抗氧化劑添加於豬精液冷凍保存之影響 (1)

章嘉潔 (2)(3)　吳昇陽 (2)

收件日期：108 年 2 月 21 日；接受日期：108 年 4 月 25 日

摘　要

本試驗探討三種抗氧化劑 α- 生育醇 (α-tocopherol)、丁羥基甲苯 (butylated hydroxytoluene, BHT) 及麩胱甘肽 (glutathione, GSH)，添加於豬冷凍稀釋液對解凍後精液品質之影響。收集 5 頭杜洛克公豬之新鮮精液，以 Lactose-egg yolk (LEY) 之冷凍精液稀釋液稀釋，精子最終稀釋濃度為 5×10^8 cells /mL。試驗一之分組分為對照組 (LEY 組)、添加 5 mM GSH、1 mM BHT 及 200 μg/mL α-tocopherol 入冷凍稀釋液等 4 組，以評估解凍後精子活力、精子前進式活力、精子活力各項移動參數，及精子頭帽完整性等項目之差異。結果顯示精液解凍後體外培養 2 至 6 小時，5 mM GSH 添加組精子活力及精子快速前進式活力均較 200 μg/mL α-tocopherol 添加組差 ($P < 0.05$)。200 μg/mL α-tocopherol 或 1 mM BHT 添加組與對照組進行比較，統計無顯著差異。在精子活力各項移動參數方面，精液解凍後培養 5 分鐘，5 mM GSH 添加組精子活力移動參數 VAP、VSL 及 VCL 均較 1 mM BHT 組呈顯著低之現象 ($P < 0.05$)。添加濃度 1 mM BHT 或 200 μg/mL α-tocopherol 於解凍後精子頭帽完整率，與對照組或 5 mM GSH 添加組比較，結果呈顯著改善 ($P < 0.05$)。試驗二之分組分為對照組 (LEY 組)、添加 300、400 或 500 μg/mL α-tocopherol 濃度加入冷凍稀釋液等 4 組，評估解凍後的精子活力、精子前進式活力及精子活力各項移動參數，結果顯示精子活力及精子快速前進式活力及精子活力等各項移動參數，於對照組與不同濃度 α-tocopherol 添加組間均無顯著差異。本研究冷凍稀釋液中添加 5 mM GSH、1 mM BHT 及 200、300、400 與 500 μg/mL α-tocopherol 組之解凍後豬精子的相關活力參數並未見顯著改善效果。

關鍵詞：豬、冷凍精液、抗氧化劑。

緒　言

精液冷凍保存過程易產生活性含氧物 (reactive oxygen species, ROS) (Chatterjee and Gagnon, 2001)，ROS 是生物氧化代謝過程之副產物，包括超氧化物、過氧化氫、氫氧自由基及過氧化物自由基等，由於存在未成對電子，化學性質相當活躍，當 ROS 過高時會對細胞結構造成氧化性傷害。豬精細胞原生質膜之不飽和脂肪酸含量較一般家畜高，且較缺乏抗氧化物，故易發生脂質過氧化現象，損傷精子活力、存活率及影響後續受精能力 (Cerolini *et al.*, 2001; Roca *et al.*, 2004; Buranaamnuay *et al.*, 2011)。許多研究探討如何改善製作冷凍精液過程所造成的氧化傷害，選擇添加天然或人工合成之抗氧化劑不失為有效緩解精液冷凍製作過程 ROS 生成所造成損傷的一種方式。抗氧化劑添加至家畜精液冷凍稀釋液者，如丁羥基甲苯 (butylated hydroxytoluene, BHT) (Bamba and Cran, 1992; Trzcińska *et al.*, 2015)、α- 生育醇 (α-tocopherol) (Satorre *et al.*, 2009; Xia *et al.*, 2012; Mendez *et al.*, 2013; Ma *et al.*, 2015) 及麩胱甘肽 (glutathione, GSH) (Estrada *et al.*, 2014; Yeste *et al.*, 2014; Giaretta *et al.*, 2015; Zhang *et al.*, 2016) 等，均可有效改善解凍後之精液品質如精子活力、精子頭帽完整性及提升卵受精之能力。

抗氧化劑依其化學組成不同，各具其理化特性及功能，與 ROS 之交互作用機制原理亦有所不同。正常公豬精液之精漿成分含有一定濃度的抗氧化劑，可有效減緩 ROS 等對精子傷害 (Strezezek *et al.*, 1999; Strezezek, 2002)。但製備冷凍精液之過程中需去除精漿，則會造成精子失去抗氧化劑之保護 (Breininger *et al.*, 2011)；此外，冷凍保存過程，精子受損及死亡數目會增加，亦造成 ROS 來源量增多，引起精子的過氧化損傷導致精子結構破壞。因此本試驗探討不同抗氧化劑添加於冷凍稀釋液，評估豬冷凍精液解凍後的精子活力、精子前進式活力及精子活力各項移動參數，及頭帽完整性等項目之差異，俾提供日後製作豬冷凍精液時，抗氧化劑添加的相關參考。

