The Thai Journal of Veterinary Medicine

Volume 40
Issue 2 *June, 2010*

Article 7

6-1-2010

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Original Article

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Abstract

The objectives of the present study were to evaluate the damage of DNA of the frozen-thawed (FT) boar spermatozoa and to investigate the effect of various concentrations of *L*-cysteine supplementation on the sperm DNA damage. A total of 104 cryopreserved semen samples from twenty-six ejaculates of 16 proven boars were analyzed. Of these samples, each semen sample contained a different concentration of *L*-cysteine i.e., 0 (n=41), 5 (n=41), 10 (n=11) and 15 (n=11) mM. All of the semen samples were cryopreserved by controlled-rate freezer. The semen was thawed at 50°C for 12 sec and the damage to the sperm DNA was determined using acridine orange (AO) staining. The results revealed that, on average, the DNA damage was observed in 0.5% of the FT boar spermatozoa. DNA damage varied among the boars from 0.0% to 4.0%. The levels of DNA damage were 0.6%, 0.4%, 0.5% and 0.9% in the extenders supplemented with 0, 5, 10 and 15 mM of *L*-cysteine, respectively (p>0.05). In conclusion, the DNA damage of the FT boar spermatozoa was relatively low. No adverse effect of *L*-cysteine supplementation up to 10 mM on the damage of the sperm DNA was found. Boar characteristic is the most important factor affecting the damage of the sperm DNA.

Keywords: antioxidant, DNA damage, pig, sperm

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บทคัดย่อ

การเสียหายของดีเอ็นเอของเซลล์อสุจิของน้ำเชื้อสุกรแช่แข็งสัมพันธ์กับคุณภาพของน้ำเชื้อสุกรแช่แข็ง สารต้านอนุมูลอิสระ และพ่อสุกร

พนิดา ชนาภิวัตน์[1] กัมพล แก้วเกษ[2] เผด็จ ธรรมรักษ์[1]*

การศึกษานี้มีวัตถุประสงค์เพื่อตรวจประเมินความเสียหายของดีเอ็นเอของเซลล์อสุจิของน้ำเชื้อสุกรแช่แข็งสัมพันธ์กับคุณภาพของน้ำเชื้อแช่แข็งหลังทำละลาย การเติมสารต้านอนุมูลอิสระ แอล-ซีสเทอีน ที่ความเข้มข้นที่แตกต่างกัน และ ความแปรปรวนระหว่างพ่อสุกรแต่ละตัว ทำการตรวจประเมินตัวอย่างน้ำเชื้อสุกรแช่แข็งจำนวน 104 ตัวอย่าง จากพ่อสุกรที่มีความสมบูรณ์พันธุ์จำนวน 16 ตัว แต่ละตัวอย่างถูกถูกแช่แข็งโดยใช้สารละลายที่มีสารแอล-ซีสเทอีน ในความเข้มข้นต่างๆ กัน ได้แก่ 0 (n=41), 5 (n=41), 10 (n=11) และ 15 (n=11) มิลลิโมลาร์ ตัวอย่างน้ำเชื้อทั้งหมดถูกนำไปผ่านกระบวนการแช่แข็งโดยเครื่องควบคุมอุณหภูมิ และถูกทำละลายที่ 50 องศาเซลเซียส นาน 12 วินาที และ ทำการตรวจประเมินความเสียหายของดีเอ็นเออสุจิโดยการใช้สีย้อมอะคริดีน ออเรนจ์ ผลการศึกษาพบว่าสัดส่วนความเสียหายของดีเอ็นเอของเซลล์อสุจิของน้ำเชื้อสุกรแช่แข็งมีค่าเฉลี่ย 0.6% โดยสัดส่วนความเสียหายของดีเอ็นเออสุจิอยู่มีความแปรปรวนระหว่างพ่อสุกรแต่ละตัว ตั้งแต่ 0.0 ถึง 4.0% การเติมสารซีสเทอีนในสารละลายเลี้ยงเชื้อทำให้เกิดความเสียหายของดีเอ็นเอของเซลล์อสุจิเพียงเล็กน้อย โดย มีค่าเฉลี่ย 0.6% 0.4% 0.5% และ 0.9% ในกลุ่มที่เสริมสารซีสเทอีนในปริมาณ 0 5 10 15 มิลลิโมลาร์ ตามลำดับ สรุปได้ว่ากระบวนการแช่แข็งน้ำเชื้อสุกรมีผลต่อความเสียหายของดีเอ็นเอของเซลล์อสุจิสุกรเพียงเล็กน้อย การเสริมสารซิสเตอีนในนำเชื้อพ่อสุกรแช่แข็ง ไม่มีผลกระทบต่อการเสียหายของดีเอ็นเอของอสุจิพ่อสุกร พ่อสุกรเป็นปัจจัยที่สำคัญที่มีผลต่อความเสียหายของดีเอ็นเออสุจิ

