Original Article

Effect of L-cysteine on Chilled Carp (*Cyprinus carpio*) Semen Qualities

Kan Kledmanee[1] Somrat Taweedet[2] Prawporn Thaijongruk[1]
Panida Chanapiwat[1] Kampon Kaeoket[1*]

Abstract

The aim of the present study was to study the effects of L-cysteine on chilled carp (*Cyprinus carpio*) semen qualities. Pooled semen samples were prepared from eight fish, and divided into five groups according to the concentrations of L-cysteine as follows: 0 (T1), 0.5 (T2), 1.0 (T3), 1.5 (T4) and 2 (T5) mM. The sperm motility, duration of sperm motility and sperm viability were evaluated at 0, 12, 24, 48, 72 hours after chilled storage. Comparing between treatment and control groups, the percentage of sperm motility, duration of sperm motility and the percentage of sperm viability in the treatment groups were significantly higher than the control group ($p < 0.05$). Considering over a period of time after chilled storage, modified Kurokura's extender plus L-cysteine groups were able to maintain carp semen qualities (motility, duration of motility and viability) up to 24 hour. Comparing all concentrations of L-cysteine, at 24 hour after chilled storage, the optimal concentration of L-cysteine was found at 1 mM. In conclusion, supplementation of L-cysteine at 1 mMol in modified Kurokura's extender can be recommended for carp semen chilled storage.

Keywords: antioxidant, carp, L-cysteine, semen extender

[1]*Semen Laboratory, Department of Clinical Sciences and Public Health, Faculty of Veterinary Science, Mahidol University, Nakhon Prathom 73170, Thailand*
[2]*The Institute of Marine Science, Burapha University, Chonburi 20131, Thailand*
***Corresponding author: E-mail:** vskkk@mahidol.ac.th, kampon.kae@mahidol.ac.th*

บทคัดย่อ

ผลของ L-cysteine ต่อคุณภาพของน้ำเชื้อปลาคาร์พ (*Cyprinus carpio*) แช่เย็น

กัญช์ เกล็ดมณี [1] สมรัฐ ทวีเดช [2] แพรวพร ไทยจงรักษ์ [1] พนิดา ชนาภิวัตน์ [1] กัมพล แก้วเกษ [1]*

การทดลองนี้มีวัตถุประสงค์เพื่อศึกษาผลของสารแอลซีสเทอีนต่อคุณภาพของน้ำเชื้อปลาคาร์พแช่เย็น ทำการเก็บน้ำเชื้อจากปลาคาร์พจำนวน 8 ตัวและนำตัวอย่างน้ำเชื้อมารวมกัน และแบ่งออกเป็น 5 กลุ่มทดลองโดยจำแนกตามความเข้มข้นของสารแอลซีสเทอีน ได้แก่ ความเข้มข้น 0 (T1), 0.5 (T2), 1.0 (T3), 1.5 (T4) และ 2 (T5) มิลลิโมลาร์ ทำการตรวจประเมินการเคลื่อนที่ของตัวอสุจิ ระยะเวลาในการเคลื่อนที่ของตัวอสุจิ และอัตราการรอดชีวิตของอสุจิ ที่ระยะเวลา 0 12 24 48 และ 72 ชั่วโมงภายหลังจากการแช่เย็น จากผลการทดลองเมื่อเปรียบเทียบระหว่างกลุ่มควบคุมและกลุ่มทดลองพบว่า กลุ่มทดลองมีเปอร์เซ็นต์การเคลื่อนที่ของตัวอสุจิ ระยะเวลาในการเคลื่อนที่ของตัวอสุจิและอัตราการรอดชีวิตของตัวอสุจิที่สูงกว่ากลุ่มควบคุมอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) เมื่อพิจารณาระยะเวลาในการแช่เย็นพบว่าสารละลายน้ำเชื้อ modified Kurokura ที่เสริมด้วยสารแอลซีสเทอีน สามารถช่วยเก็บรักษาคุณภาพของน้ำเชื้อปลาคาร์พได้ถึง 24 ชั่วโมง และจากการทดลองเสริมสารแอลซีสเทอีนที่มีความเข้มข้นระดับต่างๆ กันพบว่า ความเข้มข้นที่เหมาะสมสำหรับการเก็บรักษาน้ำเชื้อปลาคาร์พแช่เย็นคือที่ระดับความเข้มเข้น 1 มิลลิโมลาร์ จากผลการทดลองนี้สรุปได้ว่าการเสริมสารแอลซีสเทอีนที่ความเข้มข้น 1 มิลลิโมลาร์ในสารละลายน้ำเชื้อ modified Kurokura สามารถนำไปใช้ในการเก็บรักษาน้ำเชื้อปลาคาร์พแช่เย็นได้

