

**Effects of Cooling Rates, Plunge Temperatures and Warming Rates
on Survival and Fertility of Cryopreserved Swamp Buffalo Spermatozoa**
อิทธิพลของอัตราการลดอุณหภูมิ อุณหภูมิก่อนการแช่แข็งในไนโตรเจนเหลว และอัตรา
การอุ่นน้ำเชื้อแช่แข็งต่ออัตราการรอดชีวิตและความสมบูรณ์พันธุ์ของน้ำเชื้อแช่แข็งกระบือปลัก

Parishat Sukhato¹ Surajit Thongsodseang¹ Apirak Utha² Nucharin Songsasen¹

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อัตราการลดอุณหภูมิ อุณหภูมิก่อนการแช่แข็งในไนโตรเจนเหลว และอัตราการอุ่นน้ำเชื้อแช่แข็ง ต่ออัตราการรอด
ชีวิตและความสมบูรณ์พันธุ์ของน้ำเชื้อแช่แข็งกระบือปลัก. วารสารวิจัยวิทยาศาสตร์การแพทย์ 15 (1) : 55-68.

ศึกษาวิธีการที่เหมาะสมในการแช่แข็งตัวสุจิพวกระบือปลัก และความสมบูรณ์พันธุ์ของน้ำเชื้อแช่แข็ง
โดยศึกษาอิทธิพลของอัตราการลดอุณหภูมิ อุณหภูมิก่อนการแช่แข็งในไนโตรเจนเหลว และอัตราการอุ่นน้ำเชื้อ
ต่อการเคลื่อนไหวและความสมบูรณ์ของโครโมโซมของตัวสุจิ โดยรีดเก็บน้ำเชื้อจากพวกระบือ 3 ตัว แช่แข็งด้วย
อัตราการลดอุณหภูมิที่ 10°, 20° และ 30° เซลเซียสต่อนาที ด้วยเครื่องแช่แข็งแบบควบคุมอุณหภูมิ น้ำเชื้อ
แต่ละชุดลดอุณหภูมิจนถึง -40°, -80° และ -120° เซลเซียส ซึ่งเป็นอุณหภูมิก่อนการแช่แข็งในไนโตรเจนเหลว
หลังจากแช่แข็งและเก็บไว้ในไนโตรเจนเหลว 24 ชั่วโมง นำมาละลายในอัตราการอุ่นน้ำเชื้อแช่แข็งที่อุณหภูมิ 1000°
และ 200° เซลเซียสต่อนาที พบว่าอัตราการลดอุณหภูมิ อุณหภูมิก่อนการแช่แข็งในไนโตรเจนเหลว และอัตรา
การอุ่นน้ำเชื้อแช่แข็ง มีอิทธิพลต่ออัตราการรอดชีวิตของตัวสุจิอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) ในพวกระบือ
ทั้ง 3 ตัว อัตราการลดอุณหภูมิที่เหมาะสมคือ การลดอุณหภูมิจาก 4° ถึง -120° เซลเซียส ในอัตรา 20° หรือ
30° เซลเซียสต่อนาที และการอุ่นน้ำเชื้ออย่างรวดเร็ว (1000° เซลเซียสต่อนาที) ได้ผลดีกว่าการอุ่นน้ำเชื้ออย่าง
ช้าๆ (200° เซลเซียสต่อนาที)

ผลการศึกษาเปรียบเทียบความสมบูรณ์พันธุ์ของน้ำเชื้อแช่แข็งตามวิธีการที่เหมาะสมจากการทดลอง
ข้างต้น กับน้ำเชื้อแช่แข็งที่ผลิตตามมาตรฐานเดิม โดยนำน้ำเชื้อแช่แข็งไปผสมเทียมในแม่กระบือของเกษตรกร
จำนวน 178 ตัว หลังการผสม 60 วัน ตรวจท้องโดยวิธีสังคัลผ่านทวารหนัก พบว่าอัตราการตั้งท้องในแม่
กระบือที่ผสมด้วยน้ำเชื้อแช่แข็งด้วยอัตราการลดอุณหภูมิ 20° เซลเซียส และ 30° เซลเซียส ต่อนาที คือ 43%
(26/30 ตัว) และ 40% (23/58 ตัว) ตามลำดับ ในขณะที่แม่กระบือที่ผสมเทียมด้วยน้ำเชื้อแช่แข็งด้วยวิธี
มาตรฐานเดิม มีอัตราการตั้งท้อง 28% (17/60 ตัว) ทั้งสามวิธีไม่มีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติ
($p > 0.05$)

คำสำคัญ : กระบือ ตัวสุจิ กระบวนการแช่แข็ง การผสมเทียม

¹ Artificial Insemination Division, Department of Livestock Development, Pathumthani
กองผสมเทียม กรมปศุสัตว์ จังหวัดปทุมธานี

² Khon Kaen Artificial Insemination Research Centre, Tha Phra, Khon Kaen
ศูนย์วิจัยการผสมเทียมขอนแก่น ตำบลท่าพระ จังหวัดขอนแก่น

Abstract : Parishat Sukhato, Surajit Thongsodseang, Apirak Utha and Nucharin Songsasen. 2001 Effects of cooling rates, plunge temperatures and warming rates on survival and fertility of cryopreserved swamp buffalo spermatozoa. Thai J Hlth Resch 15 (1) : 55-68.