คำสำคัญ: สารต้านอนุมูลอิสระ ความเสียหายของดีเอ็นเอ สุกร ตัวอสุจิ

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Introduction

In general, the fertility rate of female pigs is largely depends on the male factors. A number of studies based on many mammalian species demonstrate that the male factors caused infertility in the female due to many problems, e.g., congenital abnormalities, gene mutation, infectious disease and the damage or fragmentation of DNA of the spermatozoa (Agarwal and Said, 2003; Guerin and Pozzi, 2005). During the last decade, one area of research interest is the studies on the sperm DNA damage that influence male fertility in either humans or animals (Agarwal and Said, 2003; Rybar et al., 2004; Boe-hansen et al., 2005; Perez-Llano et al., 2006). Earlier studies demonstrated that the sperm DNA can be damaged by various mechanisms such as reactive oxygen species (ROS) and apoptosis (Agarwal and Said, 2003). It has been revealed that the DNA damage of the boar spermatozoa depends on two main factors i.e., the tight packaging of chromatin of the spermatozoa of each boar, and the amount of antioxidant substances in seminal plasma (De Ambrogi et al., 2006). Generally, boar spermatozoa are highly susceptible to lipid peroxidation. The reason is that the plasma membranes of the boar spermatozoa contain high level of polyunsaturated fatty acids and also the cytoplasm has a low level of scavenging enzymes (Alvarez and Storey, 1995; Sharma and Agarwal, 1996). Chanapiwat et al. (2009) demonstrated that a number of sperm parameters, e.g., progressive motility, sperm viability and acrosome integrity, significantly decreased after cryopreservation. However, cryopreserved boar semen with the subjective motility of higher than 40% is still recommended to be used in the swine industry (Eriksson et al., 2000; Buranaumnuay et al., 2010). It was found that the use of FT boar semen under field conditions generally led to a reduction of 2-3 piglets per litter (Johnson et al., 2000; Roca et al., 2003; Buranaumnuay et al., 2008). This might possibly be caused by low fertilization and/or a high proportion of early embryonic loss in which sperm DNA integrity was one of the key factors for successful fertilization and embryo development (Lopes et al., 1998).

There are several methods to determine the sperm DNA damage including, for instance, the sperm chromatin structure assay (SCSA) (Evenson et al., 1994; Rybar et al., 2004), Terminal

deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (Martins et al., 2007), comet assay (Fraser and Strzezek, 2004), and acridine orange (AO) staining (Thuwanut et al., 2008). AO staining is commonly used for assessing sperm DNA damage in many mammalian species including humans, dogs, bulls and cats (Rota et al., 2005; Dejarkom and Kunathikom, 2007; Martins et al., 2007; Thuwanut et al., 2008). Using this technique, the DNA damage of the FT sperm was 48.1%, 2.5%, 0.5% and 13.3% in humans (Dejarkom and Kunathikom, 2007), dogs (Rota et al., 2005), bulls (Martins et al., 2007) and cats (Thuwanut et al., 2008), respectively.

In the boar, it has been demonstrated that a number of factors influence sperm DNA damage such as long-term storage of extended boar semen (Boe-hansen et al., 2005; Peréz-Llano et al., 2006), sex-sorted boar semen using flow cytometry (De Ambrogi et al., 2006), and the freezing and thawing process (Evenson et al., 1994; Cordova-Izquierdo et al., 2006; Hu et al., 2008). During the last decade, the use of cryopreserved boar semen under field conditions has increased (Eriksson et al., 2002; Roca et al., 2003). It was found that both the conception rate and the total number of piglets born per litter after using cryopreserved boar semen for artificial insemination (AI) was significantly lower than the use of extended boar semen for AI (Eriksson et al., 2002; Roca et al, 2003). Since the cryopreservation of the spermatozoa can induce ROS formation and leads to lipid peroxidation and DNA oxidation, the evaluation of DNA damage of the cryopreserved boar sperm should be performed. To our knowledge, few studies on sperm DNA damage have been conducted in boars (Hu et al., 2008; Fraser et al., 2009) but the influence of antioxidant supplementation in the semen extender on the sperm DNA damage has not been studied.

