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冷凍程式對豬精液冷凍解凍後品質之影響 (1)

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摘　要

本試驗探討不同冷凍降溫程式製作豬冷凍精液對解凍後精液品質之影響。採集 5 頭健康杜洛克公豬新鮮精液，以 Lactose-egg yolk (LEY) 精液稀釋液進行稀釋，最終濃度為 5×10^8 cells/mL 並分裝於 0.5 mL 麥管，分別以三組不同冷凍降溫程式進行冷凍精液製作。精液冷凍程序啟動之起始溫度皆為 5℃，第一組以 -3℃/min 之速率降至 -5℃並停留 1 min，之後繼續以 -50℃/min 速率降溫至 -140℃。第二組以 -3℃/min 速率降至 -5℃，隨後以 -40℃/min 之速率繼續降溫，至 -80℃停留 30 sec 後，以 -60℃/min 速率繼續降溫至 -140℃。第三組以 -20℃/min 速率降溫至 -8℃，後以 -70℃/min 速率降溫直至 -140℃，完成降溫之冷凍精液隨即放入液態氮內貯存。凍存精液於解凍後即進行精子品質檢查，試驗結果顯示三種不同冷凍降溫程式處理製作之冷凍精液，其解凍後之精子總活力、前進式活力、各項運動參數及頭帽完整性等性狀表現均無顯著差異。由於第三組冷凍降溫程式製作豬冷凍精液製程所需時間較短，可減少液態氮損耗，建議可提供豬精液冷凍保存採行之參考。

關鍵詞：豬、冷凍精液、冷凍程式。

緒　言

精液冷凍保存有利於種原之維護，提高優良種畜的利用，物種跨國間交流及備份，並減少疫病傳播風險，於現代化畜殖業發展極具深遠之意義 (Bailey *et al.*, 2008; Knox, 2011)。相較於其他物種，豬冷凍精液使用並不普及 (Woelders *et al.*, 2005)；因豬精子對周圍環境較為敏感 (Grossfeld *et al.*, 2008)，解凍後普遍存在活力及受胎率低下等問題，一直不易被廣泛推行 (Garcia-Olivares *et al.*, 2016)。冷凍精液之製作過程通常係於室溫下採集精液後，即進行一系列冷卻步驟，最後貯存於 -196℃液態氮桶。學者專家皆致力探討相關降溫程序，Dalal *et al.* (2018) 研究不同物種如水牛、乳牛、綿羊、豬、馬及兔精液之降溫速率對解凍後精液品質之影響，結論指出因物種不同其精子細胞膜組成亦有所不同，都各自有其合適的冷凍程序。家禽精液冷凍保存，可分為慢速冷凍、兩階段及一階段程式冷凍方式，Santiago-Moreno *et al.* (2011) 之試驗結果，兩階段程式冷凍方式可優化解凍後精液品質。Galarza *et al.* (2019) 之試驗結果指出以兩階段冷凍程式製作綿羊冷凍精液，其解凍後活力、精子細胞膜及 DNA 完整性皆比三階段冷凍程式為佳。Farhana *et al.* (2018) 比較以 5、10 或 15℃/min 三種不同冷卻速率進行牛冷凍精液製作，顯示冷卻速度 10℃/min 之方式，解凍後精子活力、存活率和精細胞膜完整性優於 15 及 5℃/min。目前文獻上有許多製作 0.5 mL 豬冷凍精液之冷凍降溫程式 (Wongtawan *et al.*, 2006; Kaeoket *et al.*, 2010; Purdy *et al.*, 2010; Tomás *et al.*, 2014; Yeste *et al.*, 2014)，但最佳的冷凍降溫程式條件並未被探討。本試驗旨在比較不同冷凍降溫程式處理製作豬冷凍精液，對解凍後包括精子活力、前進式活力、各項運動參數及頭帽完整性等性狀的影響，以期提供較佳豬精液冷凍降溫技術之參考。

材料與方法

I.　精液採集與處理

本研究選擇 5 頭年齡約 1 至 2 歲，生殖能力正常健康之杜洛克公豬供試驗，每週規律採集精液一次，採集後立即進行常規檢查，選取存活率 80% 以上與活力 75% 以上的精液進行冷凍保存試驗。精液稀釋係應用 Beltsville