(1) 行政院農業委員會畜產試驗所研究報告第 2608 號。
(2) 行政院農業委員會畜產試驗所臺東種畜繁殖場。
(3) 通訊作者，E-mail：janices@mail.tlri.gov.tw。

材料與方法

I. 精液採集與處理

本試驗選擇生殖能力正常，平均年齡 1.5 歲之杜洛克公豬 5 頭，每週採集公豬精液一次。精液採集後於 37℃ 下靜置 30 分鐘後，選取存活率 80% 以上與活力 75% 以上的精液進行冷凍保存。使用 Beltsville thawing solution (BTS: 37.0 g/L glucose, 1.25 g/L EDTA, 6.0 g/L sodium citrate, 1.25 g/L sodium bicarbonate 及 0.75 g/L potassium chloride) (Johnson *et al.*, 2000) 進行精液稀釋。冷凍精液製作參考 Westendorf *et al.* (1975) 之方式，稀釋之精液冷卻至 15℃，於 800 × g 離心 10 分鐘，去除上清液後加入冷凍稀釋液。冷凍稀釋液的組成及製備過程簡述如下：取乳糖 11 g 加入蒸餾水至 100 mL 後，試驗一分別添加以 5 mM GSH、1 mM BHT 或 200 μg/mL α-tocopherol 之不同抗氧化劑測試；試驗二分別添加以 300、400 或 500 μg/mL α-tocopherol 之不同抗氧化劑測試。新鮮精液於 4℃ 下平衡 3 小時後，按 1：2 的比例加入含不同抗氧化劑冷凍稀釋液，並添加甘油使其最終濃度為 3% (v/v)，稀釋精子最終濃度為 5×10^8 cells /mL，裝填於 0.5 mL 之麥管 (Minitüb, Tiefenbach, Germany)，並以封口粉封住末端。然後將麥管移置於電腦程式控制儀 (Ice cube 14S, GmbH, Austria) 內，以下列冷卻條件執行冷凍程式降溫進行凍存：從 5℃ 至 -5℃，降溫速率以 -6℃ /min；從 -5℃ 到 -80℃，以 40℃ /min 速率降溫；於 -80℃ 保持 30 s 後，由 -80℃ 到 -150℃，降溫速率 -60℃ /min；最後再將完成冷凍之麥管移入液氮桶內貯存 (Yeste *et al.*, 2014)。

II. 冷凍精液之解凍過程

備妥解凍用 BTS 精液稀釋液，回溫至 25℃。冷凍精液於液氮桶保存兩周後，取出所需之冷凍精液麥管，以 40℃、30 秒水浴進行解凍；隨後擦乾麥管表面，剪開麥管讓精液流至 2 mL 解凍稀釋液 (BTS) 中，並於 37℃ 5% CO_2 培養箱靜置 30 min 至 6 h，進行解凍後之精液性狀測試，所有過程儘可能避免精液受到溫度變化及光線傷害。

III. 精液性狀評估

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) 進行分析，分析校正係參考 Dziekońska *et al.* (2013) 之方法，評估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)、直線前進之比率 (linearity, LIN)、直線趨勢 (straightness, STR) 等移動能力參數。

IV. 精子頭帽完整性評估

以免疫螢光染色技術評估精子頭帽完整性，其步驟係依據 Zeng and Terada. (2001) 之方法稍作修正。取精液樣品 30 μL 塗抹於載玻片上，經空氣乾燥後以甲醇固定 10 分鐘。取 30 μL 含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，再移於可控制濕度之 37℃ 培養箱內靜置 30 分鐘後，再以 PBS 沖洗，並經空氣乾燥後使用 5 μL 的 Antifade 溶液 (Molecular Probes, Inc., Eugene, OR) 封片，以保持螢光效果。精子頭帽完整性評估使用光學螢光顯微鏡 (DM 2500, Leica) (1,000 ×，油鏡)，以激發波長 480 nm、射出波長 530 nm 進行鏡檢，隨機計數約 100 個細胞，且每一樣品重覆計算數 6 次。在顯微鏡下觀察豬精子頭帽染色及形態，其判讀方式如下：(i) 精子頭帽顯現完整密集明亮螢光表示頭帽完整；(ii) 精子頭帽僅顯現部份螢光表示頭帽部分受損；(iii) 精子頭帽未顯現螢光表示頭帽之細胞膜及外頭帽膜完全受損。

V. 統計分析

豬冷凍精液解凍後，於 37℃ 下靜置 6 h，並每間隔 2 h 評估其精子活力、精子前進式活力及精子活力各項移動參數，精子頭帽完整率則於解凍後立即評估，藉以探討上述評估值於個試驗處理間之差異；試驗資料均以平均值 ± 標準差表示，並以變異數分析 (ANOVA) 檢驗差異之顯著性，當 ANOVA 檢測出現差異時，再以鄧肯多變域分析法 (Duncan's multiple range test) 評估各處理間之差異性，所有處理組間差異性以 $P < 0.05$ 表示。