The objectives of the present study were to determine the damage to sperm DNA of the FT boar semen in relation to post-thawed sperm qualities and the influence of antioxidant (*L*-cysteine) supplementations in different concentrations in the semen extender, and variation among boars.

Materials and Methods

Semen samples: A total of 104 cryopreserved semen samples from 16 proven boars were used in the experiment. The semen was collected from 5 Pietrain, 4 Duroc, 4 Landrace and 3 Yorkshire boars. Of these samples, each semen sample contained a different concentration of *L*-cysteine i.e., 0 (n=41), 5 (n=41), 10 (n=11) and 15 (n=11) mM. All of the semen samples were obtained from boars used for routine AI in two commercial swine herds in Ratchaburi Province. The boars were kept in individual pens in a conventional open-housed system. Boars were fed twice daily and water was provided up to *ad libitum* via water nipple. Semen was collected by the gloved-hand method with an interval of at least 5-7 days. Semen qualities including semen volume, pH, subjective sperm motility, sperm concentration, sperm viability and sperm morphology were evaluated by the first author of the present study (P. Chanapiwat). Ejaculated semen with a subjective motility of at least 70%, a sperm concentration of at least 150 spermatozoa/ml, and normal sperm morphology of more than 80% were included in the experiment.

Semen freezing and thawing procedures: The cryopreservation procedure of the boar semen was carried out according to our previous study (Chanapiwat et al., 2009). Briefly, the sperm-rich fraction of ejaculated semen was diluted with extender I (Modena™, Swine Genetics International, Ltd., Iowa, USA), equilibrated for 2 hrs at 15°C, and centrifuged at 800xg for 10 min at 15°C. The semen pellet was re-suspended (about 1-2:1) with lactose-egg yolk (LEY) extender II (80% (v/v) lactose solution and 20% (v/v) egg yolk) to a concentration of $1.5x10^9$ spermatozoa/ml. The re-suspended semen was divided according to the concentration of *L*-cysteine in the semen extender into 4 groups, i.e., 0, 5, 10, 15 mM *L*-cysteine supplementation. The semen was equilibrated at 5°C for 90 min and further diluted with extender III (89.5% extender II, 9% glycerol and 1.5% Equex-STM) to obtain the final concentration of $1,000x10^6$ spermatozoa/ml and 3% (v/v) glycerol. The semen was loaded into 0.5 ml straws (Bio-Vet, Z.I. Le Berdoulet, France) and frozen using a controlled rate freezer (Icecube 14s, Sylab, Purkersdorf, Austria). The freezing rate was 3°C min-1 from +5 to -5°C, then 50°C min -1 from -5 to -140°C and plunged into liquid nitrogen (-196°C) for storage. The frozen semen was thawed at 50°C for 12 sec. The thawed semen was diluted (1:4) with extender I and incubated in water-bath at 37 °C for 15 min before sperm assessment.

Post-thawed semen evaluation

Sperm concentration and subjective motility: The concentration of the boar sperm was evaluated by a Neubauer haemocytometer (Boeco, Humburg, Germany) at a dilution of 1:100 (v/v) (Beardon and Fuquay, 1997). The subjective sperm motility was evaluated on a warm plate at 37°C under a phase contrast microscope at 200x magnification.

Sperm viability: The sperm viability was assessed using SYBR-14/Ethidiumhomodimer-1 staining technique (Fertilight®, Sperm Viability Kit, Molecular Probes Europe, Leiden, The Netherlands), which modified after Axnér et al. (2004). Ten µl of semen samples were mixed with 2.7 µl of the working solution of SYBR-14 (diluted with DMSO 1:100, v/v) and 10 µl of EthD-1. The mixture was incubated at 37°C for 20 min, 200 sperm were counted (x1000) under fluorescence microscope. The nuclei of the live spermatozoa with intact plasma membrane were stained green with SYBR-14, while those with damaged membranes were stained red-green and dead spermatozoa were stained red with EthD-1 (Axnér et al, 2004; Chanapiwat et al., 2009). The results were presented as the percentage of live spermatozoa with intact plasma membranes.

Acrosome integrity: Acrosome integrity was assessed using fluorescein isothiocyanate–labeled peanut agglutinin (FITC-PNA) staining. The method was carried out according to our previous study

(Chanapiwat et al., 2009). Ten µl of the diluted semen was mixed with 10 µl of EthD-1 and incubated at 37°C for 15 min. An aliquot of 5 µl was smeared on a pre-warmed slide and immersed in 95% ethanol for 30 sec. Fifty µl of working solution of Fit C-PNA (diluted with PBS 1:10 v/v) was spread over the slides and placed on the chamber at 4°C for 30 min. Thereafter, slides were rinsed with cold PBS and air dried. 200 spermatozoa were counted under fluorescence microscope at 1000x magnification and classified as intact acrosome, reacted acrosome, and loose acrosome (Cheng et al., 1996; Axnér et al., 2004). The results were presented as the percentage of spermatozoa with intact acrosome.