คำสำคัญ: สารต้านอนุมูลอิสระ ปลาคาร์ฟ แอลซีสเทอีน สารละลายเจือจางน้ำเชื้อ

[1] *ห้องปฏิบัติการน้ำเชื้อ ภาควิชาเวชศาสตร์คลินิกและการสาธารณสุข คณะสัตวแพทยศาสตร์ มหาวิทยาลัยมหิดล นครปฐม 73170*
[2] *สถาบันวิทยาศาสตร์ทางทะเล มหาวิทยาลัยบูรพา ชลบุรี 20131*
***ผู้รับผิดชอบบทความ E-mail:** vskkk@mahidol.ac.th, kampon.kae@mahidol.ac.th*

Introduction

Common carp (*Cyprinus carpio*) is the most cultivated carp species throughout the world. In Asia, it is an economically important cultured fish species. Artificial insemination can be provided as a management tool for genetic importance, reducing stress to male brood stock and save cost of transportation. One approach to prolonged fish sperm quality that has proven successful is the chilled-storage technique. Previous studies reported that chilled storage of semen of fresh water fish at 4^0C (such as *Pantius gonionotus, Pangasius larnaudii, Clarias gariepinus* and *Clarias macrocephalus*) improved sperm motility and viability compared to storage at room temperature (Vijittraphun and Vuttiphanchai, 2000; Duangyai et al., 2002; Pobsuk and Wuthiphandchai, 2003; Meephol et al., 2005). In addition, the semen extender supplemented with antioxidants has been shown to serve as a defense mechanism against reactive oxygen species (ROS) which occurs during chilled storage (Agarwal et al., 2003). This ROS causes physical and chemical stress on the sperm membrane, lipid peroxidation and definitely resulted in sperm damage (Mustafa et al., 2010).

Recently, L-cysteine has been successfully used as antioxidant in extended semen of boar (de Lamirande and Gagnon, 1992; Chanapiwat et al., 2009; Taylor et al., 2009; Kaeoket et al., 2010[a]), canine (Mazor et al., 1996; Michael et al., 2007) and bull (Bilodeau et al., 2001). L-cysteine, known as a precursor of glutathione, has been demonstrated to enhance intercellular glutathione production both in vivo and in vitro as well as to prevent hydrogen peroxide-mediated loss of sperm motility in bull (Bilodeau et al., 2001). In carp, Lahnsteiner (2009) reported that during chilled storage the level of amino acid (i.e. cysteine) in the seminal plasma of carp decreased significantly which indicated that the specific amino acid in chilled carp semen was utilized by carp spermatozoa for preventing the detrimental effect from ROS. This can be hypothesized that chilled carp semen requires an amount of L-cysteine in order to minimize the effect of particular ROS. However, no study has been reported on the effect of adding antioxidant (i.e. L-cysteine) on the qualities of chilled carp semen. Therefore, the aim of this study was to study the effect of L-cysteine on the qualities of chilled-storage common carp semen.

Materials and Methods

The research proposal of this project was approved by the Faculty of Veterinary Science-Animal Care and Use Committee (FVS-ACUC)-Mahidol University, No. MUVS-2011-26.

Carp semen collection: The milt (semen) was collected from 11 common carps, 3-year-old males with body weights ranging from 0.5-1 kg. Prior to sperm collection and hormone injection, the carps were anaesthetized by 60 ppm 10% clove oil in ethanol (Penprapat et al., 2007). Thereafter, they were injected with 20 µg/kg Lutenizing hormone-releasing hormone analogue (LHRHa; Suprefact®, Sanofi-Aventis Deutschland GmbH, Frankfurt am Main, Germany) (Kwantong and Bart, 2006; Irawan et al., 2010) and 10 mg/kg domperidone (Motilium-M®, Olic, Thailand) to induce spermiation (Irawan et al., 2010). The semen was obtained from broodstocks (n= 11) by abdominal massage and collected in 1-ml syringe at 12 hours after hormonal injection. Semen samples were placed in 50 ml centrifuge tube in cooling box (4^0C) until use. Samples contaminated with fecal material, urine and blood were discarded. The semen samples that contained spermatozoa having motility at 30 seconds post activation more than 70% were included in the experiment (Adeyemo et al., 2007; Irawan et al., 2010).