結果與討論

試驗一係比較冷凍稀釋液添加 5 mM GSH、1 mM BHT 及 200 μg/mL α-tocopherol，對解凍後精子活力及精子快

速前進式活力之影響，結果如表 1 及表 2 所示。精液解凍後體外培養 2 至 6 小時，添加 5 mM GSH 組精子活力及精子快速前進式活力均較添加 200 μg/mL α-tocopherol 組差，200 μg/mL α-tocopherol 或 1 mM BHT 添加組與對照組進行比較，結果無顯著差異。另就解凍後精子活力各項移動參數結果如表 3 所示，精液解凍後 30 分鐘及體外培養 6 小時，冷凍稀釋液添加 5 mM GSH 之 VAP、VCL 及 VSL 精子活力移動參數均明顯低於其他組 ($P < 0.05$)，另添加 5 mM GSH、1 mM BHT 及 200 μg/mL α-tocopherol，對解凍後精子的各項運動參數未見顯著改善。

表 1. 精液稀釋液添加不同抗氧化劑對豬精液冷凍解凍後精子活力之影響

Table 1. Effects of different type of antioxidant added to extender on sperm motility of the frozen-thawed boar semen

Types	After thawing			
	30 min	2 h	4 h	6 h
Control	80.6 ± 9.0[a*]	62.6 ± 9.1[a]	50.9 ± 7.5[ab]	40.8 ± 7.4[ab]
GSH[#]	61.6 ± 5.9[b]	51.5 ± 4.7[b]	43.3 ± 5.5[b]	35.7 ± 6.6[b]
BHT	76.1 ± 8.8[a]	63.6 ± 5.5[a]	51.2 ± 8.9[ab]	41.9 ± 6.9[ab]
α-tocopherol	73.8 ± 4.2[a]	59.9 ± 5.1[a]	51.9 ± 4.3[a]	44.3 ± 5.2[a]

[*a, b] Values with different superscripts in the same column are significantly different ($P < 0.05$).

[#]GSH, 5 mM glutathione; BHT, 1 mM butylated hydroxytoluene; α-tocopherol, 200 μg/mL α-tocopherol.

表 2. 精液稀釋液添加不同抗氧化劑對豬精液冷凍解凍後精子前進式活力之影響

Table 2. Effects of different type of antioxidant added to extender on sperm progressive motility of the frozen-thawed boar semen

Types	After thawing			
	30 min	2 h	4 h	6 h
Control	61.7 ± 9.4[a*]	47.4 ± 8.3[ab]	26.3 ± 6.5[ab]	16.8 ± 7.1[ab]
GSH	48.6 ± 7.1[b]	38.8 ± 6.2[b]	22.6 ± 6.7[b]	15.2 ± 6.9[b]
BHT	59.3 ± 8.7[a]	48.8 ± 8.0[a]	28.0 ± 7.4[a]	17.8 ± 6.3[ab]
α-tocopherol	56.6 ± 5.6[ab]	47.8 ± 7.7[a]	27.6 ± 7.7[a]	18.4 ± 7.1[a]

[*a, b] Values with different superscripts in the same column are significantly different ($P < 0.05$).

[#]GSH, 5 mM glutathione; BHT, 1 mM butylated hydroxytoluene; α-tocopherol, 200 μg/mL α-tocopherol.

精子頭帽內富含許多促進卵子結合之成分，故鑑別家畜精液品質常以頭帽完整性作為選項，以評估冷凍精液品質良窳 (Garner and Johnson, 1995)。經由顯微鏡觀察判別精子頭帽在添加不同抗氧化劑於冷凍解凍後之受損情形，精子頭帽完整性評估結果如表 4。結果顯示，冷凍精液稀釋液中添加 1 mM BHT 或 200 μg/mL α-tocopherol，其解凍後精子頭帽完整率均顯著優於對照組或添加 5 mM GSH 組 ($P < 0.05$)。

GSH 是由麩胺酸、胱胺酸和半甘胺酸所組成三胜肽鏈，為體內主要非蛋白質硫醇化物，可維持細胞內氧化還原平衡 (Jacob *et al.*, 2003)，具抗氧化及調節細胞增殖與生長等作用 (Lu, 2009)。GSH 廣泛存在於所有細胞，學者研究精液冷凍保存過程中去除精漿步驟，會造成 GSH 濃度降低達 68% (Gadea *et al.*, 2011)，恐造成精液品質不利之影響。另外，於精液冷凍過程中會引起精子活力降低，及精細胞膜結構之組成分改變，而導致 GSH 含量更顯著降低 (Gadea *et al.*, 2004; Stradaioli *et al.*, 2007)。曾有研究以 1 至 5 mM 濃度的 GSH 添加於精液之稀釋液中，結果添加 1 mM GSH 組相較添加 5 mM GSH 組，解凍後之精子的活力與存活率顯著提升，使用 CASA 分析解凍精子的各項移動參數均明顯提高，非獲能精子數目亦增加，顯示以添加 1 mM GSH 組對精子有很好的保護效果 (Gadea *et al.*, 2004; 2005)。