Experiments were conducted to determine the effects of cooling rate, plunge temperature and warming rate on the motility and the acrosome integrity of swamp buffalo spermatozoa obtained from three bulls. There were three cooling rates: at 10^o, 20^o, and 30^oC/min and each of them had -40^o, -80^o, and -120^oC as the temperature before being plunged into liquid nitrogen. The spermatozoa frozen under nine cooling conditions mentioned before were then thawed both at 1000^o and 200^oC/min. The result of this experiment was shown that cooling rate, plunge temperature and warming rate significantly affected the survival of spermatozoa (p<0.05). Optimal cooling conditions were found to be cooling spermatozoa from 4^o to -120^oC either at 20^o or 30^oC/min. Rapid warming (1000^oC/min) was superior to slow warming (200^oC/min.). In an additional study, comparing the fertility of spermatozoa frozen under optimal conditions and those frozen by a routine protocol used for semen processing were assessed. A total of 178 swamp buffalo cows were inseminated with cryopreserved spermatozoa, and their pregnancy status was assessed 60 days later by rectal palpation. There were no significant differences (p>0.05) in pregnancy rates between 43% (26/62), 40% (23/58) of cows inseminated with sperm cooled at 20^oC/min and 30^oC/min, respectively and 28% (17/60) of cows inseminated with sperm frozen by a routine protocol.

Key words : Swamp buffalo, spermatozoa, cryopreservation, artificial insemination

Tylosin 50 μ g/ml + Lincomycin 150 μ g/ml + Spectinomycin 300 μ g/ml) were also added. The extender was then divided into two fractions; Fraction A used for cooling semen to 4 $^{\circ}$ C; Fraction B containing 1.92 M glycerol, added to an equal volume to semen samples diluted in Fraction A at 4 $^{\circ}$ C. Therefore, the final concentration of glycerol was 0.96 M.

Semen Processing

Ejaculated spermatozoa were obtained from 3 swamp buffalo bulls, age around 12 years using an artificial vagina, and assessed for volume, color, sperm concentration and % motility. Each bulls was collected semen weekly during April-June 1999. Only three samples from each bulls with more than 70% motile spermatozoa were used. The semen samples were diluted with Fraction A of the semen extender at 37 $^{\circ}$ C to yield a concentration of 2.4×10^8 sperm cells/ml. Then, diluted samples were slowly cooled to 4 $^{\circ}$ C over the period of 2 hours, and cooled samples were further diluted at a 1:1 ratio with Fraction B containing glycerol, yielding a final sperm concentration of 1.2×10^8 sperm/ml. The samples were loaded into 0.25 ml straws (IMV, L'Aigle, France), and straws were cooled in a programmable biological freezer (Planer KRYO 10 series II, TS Scientific, Perkasio, PA, USA) under the cooling conditions described below.

Experiment 1: Effects of cooling rate, plunge temperature and warming rate on survival of cryopreserved buffalo spermatozoa.

The experimental design was a Factorial 3 x 3 x 2 consisting of 8 variables: 3 cooling rates (10 $^{\circ}$, 20 $^{\circ}$ and 30 $^{\circ}$ C/min), 3 plunge temperatures (-40 $^{\circ}$ C, -80 $^{\circ}$ C and -120 $^{\circ}$ C) and 2 warming rates (1,000 $^{\circ}$ C/min and 200 $^{\circ}$ C/min) with 9 replications.

Since there was only one programmable freezer available, semen samples in fractions A were divided into 9 parts and held at 4 $^{\circ}$ C until the freezer was available. This eliminated variation among groups in exposure time of spermatozoa to the glycerol. Once the freezer became available, each sample was diluted with Fraction B, and equilibrated at 4 $^{\circ}$ C for 10 min. During equilibration, samples were loaded into straws and placed into the freezer at 4 $^{\circ}$ C. After freezing and storage in liquid nitrogen for 24 hours, the post thawed motility of spermatozoa was determined.

To obtain a warming rate of 1,000 $^{\circ}$ C/min (rapid warming), straws containing cryopreserved sperm were placed in 37 $^{\circ}$ C water bath for 15 sec whereas to obtain a warming rate of 200 $^{\circ}$ C/min (slow warming), straws were allowed to thaw at room temperature air (27 $^{\circ}$ C to 30 $^{\circ}$ C) for 1 min. Thawed samples were then expelled into a centrifuge tube containing 4 ml of TALP-HEPES (Parrish *et al.*, 1986). Evaluation of cryopreserved spermatozoa was performed

Introduction

In Thailand, Artificial Insemination (AI) for swamp buffalo (*Bubalus bubalis*), established in 1979, has been used successfully as a tool for genetic improvement. The cryopreservation procedure used for routine semen processing consists of the following: dilution of semen samples in Egg yolk-Tris extender, cooling samples in the vapor phase of liquid nitrogen at -120°C for 10 min before plunging them into liquid nitrogen (Snitwong *et al.*, 1981, Snitwong *et al.*, 1982). Although cryopreservation of buffalo spermatozoa has been performed routinely, very few studies have been done to optimize cryopreservation condition for swamp buffalo spermatozoa. Improvement of existing cryopreservation protocol for swamp buffalo spermatozoa is still required.