Semen and spermatozoa evaluation

Color, volume and pH of semen: The fresh semen was evaluated for semen volume by using Eppendorf tube scale and micropipette. Semen color was observed by eyes and pH was determined with pH-Indicator strip (pH paper, Universal indikation (pH 0-14, MERCK, Darmstadt, Germany).

Sperm motility and movement duration: Drops of 10 µl of semen and 90 µl of extender were placed in Eppendorf tube. Then, a drop of 10 µl of distilled water was placed on a microscopic slide (coated with 0.1% BSA to avoid stickiness of spermatozoa) for activating sperm movement, and mixed with 5 µl of the semen-extender dilution, and closed with cover slip (Alavi et al., 2006). Sperm motility was examined under a phase contrast microscope (Olympus CX31, Japan) at x100 magnification. Percentage of sperm motility was determined at 0-30 seconds post activation and duration of sperm motility (minute) was designated as the time since fish sperm were activated with distilled water until sperm stopped moving. Immotile sperm were defined as sperm that did not show forward movement after activation.

Sperm viability: Spermatozoa were stained with eosin-nigrosin by mixing 3 µl of semen with 9 µl of eosin and 9 µl of nigrosin solution on a microscopic slide rapidly. The mixture was smeared on new microscopic slide and examined under light microscope at x1000 magnification. Live sperm exclude the eosin stain and appear in colorless, whereas dead sperm appear in pink color with a dark background of Nigrosin-stain. The percentage of live spermatozoa was determined by counting a minimum of 200 spermatozoa on the slide.

Concentration of spermatozoa

Sperm density: Sperm count was made by using a Neubauer Haemocytometer (Improved Neubauer's chamber, BOECO, Humburg, Germany). Neat semen of fish is difficult to count due to too high concentration. Therefore, the semen sample from each fish (11 fish) was diluted with extender in two steps (Step I, 10 µl of fresh semen in 90 µl of extender; Step II, 10 µl of first dilution in 990 µl of extender). Then, the experimental semen sample was diluted with extender in a 1: 100 ratio. Thus, the final sperm concentration was 1: 1000 and determined by using a Neubauer haemocytometer.

Spermatocrit measurement: Micro-hematocrit capillary tubes were filled with fresh semen (from 11 fish) and both ends were sealed with haemoseal wax. The length of semen in capillaries was measured by meter scale in millimeter and centrifuged for 30 min at 7500 rpm (Agarwal and Raghuvanshi, 2009). Spermatocrit was defined as the percentage volume of white packed cells to the total volume of semen (sharp interface between packed sperm cell and clear seminal fluid).

Sperm morphology: Spermatozoa were stained with William's stain (Williams and Utica, 1920). Three microliters of semen was smeared on microscopic slide and air dried. The smeared slide was dipped in absolute alcohol (95-100%) for 4-5 min, chloramines-T (0.5%) for 1-2 min and absolute alcohol 2-3 times, respectively. After the smeared slides were dried, they were dipped in carbol-fuchsin-eosin for 8-10 minutes then washed and air dried. At least 200 sperm were assessed at x1000 magnification with light microscope for determining sperm morphology.

Osmolarity: Osmolarity of extender and diluted semen were evaluate by Osmometer (Micro-Osmometer Model 210, Fiske®, Germany).

L-cysteine supplementation: Pooled sperm was prepared by mixing equal portions of semen collected from male fish which had a motility of equal to or greater than 70%. The pooled semen was divided into five equal portions and diluted with semen extender in a 1:9 ratio. The present semen extender, modified Kurokura's extender, was composed of 360 mg of sodium chloride (NaCl), 20 mg of sodium hydrogen carbonate ($NaHCO_3$), 1 g of potassium chloride (KCl), 8 mg of magnesium chloride ($MgCl_2$) and 22 mg of calcium chloride ($CaCl_2$) in 100 ml of distilled water (Magyary et al., 1996). After the pooled semen was diluted, L-cysteine dilution was added to each portion. The varying L-cysteine concentrations are as follows: 0 (control, T1), 0.5 (T2), 1 (T3), 1.5 (T4) and 2 (T5) mMol. Each portion was further subdivided into 3 aliquots and kept in 50 ml tube at 4^0C in micro-computer process controller freezer (Micom control system 20Q, Continental plastic CORP, Delevan, WI, USA). The diluted semen samples were evaluated at 0, 12, 24, 48 and 72 hours after chilled storage at 4^0C for percentage of sperm viability and sperm motility.