Estrada *et al.* (2014) 添加 2 mM GSH 所製作之冷凍精液經解凍培養 240 分鐘，精子頭帽完整性比例顯著改善，授精後分娩率由 67.2 ± 3.4% 提升至 92.7 ± 5.5%，產仔數由 7.5 ± 2.4 頭提升至 13.0 ± 1.0 頭 ($P < 0.05$)，可抵消解凍過程所造成精子損傷，改善公豬精液冷凍保存品質，同時也顯著提高冷凍精液的受精能力。另有研究將採集精液區分為耐凍性佳及耐凍性差精液 (Casas *et al.*, 2009)，證明添加 5 mM 的 GSH 可改善冷凍耐受性差之精液，而冷凍耐受性佳之精液添加 2 mM GSH 即可達成顯著改善精液品質之效果，顯示使用 GSH 改善精液品質之效果與 GSH 添加量及精液之冷凍耐受性有關 (Yeste *et al.*, 2014)。本試驗添加 2 mM GSH 於冷凍稀釋液之結果，解凍後之精子活力、前進式活力與精子活力各項移動參數之評估值，在統計上均無顯著改善之效果 ，或許日後可以提高 GSH 濃度再進一

步測試。

表 3.　精液稀釋液添加不同抗氧化劑對豬精液冷凍解凍精子移動參數之影響

Table 3.　Effects of different types of antioxidants added to extender on the motion characteristic of the frozen-thawed boar semen

Parameter	Freezing media (LEY)	30 min	6 h
VAP (μm/s)*	Control	47.4 ± 10.5[a]	38.2 ± 10.0[a]
	GSH	39.0 ± 8.9[b]	35.4 ± 6.0[a]
	BHT	48.7 ± 8.5[a]	35.8 ± 12.9[a]
	α-tocopherol	46.6 ± 6.6[a]	35.9 ± 8.2[a]
VSL (μm/s)	Control	23.3 ± 5.3[a]	18.8 ± 5.0[a]
	GSH	19.0 ± 4.4[b]	17.1 ± 4.3[a]
	BHT	24.0 ± 4.3[a]	17.4 ± 6.3[a]
	α-tocopherol	23.2 ± 3.2[a]	17.5 ± 4.0[a]
VCL (μm/s)	Control	74.6 ± 17.6[a]	57.3 ± 12.5[a]
	GSH	60.4 ± 16.0[b]	54.1 ± 6.8[a]
	BHT	75.8 ± 17.6[a]	53.7 ± 19.3[a]
	α-tocopherol	67.0 ± 16.1[a]	55.4 ± 6.4a
ALH (μm/s)	Control	2.5 ± 0.5	2.0 ± 0.5
	GSH	2.1 ± 0.5	1.8 ± 0.3
	BHT	2.6 ± 0.5	1.9 ± 0.7
	α-tocopherol	2.3 ± 0.5	2.0 ± 0.6
BCF (Hz)	Control	8.5 ± 0.2	8.4 ± 0.2
	GSH	8.5 ± 0.2	8.4 ± 0.3
	BHT	8.5 ± 0.3	8.4 ± 0.2
	α-tocopherol	8.5 ± 0.2	8.4 ± 0.3
STR (%)	Control	96.0 ± 1.2	96.5 ± 1.2
	GSH	97.1 ± 1.1	96.3 ± 0.6
	BHT	97.3 ± 1.5	96.0 ± 0.8
	α-tocopherol	96.2 ± 1.1	95.9 ± 0.8
LIN (%)	Control	41.1 ± 4.4	40.5 ± 3.4
	GSH	42.6 ± 6.0	39.7 ± 5.5
	BHT	44.2 ± 4.1	39.1 ± 3.6
	α-tocopherol	42.1 ± 6.5	40.2 ± 5.1

*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; SEM, standard error of the mean.

GSH, 5 mM glutathione; BHT, 1 mM butylated hydroxytoluene; α-tocopherol, 200 μg/mL α-tocopherol.

表 4.　添加不同抗氧化劑於稀釋液對豬精液冷凍解凍後頭帽完整性之評估

Table 4.　Effects of different type of antioxidant added to extender on acrosome integrity of the frozen-thawed boar semen

		Sperm frozen with		
	Control	GSH	BHT	α-tocopherol
Acrosome integrity (%)	52.3 ± 5.3[b]	54.5 ± 8.1[b]	63.3 ± 6.7[a]	66.1 ± 6.6[a]

*GSH, 5 mM glutathione; BHT, 1 mM butylated hydroxytoluene; α-tocopherol, 200 μg/mL α-tocopherol

#a, b Values with different superscripts in the row is significantly different ($P < 0.05$).