As river buffalo (*Bubalus bubalis*) and swamp buffalo have different chromosome numbers (Di Berardino and Iannuzzi, 1981): $2n = 50$ for river buffalo while $2n = 48$ for swamp buffalo, based on this difference, the optimal conditions for cryopreservation of spermatozoa from river buffalo in an extensive review by Sansone *et al.* (2000) may not be optimal for swamp buffalo.

It has been demonstrated in many cell types including spermatozoa that survival after cryopreservation is strongly dependent on cooling rate (Gao *et al.*, 1997; Leibo and Bradley, 1999; Byrne *et al.*, 2000) and the rate at which the cells are warmed. The optimal cooling rate that yields maximum survival depends on the type and concentration of cryoprotectants and the temperature to which the cells are cooled before being plunged into liquid nitrogen, the so called "intermediate sub-zero plunge temperature" or plunge temperature. The optimal warming rate is also dependent on cooling rate, as well as type and concentration of cryoprotectants.

The aim of this study was to improve the efficiency of cryopreservation by determination the effects of cooling and warming rates, plunge temperature and their interactions on survival of cryopreserved buffalo spermatozoa. Trials of AI were also conducted by comparing fertility of swamp buffalo cows using spermatozoa frozen by a routine cryopreservation protocol to those under optimal conditions.

Materials and Methods

The experiment 1 was done at the Khonkaen Artificial Insemination Research Center's laboratory while the experiment 2 was carried out in the small farm holders that raised buffaloes only dams for draught and conservation purposes and bred by AI. There were four villages in Khonkaen and Mahasarakham provinces.

Semen extender

Semen was diluted with Egg yolk-Tris extender, pH 6.7. The chemical components of the extender were 30.28 g of Tris, 17 g of citric acid monohydrate and 12.3 g of fructose per 1 liter of distilled water plus 250 ml of chicken egg yolk and antibiotics (Gentamycin 250 $\mu\text{g}/\text{ml}$ +

both immediately (0 h) after the sperm were thawed and 3 hours (h) later. Survival of cryopreserved spermatozoa was assessed on the basis of their motility and integrity of acrosomal membranes. Sperm motility was determined by microscopy by which both percentages of motile sperm and the rate of forward movement were assessed. The rate of forward movement was assessed on a scale of 0 to 4 (0 = absence and 4 = vigorous or rapid progression) as described by Mortimer (1994). For analysis of acrosomal membranes, spermatozoa were stained and assessed under a light microscope using the method described by Pope *et al.* (1991).

Experiment 2: Fertility of cryopreserved spermatozoa frozen optimal conditions.

The purpose of this experiment was to compare fertility of cryopreserved spermatozoa frozen by a routine protocol used by the AI Research Center in Thailand for over 20 years to the fertility of spermatozoa frozen by the method found to be optimum in Experiment 1.

Ejaculated spermatozoa were obtained from a single ejaculate of a buffalo bull (Bull B) and processed as described above. Then the samples were divided into 3 parts. The first part was cooled in the vapor phase above liquid nitrogen at -120°C for 10 min before being frozen in liquid nitrogen (routine method, Treatment A). The second and third portions of the semen sample were cooled from 4°C to -120°C at $20^{\circ}\text{C}/\text{min}$ (Treatment B) and $30^{\circ}\text{C}/\text{min}$ (Treatment C), respectively. In this experiment, cryopreserved sperm from all groups were warmed rapidly at $1,000^{\circ}\text{C}/\text{min}$ by placing straws in 37°C water bath and holding for 15 sec.

Fertility was assessed by inseminating cryopreserved spermatozoa to 178 estrus synchronized buffalo cows raised in small farm holders under similar conditions. Prior to estrus synchronization, the ovaries and uterine horns of the cows were examined by rectal palpation to ensure that their ovaries were active and in the stage of nonpregnant. Estrus synchronization of buffalo cows was performed by inserting a progesterone-impregnated silicone elastomer device (CIDR-B[®], InterAg NZ) into the vagina. Each cow was injected intramuscularly with 1 mg of estradiol benzoate (CIDIROL[®], InterAg, NZ.) on the day of CIDR-B insertion and 150 IU of equine chorionic gonadotropin (Follogon[®] Phamaco NZ Ltd.,) upon CIDR-B removal. During four days artificial insemination was performed by two skilfull inseminators at fixed time, between 48 to 50 h after the CIDR-B was removed. Pregnancy diagnosis was performed by rectal palpation on day 60 after AI.

Statistical Analysis

The proportional data of sperm motility and acrosome integrity were transformed using the equation $y' = \arcsin y$, in which y was the percentage of each parameter. Analyses of transformed

data were then performed by using Analysis of Variance (Axum 6.0, Mathsoft, Inc) of Factorial 3 x3 x2. The complete model included the main effects (cooling rate, plunge temperature and warming rate) and interactions (between cooling rate and plunge temperature, cooling and warming rates, and warming rate and plunge temperature). Significance level was set at 5%. Comparison of pregnancy after AI with cryopreserved spermatozoa from the three treatments was performed by using Chi-square Test (GraphPad InStat version 3.00 for Windows 95, GraphPad Software, San Diego, Ca, USA).