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thawing solution (BTS，每公升含37.0 g glucose、1.25 g EDTA、6.0 g sodium citrate、1.25 g sodium bicarbonate及0.75 g potassium chloride) 進行 (Johnson *et al.*, 2000)。

II. 精液之冷凍過程

冷凍精液之製作係參考 Westendorf *et al.* (1975) 之方式，精液緩慢添加 BTS 稀釋液，以 1：1 比例進行稀釋，後冷卻至 15℃以 800 x g 離心 10 分鐘，去除上清液後加冷凍稀釋液 (I) (11% lactose 及 20% egg yolk) 稀釋，然後冷卻至 5℃；再添加冷凍稀釋液 (II) (11% lactose, 20% egg yolk, 9% glycerol 及 1.5% Equex STM)，稀釋最終濃度為 5×10^8 cells/mL 後裝填入 0.5 mL 麥管 (Minitüb, Tiefenbach, Germany)，將麥管封口後移置於電腦程式控制儀 (Ice cube 14S，GmbH) 內，分別以三組冷凍降溫程式進行；其中，第一組係參考 Wongtawan *et al.* (2006) 及 Kaeoket *et al.* (2010) 之文獻，起始溫度為 5℃，以 -3℃/min 之速率降至 -5℃並停留 1 min，再以 -50℃/min 速率降溫至 -140℃，製程共約 8 min。第二組係引用 Yeste *et al.* (2014) 及 Tomás *et al.* (2014) 文獻，起始溫度為 5℃，以 -3/min 的速率降至 -5℃，再以 -40℃/min 速率降溫至 -80℃並停留 30 sec，隨即則以 -60℃/min 速率降至 -140℃，製程共約 8 min。第三組係參考 Purdy *et al.* (2010) 文獻，起始溫度 5℃，以 -20℃/min 之速率降至 -8℃，繼以 -70℃/min 之速率降溫至 -140℃，製程共約 3 min。各組精液麥管降溫至 -140℃時，隨即移入液氮桶內貯存。

III. 精液性狀評估

先將解凍用 BTS 精液稀釋液備妥，並回溫至 25℃；迅速從液氮桶內取出所需冷凍精液麥管，浸入 40℃溫水中 30 sec 進行解凍。隨即擦拭麥管表面水分，剪開麥管讓精液流出，並與解凍用 BTS 稀釋液，稀釋最終濃度為 50×10^6 cells/mL，隨後靜置於 37℃培養箱中培養 6 h，期間儘可能避免精液受到溫度變化及光線傷害。解凍後精液以電腦輔助精子分析 (Computer-assisted sperm analysis, CASA) 系統 (VideoTesT-Sperm 2.1, Russia) 進行評估，分析校正係參考 Dziekoska *et al.* (2013) 之方式，分析項目包括精子活力 (Motility)、前進式活力 (Progressive motility, PM)、平均移動路徑 (Velocity average path, VAP)、直線移動速率 (Velocity straight line, VSL)、曲線移動速率 (Curvilinear velocity, VCL)、精子頭部擺動振幅 (Lateral head displacement, ALH)、精子頭部擺動與平均路徑交叉的次數 (Beat cross frequency, BCF)、直線趨勢 (Straightness, STR) 及直線前進之比率 (Linearity, LIN) 等運動能力參數。

IV. 精子頭帽完整性評估

評估精子頭帽完整性採用免疫螢光染色法，步驟係參考Zeng and Terada (2001)之方式操作，取精液樣品30 μL塗抹於預熱之載玻片上，於空氣中自然乾燥，後用 100% 甲醇固定 10 min。取 30 μL 含 100 μg/mL 螢光素異硫氰酸鹽結合花生凝集素 (Fluorescein isothiocyanate-conjugated peanut agglutinin, FITC-PNA) (Sigma -Aldrich, St, Louis, MO, USA) 之 PBS 溶液，滴置於載玻片上，再移於飽和濕度之 37℃培養箱內靜置 30 min，後以 PBS 沖洗再經由空氣乾燥，滴上 5 μL 的 Antifade 溶液 (Molecular Probes, Inc., Eugene, OR) 並蓋上封片，以保持螢光活性。迅速使用光學螢光顯微鏡 (DM 2500, Leica)，以激發波長 480 nm 及射出波長 530 nm 進行 (1,000 ×，油鏡) 鏡檢，隨機計數至少 100 個細胞，且每一樣品重覆計算數 6 次。豬精子頭帽染色及形態判讀方式如下：(1) 精子頭帽出現完整密集螢光，表示頭帽完整；(2) 精子頭帽僅顯現部分螢光，表示頭帽部分受損；(3) 精子頭帽未顯現螢光，表示頭帽之細胞膜及頭帽外膜完全受損。