BHT 是一種人工合成的脂溶性抗氧化劑，其作用為防止不飽和脂肪及脂類氧化。冷凍稀釋液中添加 BHT 可減少 ROS 形成，阻止 ROS 對精子之不利影響 (Ghorbani *et al.*, 2015)，可改善牛 (Shoae and Zamiri, 2008)、豬 (Roca *et al.*, 2004) 及山羊 (Memon *et al.*, 2011) 等之冷凍精液品質。Bamba and Crank (1992) 於豬精液稀釋液中添加 0.05 至 0.10 mM 的 BHT 冷藏 5℃保存，可顯著提高精子之活力及頭帽完整性，但添加 2 mM 的 BHT 於精液稀釋液則造成精子活力下降，評估頭帽完整性亦未具改善。Roca *et al.* (2004) 研究 BHT 添加對豬冷凍精液解凍後之影響，檢測解凍後 0.5 小時與 2.5 小時之精液，結果發現稀釋液中添加 BHT 濃度為 0.2、0.4 及 0.8 mM 組之精子存活率顯著提升 ($P < 0.05$)；其中添加 0.4 mM BHT 可使受精卵發育到囊胚比率由 16% 顯著提升至 29% ($P < 0.05$)。Trzcińska *et al.* (2015) 於冷凍稀釋液中分別添加 BHT 0.5、1.0 及 2.0 mM，解凍後 20 分鐘培養觀察精子活力、前進式活力，及頭帽完整比率均顯著改善 ($P < 0.001$)，其中 1.0 mM BHT 者分娩率由 45.4% 提升至 86.7%，產仔數由 8.2 ± 2.2 頭提升至 10.8 ± 1.6 頭具顯著改善；而添加 BHT 1.0 及 2.0 mM 兩組，其分娩率及產仔數並無顯著差異。而本研究於豬精液稀釋液中添加 1 mM BHT，冷凍解凍後精子之活力、前進式活力、精子活力各項移動參數評估值等方面，與對照組比較均無顯著之改善效果。冷凍精液稀釋液中添加 1 mM BHT 其解凍後精子頭帽完整率均顯著優於與對照組或添加 5 mM GSH 組 ($P < 0.05$)；添加 BHT 可能有利於減少 ROS 的形成，和防止 ROS 對精子的不利影響功能 (Ghorbani *et al.*, 2015)，是否與改善精子頭帽完整性有關，仍待更多探討。

α-tocopherol 為常見之抗氧化劑，可保護精細胞膜抵抗脂質過氧化反應 (Jeong *et al.*, 2009; Breininger *et al.*, 2011)。α-tocopherol 使用於改善精液品質方法通常為下列兩種，其一為直接添加在家畜之飼料中，如研究指出公豬給予 400 mg/kg α-tocopherol (α-tocopherol acetate)，可顯著改善精子前進式活力 ($P < 0.05$) (Liu *et al.*, 2015)。另一種為直接添加豬精液冷凍稀釋液，最早 Polge (1956) 測試使用 α-tocopherol 添加濃度範圍 200 至 1,000 μg/mL ，結果指出以 200 μg/mL α-tocopherol 的添加量可顯著改善解凍後精子活力。Satorre *et al.* (2009) 添加 200 μg/mL α-tocopherol 於冷凍稀釋液，解凍後 10 分鐘觀察可顯著改善精子活力和頭帽完整性，並降低精子之類獲能反應現象。Breininger *et al.* (2011) 添加相同濃度之精子解凍後培養 4 小時觀察，可見活力顯著改善，但其精子存活率和頭帽完整性評估值並未有顯著差異。

Jeong *et al.* (2009) 於豬冷凍精液解凍後培養 3 小時觀察，指出冷凍稀釋液添加 100 及 200 μg/mL α-tocopherol 組，較 400、600 或 800 μg/mL α-tocopherol 組可顯著改善精子活力及存活率，添加 100 μg/mL α-tocopherol 組精子活力移動 VCL 參數值顯著降低 ($P < 0.05$)，添加 α-tocopherol 800 μg/mL 組精子活力移動 VCL 及 VSL 參數值明顯高於其他組 ($P < 0.05$)。添加 400 和 800 μg/mL α-tocopherol 組，精子活力移動 VAP 參數值與新鮮精液未見顯著差異，另評估精子頭帽完整添加 α-tocopherol 不同劑量均有助於改善。本研究在精液之稀釋液中添加 200 μg/mL 之 α-tocopherol，試驗結果在解凍後之精子活力、精子前進式活力及精子活力各項移動參數等項目並無統計上之差異。後續於冷凍稀釋液中分別提高添加 α-tocopherol 濃度至 300、400 及 500 μg/mL，試驗結果評估解凍後精子活力如表 5、精子前進式活力如表 6 及精子活力各項移動參數如表 7 所示，統計上均無顯著之差異。結果顯示，冷凍精液稀釋液中添加 200 μg/mL α-tocopherol，其解凍後精子頭帽完整率均顯著優於與對照組或添加 5 mM GSH 組 ($P < 0.05$)。研究添加 α-tocopherol 會破壞 ROS 與細胞膜脂肪酸側鏈形成之共價連接，減少冷凍保存期間過量 ROS 生成所造成細胞膜損傷 (Jeong *et al.*, 2009)，推測目前添加 200 μg/mL α-tocopherol 其解凍後有益於精子頭帽完整可能為改善之因素。

綜合言之，本研究所探討不同抗氧化劑之添加未能提升豬精液冷凍解凍後之品質，可能導因於豬精子之原生質膜所含有的不飽和脂肪酸較其他家畜高，且較缺乏抗氧化物，致使本研究所使用之抗氧化劑種類及濃度並未達最適化，因此，應再深入探討抗氧化劑之添加策略，如多種抗氧化劑之搭配及不同抗氧化劑搭配濃度之調整，此或是改善豬精液冷凍保存效率之重要方向。