Results

Experiment 1: Effects of cooling rate, plunge temperature and warming rate on survival of cryopreserved buffalo spermatozoa

Figures 1 to 3 show motility of spermatozoa from 3 bulls after being frozen and thawed under various conditions. Although motility of cryopreserved spermatozoa varied among bulls, spermatozoa of each bull responded to the three cryobiological variables in similar ways. It appeared that cooling rate, plunge temperature and warming rate significantly affected sperm motility after freezing and thawing.

The optimal cooling rate for buffalo spermatozoa was between 20°C/min and 30°C/min. Cooling sperm at 10°C/min resulted in lower motility of spermatozoa from the three bulls, regardless of the plunge temperature and the warming rate. Although cooling rate did not exert significant influence on survival of cryopreserved sperm from bulls A and B (Figures 1 and 2), it significantly affected survival of spermatozoa from bull C (Figure 3, $p < 0.05$). Cryopreserved spermatozoa from a given treatment were able to maintain their motility after being incubated at 37°C for 3 h. There were no significant differences in motility of samples from a given treatment assessed immediately after being thawed and incubated for 3 h.

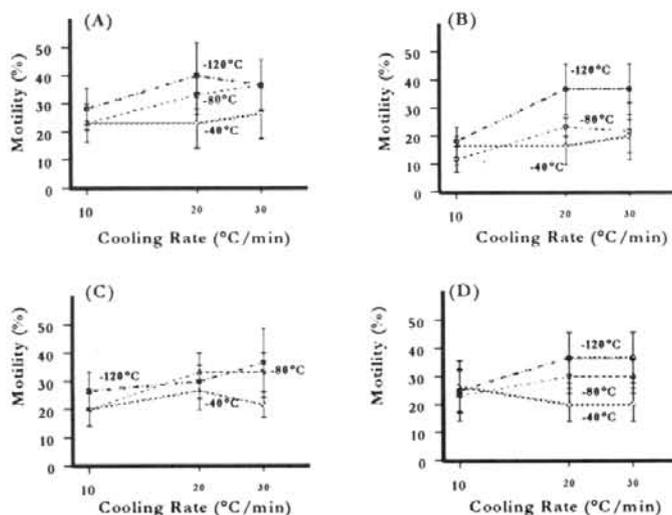


Figure 1. Motility of cryopreserved spermatozoa from Bull A:

- (A) 0 h post-thawed with 1000°C/min warming rate
- (B) 0 h post-thawed with 200°C/min warming rate
- (C) 3 h post-thawed with 1000°C/min warming rate
- (D) 3 h post-thawed with 200°C/min warming rate

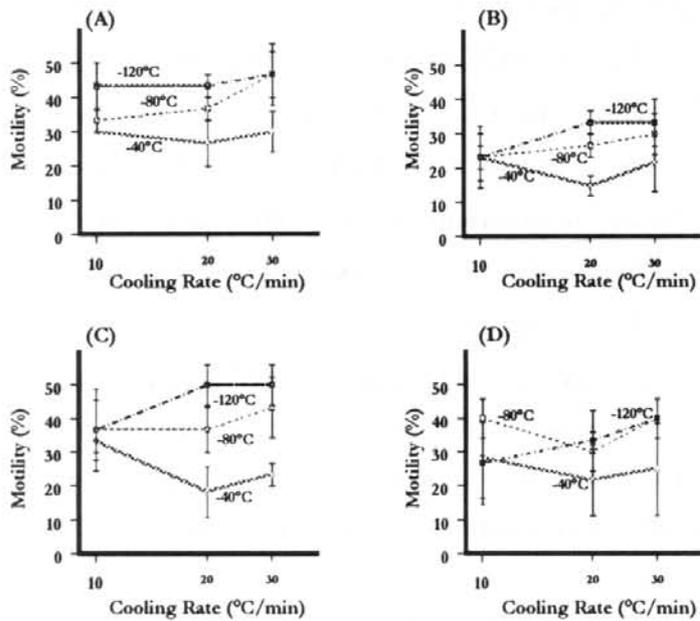


Figure 2. Motility of cryopreserved spermatozoa from Bull B:
(A) 0 h post-thawed with 1000°C/min warming rate
(B) 0 h post-thawed with 200°C/min warming rate
(C) 3 h post-thawed with 1000°C/min warming rate
(D) 3 h post-thawed with 200°C/min warming rate