Statistical analysis: Prior to analysis of variance (ANOVA), normal distribution of all data in each parameter, was checked by Shapiro Wilk test. All the data were subjected to repeated measures ANOVA according to Completely Randomized Design (CRD). When the effect of treatment was significant, multiple comparisons such as a Duncan's new multiple range test, were used to compare means with different

effects of those parameters observed. All the statistical analyses were performed as a General Linear Model using Univariate procedure of SPSS version 18.0 (SPSS 18.0; SPSS Inc, Chicago, IL, USA). Data of parameters observed for sperm motility, duration of sperm motility and viability were reported as mean of percentages. Overall differences between means were considered significant when $p < 0.05$.

Results

Fresh semen analysis

The preliminary evaluations of carp semen are presented in Table 1. The sperm motility was used as criteria to select the semen samples before pooling them. Altogether 8 semen samples were selected and pooled. Semen samples from carp No. 4, 8 and 10 were excluded from the study because their semen samples had a motility of less than 70% had small volume and were contaminated with urine.

Fresh semen analysis

The sperm density ranged from 16.14±1.30 to 18.31±1.71 cells x 10^9/ml. The average osmolarity of pooled sperm and Modified Kurokura's extender was 275.50±0.71 and 370.50±0.71 mOsm, respectively. All fresh semen samples had pH around 7. The abnormal morphology of sperm (e.g. two heads, three heads, multiflagellum, short tail, fold tail and big head sperm) was observed for 21.67±3.01%.

Chilled semen analysis

Sperm motility: The higher percentage of sperm motility at 0, 12, 24 hour were found in groups T1 (88.33±3.54%), T2 (84.17±3.95%) and T3 (73.89±4.17%), respectively. However, the percentage of sperm motility was less than 70% after 24 hours of chilled storage (Fig 1).

Duration of sperm motility

The highest duration of motility at 0, 12, 24 hour was found in groups T3 (3.61±0.33 min), T3 (3.39±0.33 min) and T3 (2.89±0.33 min), respectively ($p < 0.05$). Although the highest duration of motility at 48 (1.50±0.35 min) and 72 (1.06±0.17 min) hour was found in group T3, the statistical analysis was not significant (Fig 2).

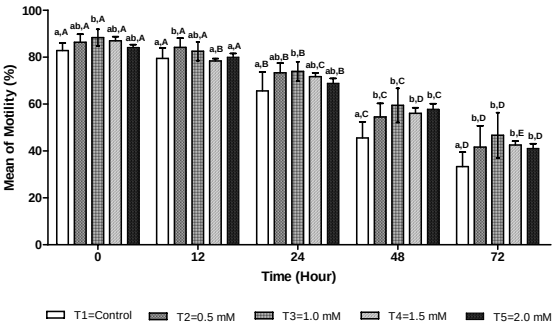

Figure 1 Percentages of sperm motility before and after chilled storage (4^0C). Bars are expressed as mean±SD. Small letters (a and b) are significantly different among treatments ($p < 0.05$). Capital letters (A, B, C and D) are significantly different at each time ($p < 0.05$).

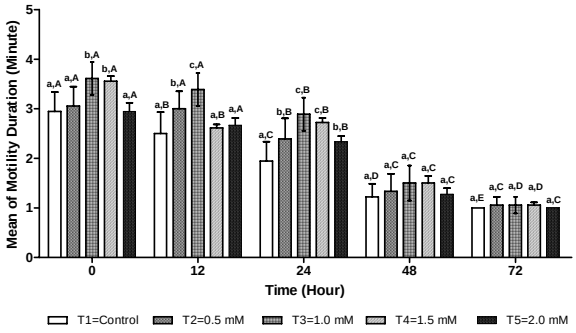

Figure 2 Sperm motility duration (minute) before and after chilled storage (4^0C). Bars are expressed as mean ± SD. Small letters (a, b and c) are significantly different among treatments ($p < 0.05$). Capital letters (A, B, C and D) are significantly different at each time ($p < 0.05$).

Table 1 Sperm density, spermatocrit, sperm motility and duration of motility of carp fresh semen (milt)

Parameters	Carp ID										
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Sperm density (Cells x 10^9/ml)	21.25	2.50	5.98	39.48	16.65	18.30	19.98	13.03	9.18	18.93	25.78
Spermatocrit (%)	29.00	38.50	33.00	50.50	25.50	30.50	46.00	17.00	16.50	28.50	34.00
Sperm motility (%)	95.00	97.50	92.50	98.75	93.75	96.25	96.25	76.25	90.00	87.50	96.25
Motility duration (minute)	4.25	5.75	3.00	2.25	4.00	4.25	3.75	2.25	4.00	3.00	4.50

Table 2 Sperm density after dilution, osmolarity of Modified Kurokura's extender and osmolarity of L-cysteine plus extender