表 5. 精液稀釋液添加不同濃度 α-tocopherol 對豬精液冷凍解凍後精子活力之影響

Table 5. Effects of different concentrations of α-tocopherol added to extender on sperm motility of the frozen-thawed boar semen

α-tocopherol conc. (μg/mL)	After thawing			
	30 min	2 h	4 h	6 h
Control	78.0 ± 8.7	65.6 ± 9.2	54.6 ± 7.3	45.2 ± 5.9
300	80.7 ± 6.8	67.0 ± 6.4	55.4 ± 5.7	43.0 ± 6.8
400	78.5 ± 6.6	64.5 ± 5.8	53.8 ± 6.4	44.4 ± 6.2
500	79.7 ± 5.8	68.7 ± 5.9	57.9 ± 5.1	46.5 ± 4.7

No significant differences between treatments.

表 6.　精液稀釋液添加不同濃度 α-tocopherol 對豬精液冷凍解凍後精子前進式活力之影響

Table 6.　Effects of different concentrations of α-tocopherol added to extender on sperm progressive motility of the frozen-thawed boar semen

α-tocopherol conc. (μg/mL)	After thawing			
	30 min	2 h	4 h	6 h
Control	60.5 ± 9.8	50.9 ± 8.2	29.3 ± 5.3	18.9 ± 3.4
300	63.8 ± 6.4	50.7 ± 5.9	30.2 ± 3.9	18.1 ± 3.7
400	62.1 ± 7.4	48.1 ± 5.9	28.0 ± 3.6	18.2 ± 2.9
500	62.3 ± 5.5	52.2 ± 6.6	30.0 ± 2.5	19.0 ± 2.7

No significant differences between treatments.

表 7.　精液稀釋液添加不同濃度 α-tocopherol 對豬精液冷凍解凍精子移動參數之影響

Table 7.　Effects of different concentrations of α-tocopherol added to extender on the motion characteristic of the frozen-thawed boar semen

Parameter	α-tocopherol conc. (μg/mL)	30 min	6 h
VAP (μm/s)	Control	51.9 ± 5.9	40.8 ± 6.2
	300	56.0 ± 7.3	40.9 ± 4.6
	400	54.8 ± 6.6	41.3 ± 3.2
	500	53.8 ± 7.9	41.3 ± 3.8
VSL (μm/s)	Control	25.3 ± 2.9	19.8 ± 3.1
	300	27.3 ± 3.8	19.8 ± 2.3
	400	27.7 ± 3.3	20.2 ± 1.5
	500	26.4 ± 3.9	20.2 ± 2.0
VCL (μm/s)	Control	76.3 ± 5.8	63.3 ± 9.9
	300	77.7 ± 13.7	64.8 ± 9.8
	400	80.4 ± 15.6	64.2 ± 12.0
	500	78.3 ± 14.4	68.7 ± 10.4
ALH (μm/s)	Control	2.7 ± 0.2	2.2 ± 0.3
	300	2.9 ± 0.3	2.3 ± 0.2
	400	2.9 ± 0.4	2.2 ± 0.3
	500	2.7 ± 0.4	2.3 ± 0.3
BCF (Hz)	Control	8.2 ± 0.2	8.3 ± 0.3
	300	8.1 ± 0.3	8.3 ± 0.2
	400	8.2 ± 0.2	8.4 ± 0.2
	500	8.3 ± 0.2	8.5 ± 0.2
STR (%)	Control	96.0 ± 0.7	95.9 ± 0.8
	300	96.0 ± 1.8	95.7 ± 0.8
	400	96.4 ± 1.5	96.0 ± 0.8
	500	97.1 ± 0.7	96.3 ± 0.7
LIN (%)	Control	42.8 ± 4.9	40.0 ± 4.2
	300	44.5 ± 6.9	39.3 ± 2.7
	400	42.9 ± 5.8	38.0 ± 6.4
	500	42.5 ± 4.5	36.4 ± 5.4

*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; SEM, standard error of the mean.

#No significant differences between treatments.

誌　謝

本試驗承農委會科技計畫 (106 農科 -2.1.1- 畜 -L3) 經費補助，試驗期間並承遺傳育種組吳明哲組長商借儀器設備，特此一併致謝。

參考文獻

Bamba, K. and D. G. Cran. 1992. Effects of treatment with butylated hydroxytoluene on the susceptibility of boar spermatozoa to cold stress and dilution. J. Reprod. Fertil. 95: 69-77.

Breininger, E., A. Descalzo, L. Rossetti, D. Abramovich and M. T. Beconi. 2011. Boar sperm functionality is related to α-tocopherol content after freezing-thawing. Andrologia 43: 409-415.

Buranaamnuay, K., R. Grossfeld, C. Struckmann and D. Rath. 2011. Influence of cryoprotectants glycerol and amides, combined with antioxidants on quality of frozen-thawed boar sperm. Anim. Reprod. Sci.127: 56-61.