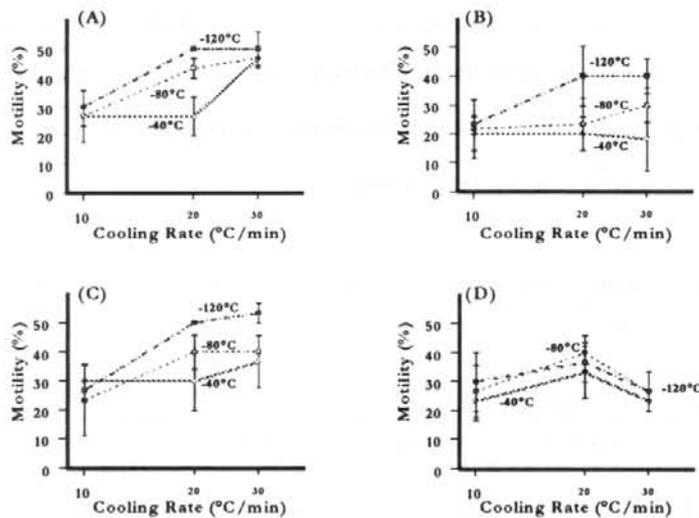


Figure 3. Motility of cryopreserved spermatozoa from Bull C:
(A) 0 h post-thawed with 1000°C/min warming rate
(B) 0 h post-thawed with 200°C/min warming rate
(C) 3 h post-thawed with 1000°C/min warming rate
(D) 3 h post-thawed with 200°C/min warming rate.

Plunge temperature significantly affected survival of cryopreserved spermatozoa from the three bulls ($p < 0.05$). Highest survival of spermatozoa from all bulls was obtained when samples were plunged at -120°C , rather than -40°C or -80°C (Figures 1-3). Moreover, interaction between cooling rate and plunge temperature also influenced sperm motility after cryopreservation. Cooling spermatozoa to -40°C and then plunging them into liquid nitrogen resulted in reduction of overall motility. However, survival of spermatozoa cooled to -80°C or -120°C depended on the rate at which spermatozoa were cooled. For example, motility of spermatozoa from bull A (Figure 1) cooled at $10^{\circ}\text{C}/\text{min}$ to -80°C were 23% and 12% for rapid and slow warming, respectively.

The respective values for spermatozoa that were cooled at 10°C to -120°C were 28% and 18%. However, motility of spermatozoa from the same bull increased with increasing cooling rate. Motility of spermatozoa cooled at 20°C/min to -80°C were 33% and 23% for rapid and slow warming, respectively. The respective values for spermatozoa that were cooled at the same cooling rate to -120°C were 40% and 36%. Motility of spermatozoa cooled at 30°C/min -80°C were 37% and 22% for rapid and slow warming, respectively. The respective values for spermatozoa cooled at the same rate to -120°C were 37% for both rapid and slow warming.

Rapid warming (1,000°C/min) was superior to slow warming (200°C/min) for bulls B and C (Table 1, $p < 0.05$). However, there were no significant effects of warming rate on survival of spermatozoa from bull A (Figure 1). By coincidence, survival of spermatozoa from bull A was slightly lower (range from 12 to 40%) than those obtained from the other two bulls (range from 15 to 50%).

Although motility of cryopreserved spermatozoa depended on the conditions in which spermatozoa were frozen and thawed, cooling and thawing conditions did not affect integrity of acrosomal membranes. Table 2 shows the results of acrosome integrity after spermatozoa from the three bulls were frozen and thawed under various conditions. Although there were no significant differences ($p > 0.05$) in acrosome integrity among treatments, the plunge temperatures of -80°C and -120°C appeared to be superior to -40°C for a given cooling rate.

Table 1 Motility of cryopreserved spermatozoa after rapid (1000°C/min) and slow warming (200°C/min)

Cooling Rate (°C/min)	Plunge Temperature (°C)	Bull B (% motility, mean ± SE)		Bull C (% motility, mean ± SE)	
		Rapid Warming	Slow Warming	Rapid Warming	Slow Warming
		10	-40	30 ± 0	23 ± 7
10	-80	33 ± 3	23 ± 9	27 ± 9	22 ± 10
10	-120	43 ± 7 ^a	23 ± 3 ^b	30 ± 0	23 ± 3
20	-40	27 ± 7	15 ± 3	27 ± 7	20 ± 6
20	-80	37 ± 3	27 ± 3	43 ± 3	23 ± 9
20	-120	43 ± 3 ^a	33 ± 3 ^b	50 ± 0	40 ± 10
30	-40	30 ± 6	21 ± 9	47 ± 3 ^a	18 ± 11 ^b
30	-80	47 ± 9	30 ± 6	47 ± 3	30 ± 6
30	-120	47 ± 7	33 ± 7	50 ± 6	40 ± 6

^{a,b} Differences letter within the same bull and cooling conditions indicate significance differences ($p < 0.05$)

