlated with the fertilization rate, where the higher the percentage of fertilization rate, the higher the hatching rate value was found [14]. The hatching rate is the total number of eggs that successfully hatched from the number of fertilized eggs [49]. The hatching rate is also positively correlated with spermatozoa motility and fertilization rate, where a high percentage of spermatozoa motility can increase the possibility of successful fertilization and increase egg hatchability [46]. A study by Pangaribuan et al. showed that the addition of 70% young coconut water in 30% physiological NaCl resulted in a hatching rate of 88.5% after a storage period of 12 hours [53].

CONCLUSION

The storage time significantly affects the reduction of *C. carpio* sperm quality. The addition of coconut water in the NaCl solution extender for the 3-hour storage time showed an increase in sperm viability compared with the group that only used the NaCl solution extender. Adding coconut water for the 3, 6, and 9-hour storage time also increases the fertilization and hatching rate compared with groups without coconut water. It can be shown that the addition of *Cocos nucifera* as a natural extender has the essential role of maintaining *C. carpio* sperm quality after a short storage period.

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