Casas, I., S. Sancho, M. Briz, E. Pinart, E. Bussalleu, M. Yeste and S. Bonet. 2009. Freezability prediction of boar ejaculates assessed by functional sperm parameters and sperm proteins. Theriogenology 72: 930-948.

Cerolini, S., A. Maldjian, F. Pizzi and T. M. Gliozzi. 2001. Changes in sperm quality and lipid composition during cryopreservation of boar semen. Reproduction 121: 395-401.

Chatterjee, S. and C. Gagnon. 2001. Production of reactive oxygen species by spermatozoa undergoing cooling, freezing and thawing. Mol. Reprod. Dev. 59: 451-458.

Dziekońska, A., L. Fraser, A. Majewska, M. Lecewicz, Ł. Zasiadczyk and W. Kordan. 2013. Effect of commercial long-term extenders on metabolic activity and membrane integrity of boar spermatozoa stored at 17ºC. J. Vet. Sci. 16: 517-525.

Estrada, E., J. E. Rodríguez-Gil, L. G. Rocha, S. Balasch, S. Bonet and M. Yeste. 2014. Supplementing cryopreservation media with reduced glutathione increases fertility and prolificacy of sows inseminated with frozen-thawed boar semen. Andrology 2: 88-99.

Gadea J., E. Selles, M. A. Marco, P. Coy, C. Matas, R. Romar and S. Ruiz. 2004. Decrease in glutathione content in boar sperm after cryopreservation. Effect of the addition of reduced glutathione to the freezing and thawing extenders. Theriogenology 62: 690-701.

Gadea, J., F. García-Vazquez, C. Matás, J. C. Gardón, S. Cánovas and D. Gumbao. 2005. Cooling and freezing of boar spermatozoa: supplementation of the freezing media with reduced glutathione preserves sperm function. J Androl. 26: 396-404.

Gadea J., M. Molla, E. Selles, M. A. Marco, F. A. Garcia-Vazquez and J. C. Gardon. 2011. Reduced glutathione content in human sperm is decreased after cryopreservation: effect of the addition of reduced glutathione to the freezing and thawing extenders. Cryobiology 62: 40-46.

Garner, D. L. and L. A. Johnson. 1995. Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. Biol. Reprod. 53: 276-284.

Ghorbani, M., I. Amiri, I. Khodadadi, A. Fattahi, M. Atabakhsh and H. Tavilani. 2015. Influence of BHT inclusion on post-thaw attributes of human semen. Syst. Biol. Reprod. Med. 61: 57-61.

Giaretta, E., E. Estrada, D. Bucci, M. Spinaci, J. E. Rodríguez-Gil and M. Yeste. 2015. Combining reduced glutathione and ascorbic acid has supplementary beneficial effects on boar sperm cryotolerance. Theriogenology 83: 399-407.

Jacob, C., G. I. Giles, N. M. Giles and H. Sies. 2003. Sulfur and selenium: the role of oxidation state in protein structure and function. Angew. Chem. 42: 4742-4758.

Jeong, Y. J., M. K. Kim, H. J. Song, E. J. Kang, S. A. Ock, B. M. Kumar, S. Balasubramanian and G. J. Rho. 2009. Effect of alpha-tocopherol supplementation during boar semen cryopreservation on sperm characteristics and expression of apoptosis related genes. Cryobiology 58: 181-189.

Johnson, L. A., K. F. Weitze, P. Fiser and W. M. Maxwell. 2000. Storage of boar semen. Anim. Reprod. Sci. 62: 143-172.

Liu, Q., Y. F. Zhou, R. J. Duan, H. K. Wei, S.W. Jiang and J. Peng. 2015. Effects of dietary n-6: n-3 fatty acid ratio and vitamin E on semen qulity, fatty acid composition and antioxidant statusin boars. Anim. Reprod. Sci. 162: 11-19.

Lu, S. C. 2009. Regulation of glutathione synthesis. Mol. Asp. Med. 30: 42-59.

Ma, H., D. Liu, W. Wang, L. Wang, B. Fu, Z. Li and X. He. 2015. Effect of semen extender supplementation with trehalose, vitamin c and e on post-thaw min pig sperm qualities. Cryo. Letters. 36: 308-312.

Memon, A. A., H. Wahid, Y. Rosnina, Y. M. Goh, M. Ebrahimi, F. M. Nadia and G. Audrey. 2011. Effect of butylated hydroxytoluene on cryopreservation of boer goat semen in tris egg yolk extender. Anim. Reprod. Sci. 129: 44-49.

Mendez, M. F., M. G. Zangeronimo, L. G. Rocha, B. G. Faria, B. A. Pereira, C. D. Fernandes, B. R. Chaves, L. D. Murgas and R.V. Sousa. 2013. Effect of the addition of IGF-I and vitamin E to stored boar semen. Animal 7: 793-798.

Polge, C. 1956. Artificial insemination in pigs. Vet. Rec. 68: 62-76.

Roca, J., M. A. Gil, M. Hernandez, I. Parrilla, J. M. Vazquez and E. A. Martinez. 2004. Survival and fertility of boar spermatozoa after freeze-thawing in extender supplemented with butylated hydroxytoluene. J. Androl. 25: 397-405.