Table 2 Acrosome integrity of cryopreserved spermatozoa from 3 bulls

Cooling rate (°C/min)	Plunge temperature (°C)	Warming rate (°C/min)	Acrosome integrity (% , mean \pm SE)		
			Bull A	Bull B	Bull C
10	-40	1000	65 \pm 3	72 \pm 6	68 \pm 4
10	-80	1000	67 \pm 1	77 \pm 3	72 \pm 6
10	-120	1000	73 \pm 2	64 \pm 8	74 \pm 2
20	-40	1000	71 \pm 1	64 \pm 4	76 \pm 6
20	-80	1000	68 \pm 2	73 \pm 7	69 \pm 4
20	-120	1000	78 \pm 0	73 \pm 8	76 \pm 1
30	-40	1000	71 \pm 3	64 \pm 10	63 \pm 8
30	-80	1000	70 \pm 3	75 \pm 7	75 \pm 6
30	-120	1000	77 \pm 1	73 \pm 10	75 \pm 3
10	-40	200	74 \pm 3	68 \pm 1	70 \pm 2
10	-80	200	73 \pm 2	70 \pm 4	71 \pm 1
10	-120	200	73 \pm 1	71 \pm 1	73 \pm 3
20	-40	200	75 \pm 3	74 \pm 1	73 \pm 2
20	-80	200	74 \pm 2	72 \pm 2	75 \pm 1
20	-120	200	78 \pm 2	72 \pm 5	77 \pm 2
30	-40	200	75 \pm 2	72 \pm 3	69 \pm 3
30	-80	200	74 \pm 3	75 \pm 1	74 \pm 2
30	-120	200	77 \pm 2	78 \pm 2	81 \pm 3

There were no significant differences in acrosome integrity among treatments ($p>0.05$)

Experiment 2: Fertility of cryopreserved spermatozoa frozen in liquid nitrogen vapor and a controlled-rate freezer

According to the results obtained in the Experiment 1, spermatozoa obtained from a single ejaculate of Bull B were cryopreserved using 3 cryopreservation protocols; Treatment A, Treatment B, Treatment C. The samples from the three groups were frozen and kept in liquid nitrogen. For AI, the samples were thawed rapidly by holding the samples in 37°C water bath for 15 sec. Pregnancy results after artificial insemination with cryopreserved spermatozoa from the three groups are shown in Table 3

Pregnancy rates of cows inseminated with spermatozoa frozen Treatments B and C were not significantly higher than those inseminated by spermatozoa cryopreserved in the vapor phase of liquid nitrogen (Treatment A). However, numbers of cows becoming pregnant after AI with spermatozoa cryopreserved using Treatments B and C were higher than those cryopreserved by Treatment A. The pregnancy rates after AI with spermatozoa cryopreserved by Treatments B and C

were 43% and 40%, respectively, whereas that of treatment A was 28%. There were no significant differences ($p>0.05$).

Table 3 Pregnancy results after artificial insemination with buffalo spermatozoa cryopreserved in various cooling conditions

Cooling methods (from 4°C to -120°C)	No. of cows inseminated	No. of cows pregnant	Percent pregnancy
Liquid nitrogen vapor (Treatment A)	60	17	28
20°C/min (Treatment B)	60	26	43
30°C/min (Treatment C)	58	23	40

Pregnancy rate among the treatments were not significant differences ($p>0.05$)

Discussion

The principle objective of the present study was to derive a better procedure for cryopreservation of swamp buffalo spermatozoa. To achieve that goal, the effects of cooling and warming rates and intermediate sub-zero plunge temperature on survival of cryopreserved spermatozoa were determined. Since these parameters have been reported to affect survival after cryopreservation of many cell types (Leibo *et al* 1970; Mazur, 1985; Songsasen, 1997), all cryobiological variables investigated in this study exerted significant effects on survival, judged by motility of cryopreserved buffalo spermatozoa. The cooling conditions found to be optimum for buffalo spermatozoa was to cool samples at 20°C/min or 30°C/min to -120°C before plunging them into liquid nitrogen. To obtain highest motility, cryopreserved spermatozoa needed to be warmed rapidly. Spermatozoa cryopreserved under optimal conditions were fertile; considerably high pregnancy rates were obtained after AI of cows with such spermatozoa.

In the present study, the survival of cryopreserved buffalo spermatozoa depended on cooling rate. The presumptive optimal cooling rate for buffalo spermatozoa observed was between 20°C/min and 30°C/min. This was similar to that of cattle spermatozoa reported by Rodriguez *et al.* (1975), but lower than the optimal cooling rate of 76°C to 146°C/min recently reported for spermatozoa of the same species (Woelders *et al.*, 1997). This discrepancy may be due to differences in composition of freezing media. In the former study, egg yolk-sodium citrate buffer without sugar supplementation was used, whereas the later investigators frozen bull spermatozoa in Egg yolk-Tris containing 0.2 M trehalose or sucrose. The presence of sugar in the freezing medium may protect spermatozoa against damage occurring at a high cooling rate (Leibo *et al.*, 1970; Watson, 1990; Woelders *et al.*, 1997).

It is the first report to determine effect of cooling and warming rates for swamp buffalo spermatozoa at the present. Cooling samples in vapor phase of liquid nitrogen at various temperatures has been a routine protocol for cryopreservation of buffalo spermatozoa for many years (Snitwong *et al.*, 1982; Sansone *et al.*, 2000). Snitwong *et al.* (1982) cooled buffalo spermatozoa in vapor phase of liquid nitrogen and demonstrated that cooling samples to temperatures range between -80°C and -120°C was superior to cooling samples to -60°C . Del Sorbo (1995 cited by Sansone *et al.*, 2000) reported that step-wise cooling was superior to continuous cooling.