V. 統計分析

精液解凍後於 37℃下靜置 6 h，並每隔 2 h 檢查其精子活力、精子前進式活力，精子活力等各項移動參數於解凍後體外培養 5 min 及 6 h 檢測，精子頭帽完整率則於解凍後立即進行評估，以探討試驗各組間之差異。所得資料以變異數分析 (ANOVA) 及鄧肯多變域分析法 (Duncan's multiple range test) 比較各組間差異之顯著性，以 $P < 0.05$ 為差異顯著水準。

結果與討論

本試驗比較三種不同冷凍降溫程式製作豬冷凍精液對解凍後精液品質的影響，試驗結果如表 1 所示，各不同的冷凍降溫程式組所製作之豬冷凍精液，解凍後體外培養各時段之精子活力、精子快速前進式活力，於各處理分析相同培養時段間均無顯著差異。採 CASA 方式進行公豬繁殖能力評估已被廣泛探討及應用 (Broekhuijse *et al.*, 2012)，藉由精子運動參數與相關的形態變化進行分析，亦有研究證實與受胎率呈相關性 (Didion, 2008; Vyt *et al.*, 2008)。

精子活力各項運動參數之分析結果如表 2 所示，以三種不同的冷凍降溫程式製作之豬冷凍精液，解凍後體外培養 5 min 及 6 h 的精子活力各項運動參數均無顯著差異。

表 1. 不同冷凍程式對豬冷凍精液解凍後精子活力及前進式活力率之影響

Table 1. The influence of different freezing programs on sperm motility and progressive motility of boar frozen-thawed semen

Freezing programs	Post thawed incubation time				
	Fresh	30 min	2 h	4 h	6 h
	---------- Total sperm motility (%) ----------				
1	91.1 ± 2.5	78.0 ± 6.9	73.2 ± 6.1	50.4 ± 5.7	33.1 ± 7.8
2	90.2 ± 2.1	78.3 ± 4.8	74.8 ± 4.4	52.6 ± 6.8	32.7 ± 6.1
3	89.6 ± 2.1	80.8 ± 4.6	74.8 ± 6.2	53.0 ± 7.3	33.8 ± 6.2
	---------- Progressive sperm motility (%) ----------				
1	79.5 ± 2.7	55.3 ± 7.9	37.9 ± 7.0	23.2 ± 6.2	10.9 ± 6.6
2	78.3 ± 4.3	56.0 ± 4.6	38.5 ± 4.6	24.6 ± 7.0	11.1 ± 5.6
3	78.9 ± 4.3	58.5 ± 5.8	38.6 ± 7.2	24.3 ± 6.9	11.2 ± 7.3

No significant difference was detected among treatment group ($P > 0.05$). Data shown all mean ± S.D. (n = 12). Three types of freezing programs: 1. Cooling rate 3℃/min from 5 to -5℃, 1 min holding at -5℃ and then freezing rate of 50℃/min from -5 to -140℃, 2. Cooling rate 3℃/min from 5 to -5℃, and then freezing rate of 40℃/min from -5 to -80℃, 30 sec holding at -80℃ and then freezing rate of 60℃/min from -80 to -140℃ and 3. Cooling rate 20℃/min from 5 to -8℃, and then freezing rate of 70℃/min from -8 to -140℃.