Treatments	Sperm Density (Cells x 10^9/ml)	Modified Kurokura (mOsm/kg)	L-cysteine plus (mOsm/kg)
T1 (control)	17.62±1.43	368.00±0.00	361.50±0.71
T2 (0.5 mM L-cysteine)	16.35±9.68	367.00±2.83	362.00±0.00
T3 (1.0 mM L-cysteine)	18.31±1.71	369.00±0.00	360.50±2.12
T4 (1.5 mM L-cysteine)	16.14±1.30	370.50±0.71	361.50±0.71
T5 (2.0 mM L-cysteine)	16.30±2.31	368.00±2.83	361.50±2.12

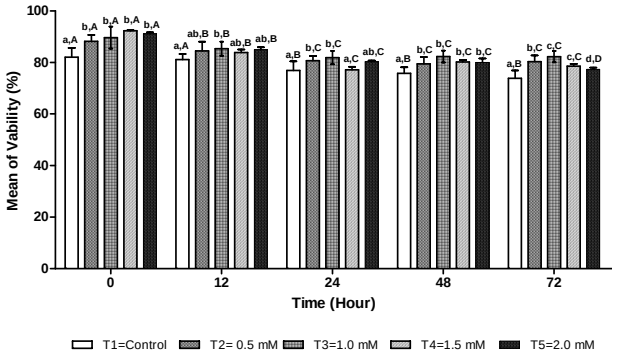

Figure 3 The percentages of sperm viability before and after chilled storage (4^0C). Bars are expressed as mean ± SD. Small letters (a, b, c and d) are significantly different among treatments ($p < 0.05$). Capital letters (A, B, C and D) are significantly different at each time ($p < 0.05$).

Sperm viability

The highest percentage of sperm viability at 0, 12, 24, 48, 72 hour was found in groups T4 (92.22±0.83%), T3 (85.28±2.72%), T3 (81.89±2.57%), T3 (82.28±2.28%) and T3 (82.22±2.17%), respectively (Fig 3).

Discussion

Significant parameters such as percentage of motility, movement duration and percentage of viability have been used in order to evaluate the quality and mirror the fertilizing ability of carp semen (Chambeyon and Zohar, 1990). Lahnsteiner et al. (1996) reported that highly motile semen was characterized by high percentages of linear swimming spermatozoa tend to have a higher fertilizing capacity since these spermatozoa could reach the micropyle of the fish egg in a short time. In the present study, it is clearly showed that supplementing L-cysteine at a concentration of 1.0 mMol in carp semen (group T3) could maintain a motility of ≥ 70% for 24 hours after chilled storage, indicating their ability to reach the micropyle and fertilize eggs (Akçay et al., 2004; Bozkurt and Secer, 2005). This is in agreement with other studies (Lahnsteiner, 2009, 2010; Metwally and Fouad, 2009) in that delivering antioxidants either by oral administration or direct supplementation in extended semen improved fish sperm motility, movement duration and viability. In addition, L-cysteine, with its antioxidant activities, has been shown to improve viability of spermatozoa by minimizing lipid peroxidation of sperm plasma membrane and preventing DNA protein damage of spermatozoa from ROS during chilled and frozen storage in fish and pigs (Darkwa et al., 2004; Stejskal et al., 2008; Kaeoket et al., 2010[a]). Consistently, chilled carp semen quality in the present study and in other freshwater fish (Adeyemo et al., 2007; Asturiano et al., 2007; Abinawanto et al., 2012) had a high percentage of viability and ranged between 73-90%.

It is worth noting that the effect of L-cysteine on the qualities of chilled carp semen in the present study seemed to be in a dose dependent manner which can be seen in boar semen (Chanapiwat et al., 2009) as well as in other antioxidants, e.g. DHA and gamma-Oryzanol (Kaeoket et al., 2010[b]; 2012). Nonetheless, too high concentration of L-cysteine may not be beneficial if carp spermatozoa have limiting L-cysteine uptake (White, 1993) which has also been reported in fresh (Funahashi and Sano, 2005) and frozen boar semen (Kaeoket et al., 2010a). The toxic effects of too high amino acid concentration such as osmotic toxicity and hyper tonicity have been described in stallion spermatozoa (Trimèche et al., 1999; Khlifaouia et al., 2005). In the present results, relatively low semen qualities found in group T5 after chilled storage, may explain by these particular toxic effects.

Taken all the results of chilled semen qualities together, supplementation of L-cysteine at a concentration of 1 mMol in modified Kurokura's extender can be recommended for carp semen chilled storage at 4^0C for up to 24 hour.

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