The temperature at which spermatozoa were cooled before being plunged into liquid nitrogen did affect the survival after cryopreservation, with a significant interaction of cooling rate and the plunge temperature. This agrees with a previous report for mouse spermatozoa (Songsasen, 1997). Cooling buffalo spermatozoa at $10^{\circ}\text{C}/\text{min}$ resulted in low motility after freezing and thawing, regardless of the temperature to which spermatozoa were cooled before being plunged into liquid nitrogen. Cooling spermatozoa at 20° and $30^{\circ}\text{C}/\text{min}$ resulted in high survival if spermatozoa were cooled to -120°C before being plunged into liquid nitrogen.

The critical factor in survival of cells after freezing is to avoid the formation of intracellular ice crystals. To minimize intracellular ice formation, cells must dehydrate sufficiently before they are cooled in liquid nitrogen (Mazur, 1970). The degree of dehydration during cooling depends mainly on cooling rate and the intermediate subzero plunge temperature. In the present study, high survival based on motility was obtained when spermatozoa were cooled at a high cooling rate ($-20^{\circ}\text{C}/\text{min}$ or $-30^{\circ}\text{C}/\text{min}$) to a relatively low subzero temperature (-120°C). Low survival was obtained when spermatozoa were cooled at a high rate to relatively high subzero temperature (-40°C); with these cooling conditions, spermatozoa probably did not have sufficient time to lose water, and cell damage most likely occurred as a result of intracellular ice formation. However, cooling spermatozoa at $10^{\circ}\text{C}/\text{min}$ was apparently too slow, and cell damage was probably due to solution effects (Watson and Duncan, 1988; Watson *et al.*, 1992; Leibo and Bradley, 1999).

Warming rate can also exert significant effects on survival of cells after cryopreservation (Mazur, 1985). The effects depend on whether the cooling rate had been high enough to induce intracellular freezing or low enough to produce cell dehydration. In the present study, rapid warming ($1000^{\circ}\text{C}/\text{min}$) appeared to be superior to slow warming ($200^{\circ}\text{C}/\text{min}$). This agrees with studies in bull spermatozoa (Robbins *et al.* 1976; Arriola and Foote, 1987). Slow warming did not improve motility of spermatozoa cooled at the lowest rate ($10^{\circ}\text{C}/\text{min}$). It has been

reported for human and boar spermatozoa that the influence of thawing on sperm survival depends on the rate at which the spermatozoa had been frozen (Fiser *et al.*, 1993; Henry *et al.*, 1993).

Comparison of fertility between sperm of the Bull B cryopreserved under optimal conditions ($20^{\circ}\text{C}/\text{min}$ and $30^{\circ}\text{C}/\text{min}$ to -120°C) found in the first part of this study and those cryopreserved using a routine protocol (that is, cooling in liquid nitrogen vapor at -120°C for 10 min) was performed. Pregnancy rates after AI with spermatozoa frozen using optimal protocol and standard protocol were about 40% and 28%, respectively. Although, these numbers were not statistically different, a 12 percent increase in pregnancy rate seems to be promising.

Since buffalo often exhibit silent heat or unclear estrous signs, estrus detection in this species seems to be very difficult (Chantaraprateep, 1987; Singh *et al.*, 2000). This results in low pregnancy rate after AI in buffalo compared to that of cattle (Sansone *et al.*, 2000). In the present study, a progesterone-impregnated silicone elastomer device was used to induced estrus of buffalo cows. Since buffalo cows were allowed to graze freely in a rice paddy field all day, estrus detection after removal of progesterone device was very difficult. Due to such logistical reasons, fixed-time inseminations were performed in this study between 48 h to 50 h after progesterone withdrawal. It has been previously reported that estrus occurred approximately 50 h after removal of CIDR-B (Hill *et al.*, 1992). However, there were large variations of 31 h to 70 h (Hill *et al.*, 1992). Based on the previous study, some of the buffalo cows in the present study might have not been inseminated at the optimal time. Therefore, it is reasonable to expect that pregnancy rate in the present study could be improved by determining the actual onset of estrus before insemination.

By studying factors that affect cell survival after cryopreservation, procedures for cryopreservation of buffalo spermatozoa could be derived. These procedures consist of cooling spermatozoa from 4°C to -120°C at 20°C or $30^{\circ}\text{C}/\text{min}$ before plunging them into liquid nitrogen. Spermatozoa cryopreserved this way were fertile and were able to fertilize oocytes *in vivo* at an acceptable rate. The numerical value of pregnancy rate was increased after AI with spermatozoa frozen by the protocols described here. The obvious impact of this study will be its practical application to improve viability and fertilizing ability of cryopreserved spermatozoa used for AI, which in turn will be beneficial to genetic improvement, productivity and conservation of swamp buffalo in Thailand.