Satorre, M. M., E. Breininger, M. T. Beconi and N. B. Beorlegui. 2009. Protein tyrosine phosphorylation under capacitating conditions in porcine fresh spermatozoa and sperm cryopreserved with and without alpha tocopherol. Andrologia 41: 184-192.

Shoae, A. and M. J. Zamiri. 2008. Effect of butylated hydroxytoluene on bull spermatozoa frozen in egg yolk-citrate extender. Anim. Reprod. Sci. 104: 414-418.

Stradaioli, G., T. Noro, L. Sylla and M. Monaci. 2007. Decrease in glutathione (GSH) content in bovine sperm after cryopreservation: comparison between two extenders. Theriogenology 67: 1249-1255.

Strezezek, J., S, Lapkiewicz and M. Lecewicz. 1999. A note on antioxidant capacity of boar seminal plasma. Anim. Sci. Pap. Rep. 17: 181-188.

Strezezek, J. 2002. Secretory activity of boar seminal vesicle glands. Reprod. Biol. 2: 243-266.

Trzcińska, M., M. Bryła, B. Gajda and P. Gogol. 2015. Fertility of boar semen cryopreserved in extender supplemented with butylated hydroxytoluene. Theriogenology 83: 307-313.

Westendorf, P., L. Richter and H. Treu. 1975. Zur Tiefgefrierung von ebersperma labor-und besamungsergebnisse mit dem hulsenberger Pailetten-Verfahren. Dtsch. Tierarztl. Wschr. 82: 261-267.

Xia, C., W. Xia, S. Yang, L. An, X. Li, Z. Wu, J. Zhang, Z. Wang and J. Tian. 2012. Effect of antioxidant supplementation on function and fertility of sex-sorted boar spermatozoa. Anim. Reprod. Sci.136: 108-114.

Yeste, M., E. Estrada, E. Pinart, S. Bonet, J. Miró and J. E. Rodríguez-Gil. 2014. The improving effect of reduced glutathione on boar sperm cryotolerance is related with the intrinsic ejaculate freezability. Cryobiology 68: 251-226.

Zeng, W. X. and T. Terada. 2001. Protection of boar spermatozoa from cold shock damage by 2-hydroxypropyl-beta-cyclodextrin. Theriogenology 55: 615-627.

Zhang, X. G., Q. Liu, L. Q. Wang, G. S. Yang and J. H. Hu. 2016. Effects of glutathione on sperm quality during liquid storage in boars. Anim. Sci. J. 87: 1195-1201.

Effect of antioxidants supplementation on boar semen cryopreservation [1]

Chia-Chieh Chang [2] [3] and Sheng-Yang Wu [2]

Received: Feb. 21, 2019; Accepted: Apr. 25, 2019

Abstract

The aim of this study was to investigate the effects of three antioxidants α-tocopherol, butlated hydroxytoluene (BHT) and glutathione (GSH) were added to freezing extender on the quality of frozen-thawed boar semen. Semen collected from five Duroc boars were diluted with Lactose-egg yolk (LEY) extender as control group, which it brought to 5×10^8 cell/mL in the final concentration. In the first experiment, four groups were divided with adding 5 mM GSH, 1mM BHT and 200 μg/mL α-tocopherol into freezing extenders. The percentage of sperm motility, rapid progressive motility, motility kinetic variables parameters and acrosome integrity were evaluated. The results showed that the percentage of total motility and rapid progressive motility in 5mM GSH supplemented freezing extender after thawing for 2-6 hrs were lower than in 200 μg/mL α-tocopherol group ($P < 0.05$). No significant differences in the percentage of total motility and rapid progressive motility were observed either between control group and 200 μg/mL α-tocopherol or 1mM BHT supplemented. The results showed that the percentage of sperm motion parameters (VAP, VSL and VCL) of semen cryopreserved with freezing extender after thawing for 5min were lower in 5 mM GSH group than in the 1mM BHT group ($P < 0.05$). Acrosome integrity results demonstrated that the intact acrosome was significantly higher in the 1mM BHT or 200 μg/mL α-tocopherol group than that in the control or than that in 5 mM GSH group ($P < 0.05$). In the second experiment, the experimental designs are separated into 4 groups, inclusive of control group, and the 300, 400 or 500 μg/mL α-tocopherol supplementation group in the boar semen extender during cryopreservation on post-thawed sperm motility, rapid progressive motility and motility kinetic variables parameters. There is no significant difference between the α-tocopherol supplemented and control group. In conclusion, the addition of 5 mM GSH, 1 mM BHT and 200, 300, 400 or 500 μg/mL α-tocopherol to the freezing extender demonstrate no any improvement in sperm motility parameters.

Key words: Boar, Frozen Semen, Antioxidant.

(1) Contribution No. 2608 from Livestock Research Institute, Council of Agriculture, Executive Yuan.
(2) Taitung Animal Propagation Station, COA-LRI, Taitung 95444, Taiwan, R. O. C.
(3) Corresponding author, E-mail: janices@mail.tlri.gov.tw.