表 2. 不同冷凍程式對豬冷凍精液解凍後精子運動參數率之影響

Table 2. The influence of different freezing programs on sperm motion characteristics of boar frozen-thawed semen

Freezing programs		Post thawed incubation time	
		5 min	6 h
VAP (μm/s)	1	52.0 ± 7.8	45.0 ± 9.1
	2	49.1 ± 6.5	45.6 ± 6.6
	3	51.8 ± 9.0	42.6 ± 6.3
VSL (μm/s)	1	25.4 ± 3.6	22.0 ± 4.4
	2	23.8 ± 3.2	22.2 ± 3.3
	3	25.3 ± 4.5	20.8 ± 3.0
VCL (μm/s)	1	75.9 ± 9.3	71.8 ± 8.7
	2	72.7 ± 7.5	75.8 ± 8.9
	3	75.5 ± 9.3	68.8 ± 9.3
ALH (μm/s)	1	2.9 ± 0.5	2.5 ± 0.4
	2	2.5 ± 0.3	2.5 ± 0.3
	3	2.7 ± 0.5	2.3 ± 0.3
BCF (Hz)	1	8.3 ± 0.3	8.4 ± 0.1
	2	8.2 ± 0.3	8.5 ± 0.1
	3	8.2 ± 0.1	8.5 ± 0.2
STR (%)	1	96.6 ± 1.3	96.7 ± 0.7
	2	96.0 ± 0.8	96.5 ± 0.6
	3	96.3 ± 0.8	95.9 ± 0.7
LIN (%)	1	39.6 ± 6.3	38.9 ± 5.4
	2	43.0 ± 6.7	37.5 ± 3.6
	3	42.2 ± 3.0	37.5 ± 3.9

VAP, average path velocity; VSL, straight line (progressive) velocity; VCL, curvilinear velocity; ALH, lateral head displacement; BCF, cross-beat frequency; STR, straightness; LIN, linearity; CASA, computer-assisted sperm analysis. No significant difference was detected among treatment group ($P > 0.05$). Data shown all mean ± S.D. (n = 12).
1, 2 and 3 as footnote as Table 1.

精子穿越卵母細胞透明帶後結合完成受精，此過程之成功率與頭帽完整性呈正相關 (Singh *et al.*, 2017)，故進行冷凍條件評估時，有必要檢測解凍後精子頭帽完整性，以辨別冷凍之成效。利用三種不同的冷凍降溫程式製作豬冷凍精液，經解凍後精子頭帽完整性之分析如表 3 所示。結果顯示解凍後三組冷凍精液之精子頭帽完整率分別為 64.3 ± 3.3、66.4 ± 2.7 及 65.8 ± 5.0%，部分頭帽受損率分別為 20.7 ± 6.1、16.1 ± 4.8 及 16.1 ± 3.2%，而頭帽完全受損率為 15.0 ± 2.4、17.5 ± 3.7 及 18.1 ± 5.5%；顯示三種不同的冷凍降溫程式製作冷凍精液的製程，對冷凍精子解凍後頭帽性狀的影響並無顯著差異。

表 3.　不同冷凍程式對豬冷凍精液解凍後精子頭帽完整率之影響

Table 3.　The influence of different freezing programs on the acrosome integrity of boar frozen-thawed sperm

Freezing programs	Intact acrosome (%)	Partially damaged acrosome (%)	Lost acrosome (%)
1	64.3 ± 3.3	20.7 ± 6.1	15.0 ± 2.4
2	66.4 ± 2.7	16.1 ± 4.8	17.5 ± 3.7
3	65.8 ± 5.0	16.1 ± 3.2	18.1 ± 5.5

No significant difference was detected among treatment groups ($P > 0.05$).
Data shown all mean ± S.D. (n = 12).
1, 2 and 3 as footnote as Table 1.