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References

- Arriola J. and Foote RH. 1987. Glycerolation and thawing effects on bull spermatozoa frozen in detergent-treated egg yolk and whole egg extenders. *J. Dairy Sci.* 70: 1664-1670.
- Byrne GP., Lonergan P., Wade M., Duffy P., Donovan A., Hanrahan JP. And Boland MP. 2000. Effect of freezing rate of ram spermatozoa on subsequent fertility in vivo and in vitro. *Anim. Reprod. Sci.* 62: 265-275.
- Chantaraprateep P. 1987. Oestrous synchronization in buffalo. *Buffalo J. (Suppl)* 1: 115-126.
- Di Bernardino D. and Iannuzzi L. 1981. Chromosome banding homologies in swamp and murrab buffalo. *J. Hered.* 72:183-188.
- Fiser PS., Fairfull RW., Hansen C., Panich PL., Shrestha JN. and Underhill L. 1993. The effect of warming velocity on motility and acrosomal integrity of boar sperm as influenced by the rate of freezing and glycerol level. *Mol. Reprod. Dev.* 34: 190-195.
- Gao D., Mazur P. and Critser JK. 1997. Fundamental cryobiology of mammalian spermatozoa. In: *Reproductive Tissue Banking*. Karow, A. M. and Critser, J. K. (eds.), Academic Press, San Diego, p. 263-328.
- Henry MA., Noiles EE., Gao D., Mazur P. and Critser JK. 1993. Cryopreservation of human spermatozoa. IV. The effects of cooling rate and warming rate on the maintenance of motility, plasma membrane integrity, and mitochondrial function. *Fertil. Steril.* 60: 911-918.
- Hill FI., Knight TW., Death AF., Wyeth TK. and Ridland M. 1992. Oestrous synchronisation and oestrus detection in swamp buffaloes (*Bubalus bubalis*). *Proc. New Zeal. Soc. of Anim. Prod.* 52: 25-27.
- Leibo SP., Farrant J., Mazur P., Hanna MG. and Smith LH. 1970. Effects of Freezing on marrow stem cell suspensions: interactions of cooling and warming rates in the presence of PVP, sucrose or glycerol. *Cryobiology* 6: 315-332.
- Leibo SP. and Bradley L. 1999. Comparative cryobiology of mammalian spermatozoa. In: *The Male Gamete: From Basic Science to Clinical Applications*. Gagnon, C. (ed.), Cache Raven Press, Vienna, p. 501-516.
- Mazur P. 1970. Cryobiology: The freezing of biological systems. *Science* 168: 939-949.
- Mazur P. 1985. Basic concepts in freezing cells. In: *Proceedings First International Conference on Deep Freezing of Boar Semen*, Johnson, L.A., Larsson, K.L. (eds.) Swedish University of Agricultural Sciences, Uppsala, Sweden, p. 91-112.
- Mortimer D. 1994. *Practical Laboratory Andrology*. New York, Oxford University Press.

- Parrish JJ., Susko-Parrish J., Leibfried-Rutledge ML., Critser ES., Eyestone WH. and First NL. 1986. Bovine in vitro fertilization with frozen-thawed semen. *Therigenology* 25: 591-600.
- Pope CE., Zhang YZ. and Dresser BL. 1991. A simple method for evaluating acrosomal status of cat spermatozoa. *J. Zoo Wildlife Med.* 22: 87-95.
- Robbins RK., Saacke RG. and Chandler PT. 1976. Influence of freeze rate, thaw rate and glycerol level on acrosomal retention and survival of bovine spermatozoa frozen in French straws. *J. Anim. Sci.* 42: 145-146.
- Rodriguez OL. Berndtson WE., Ennen BD. and Pickett BW. 1975. Effect of rate of freezing, thawing and level of glycerol on the survival of bovine spermatozoa in straws. *J. Anim. Sci.* 41: 129-136.
- Sansone G., Nastri MJF. and Fabbrocini A. 2000. Storage of buffalo (*Bubalus bubalis*) semen. *Anim. Reprod. Sci.* 62: 55-76.
- Singh J., Nanda AS. and Adams GP. 2000. The reproductive pattern and efficiency of female buffaloes. *Anim. Reprod. Sci.* 60-61: 593-604.
- Snitwong B., Shanitwong M., Boonkoom A., Punprapa M. and Thongsodsang S. 1981. Preliminary report of swamp buffalo deep frozen semen. *Thai J. Vet. Med.* 11(1): 1-9
- Snitwong B., Shanitwong M., Boonkoom A., Chanatinart V., Abhisingha I. and Sukhato P. 1982. Effect of diluents and freezing temperatures on the post thaw motility of swamp buffalo semen. *Thai J. Vet. Med.* 12(4): 246-256.
- Songsasen N. 1997. Cryobiology of mouse spermatozoa: Fundamental and applied studies. Ph. D Thesis University of Guelph, Canada , 206 pp.
- Watson PF. 1990. Artificial insemination and preservation of semen. In: Marshall's Physiology of Reproduction. Laming, G. E. (ed.), Churchill LivingStone, New York, p. 747-869.
- Watson PF. and Duncan AE. 1988. Effect of salt concentration and unfrozen water fraction on the viability of slowly frozen ram spermatozoa. *Cryobiology* 25: 131-142.
- Watson PE., Crister JK. and Mazur P. 1992. Sperm preservation : fundamental cryobiology and practical implication. In : Infertility. Templeton AA. and Drife JO. (eds), Springer-Verlag publ., London, p102-114.
- Woelders H., Matthijs A., Engel B. 1997. Effects of trehalose and sucrose, osmolality of the freezing medium, and cooling rate on viability and intactness of bull sperm after freezing and thawing. *Cryobiology* 35: 93-105.