製作冷凍精液的過程可能損害精子、影響精子活力、存活力和受精能力 (Watson, 1990)，理想降溫程序必須能預防冰晶形成，又避免「溶液效應」所產生之細胞脫水與一系列冷凍損傷 (Mazur *et al.*, 1970)，如能減少與抗凍劑接觸時間，則可降低所造成細胞之傷害 (Thurston *et al.*, 2003)，又所採用降溫條件非最佳化，可能導致精子不可逆的損害 (Fiser and Fairfull, 1990; Mazur, 1970)。另冷凍成效也受稀釋液成分、凍存細胞種類及細胞膜通透性等因素影響，皆必須併入考量 (Woelders and Chaveir, 2004)。本研究檢視國外製作豬冷凍精液常用冷凍降溫程式，希望比較確認合適方式，進一步改善程序並簡化流程、減少製作所需時間，同時維持解凍後之精液品質。本試驗比較三組冷凍降溫程式，第一組及第二組製程均約 8 min，第三組因於製程過程中未設置短暫平衡時間，及較快速降溫程序因此只約 3 min。故第三組之冷卻速度較快，因此可以減少電腦程式控制儀液態氮充填量。第一組冷凍程式於 -5℃設定停留 1 min 進行短暫平衡，此設計用以誘導冰晶之形成，文獻提出此步驟之設計原理，為精液樣品冷凍期間會釋出融化之潛熱，導致溫度急劇升高，破壞「最佳」冷卻曲線 (Bwanga *et al.*, 1991; Medrano *et al.*, 2003) ，透過誘導樣品中的冰晶形成，可抵消融合過程所生成之潛熱，改善解凍後精子活力 (Critser *et al.*, 1987)。測試第二組製程於 -80℃停留 30 sec，進行短暫平衡，以維持程式控制儀內部環境與麥管溫度一致。第三組省略此降溫平衡步驟，並採取快速直接凍存，其解凍後活力、前進式活力、精子各項運動參數及精子頭帽完整率等參數，與其他二組並無顯著差異。早期研究公豬精液冷凍理想降溫程序，自 5℃降至 -5℃以 -3 至 -5℃/min 之速率進行，接著則以 -20 至 -50℃/min 之速率降溫至 -196℃為最適條件 (Fisher and Fairfull, 1990; Bwanga *et al.*, 1991)。Kumer *et al.* (2003) 及 Devireddy *et al.* (2004) 認為以降溫速率 -30 或 -50℃/min 製作豬冷凍精液優於 -1℃/min，其中又以 -30℃/min 的降溫速率為佳。Hernandez *et al.* (2007) 等測試降溫速率為 10、40 或 60℃/min，製作豬 0.5 mL 麥管式冷凍精液，結果發現降溫速度並未影響精子品質相關參數。本研究之第三組程式降溫方式係參考自 Purdy *et al.* (2010)，解凍後之精子品質與其他二組比較並無顯著差異。

精子品質體外分析如活力及頭帽完整評估，於後續受精之能力預測非常重要 (Didion *et al.*, 2008; Vyt *et al.*, 2008; Broekhuijse *et al.*, 2012)。但體內受精過程仍須先後達成幾個步驟才可實現，如包括精子進入至輸卵管、精子獲能並啟動頭帽反應，順利穿透卵母細胞及完成激活等過程，所以本研究後續仍待體內受精測試予以印證。本研究利用三組不同的冷凍降溫程式製作豬冷凍精液，其解凍後精子之活力、快速前進式活力、各項運動參數及頭帽完整率等均無顯著差異。惟第三組之冷凍降溫程式處理製程所需時間較短，且可減少液態氮之損耗，建議可提供作為豬精液冷凍保存製作方式之參考。

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Effect of freezing programs on the sperm quality of frozen-thawed boar semen [1]

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Abstract

The objective of this study was to evaluate the effects of different freezing programs on the quality of frozen-thawed boar semen. Semen collected from five Duroc boars were diluted with Lactose-egg yolk extender, which were brought to 5×10^8 cell/mL as the final concentration, and packaged into 0.5 mL plastic straws. Three freezing programs for boar semen cryopreservation were applied and compared: (1) cooling rate -3℃/min from 5 to -5℃, holding at -5℃ for 1 min and then frozen at -50℃/min rate to -140℃, (2) cooling rate -3℃/min from 5 to -5℃, and then frozen at -40℃/min rate to -80℃, holding at -80℃ for 30 sec and then frozen at -60℃/min rate to -140℃, and (3) cooling rate -20℃/min from 5 to -8℃, and then frozen at -70℃/min rate to -140℃ after reaching -140℃, the straws were then plunged into liquid nitrogen. Analysis of sperm quality after thawing showed that the percentage of motility, rapid progressive motility, motility kinetic variables parameters and acrosome integrity were not affected by the different freezing programs. In conclusion, the 3rd freezing program is recommended for boar semen cryopreservation due to the shorter processing time and reduction of liquid nitrogen consumption.

Key words: Boar, Frozen semen, Freezing program.

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