DNA damage: DNA damage was evaluated by Acridine orange (AO) staining. The technique was modified after Thuwanut et al. (2008). Briefly, two smears from each sample were prepared on glass slide and air-dried. Each slide was fixed overnight in freshly prepared Carnoy's solution (methanol: glacial acetic acid, 3:1 v/v). Thereafter, slides were air-dried, and stained with 1% (100 mg/ml) AO (Sigma) for 10 min. The AO working solution was daily prepared by mixing 10 ml of 1% AO with 40 ml of 0.1 M citric acid and 2.5 ml of 0.3 M $Na_2HPO_4.7H_2O$ (Merck, Darmstadt, Germany), pH 2.5 and stored in the dark at room temperature. After staining, the slides were gently rinsed by distilled water and air-dried. At least one thousand spermatozoa were counted under the fluorescence microscope. The spermatozoa showing the green fluorescence was considered normal DNA (double-stranded) while those spermatozoa showed orange or red fluorescence were considered damaged DNA (Figure 1). The results were presented as the proportion of the damage/single stranded DNA per 1,000 counted spermatozoa.

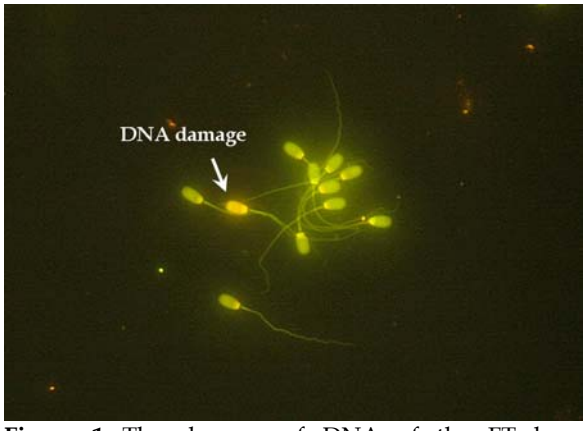

Figure 1 The damage of DNA of the FT boar spermatozoa stained with orange (arrow)

Statistical analysis: The statistical analysis was performed using the Statistical Analysis Systems software package version 9.0 (SAS, 1996). All parameters were evaluated for normality by using the UNIVARIATE procedure option NORMAL PLOT. Pearson's correlation was used to analyze the relationships among the post-thawed sperm qualities and the sperm DNA damage. The semen samples were classified as "good" and "poor" freezability according to post-thawed motility. If the post-thawed motility > 35%, the sperm sample was classified as "good freezability." If post-thawed motility < 35%, the sperm sample was classified as "poor freezability". The sperm DNA damage was compared between good and poor freezability semen by using Student's *t*-test. The influence of boar characteristics and *L*-cysteine supplementation on subjective motility, sperm viability, acrosome integrity and DNA damage were analyzed using the General Linear Model (GLM) procedure of the SAS. The model included boar (16 boars) and concentrations of *L*-cysteine supplementation (0, 5, 10 and 15 mM) as fixed effects. In the results, least significant difference (LSD) test were used to compared least-square means from each class of the factors. $p < 0.05$ was considered as statistically significant.

Results

Descriptive statistics: Subjective motility, sperm viability, acrosome integrity and sperm DNA damage of FT boar semen are demonstrated in Table 1. On average, the sperm DNA damage was 0.5% (Table 1). A negative correlation was found between the sperm DNA damage and the subjective motility (r=-0.21, p=0.03) and the sperm viability (r=-019, p=0.04).

Table 1 Descriptive statistics for sperm parameters measurements of FT boar sperm (n=104)

Sperm Parameters	Mean ± SD	Range
Subjective motility (%)	29.3±13.5	5-55
Sperm viability (%)	45.5±13.8	10-83
Acrosome integrity (%)	39.4±16.1	9-80
Sperm DNA damage (%)	0.5±0.6	0-4

Effect of boar and breed: Among the boars, the sperm DNA damage varied between 0.0% and 4.0% (p=0.05). The sperm DNA damage in the 16 boars is presented in Figure 2. Comparing among breeds, the sperm DNA damage was 0.9%, 0.3% and 0.4% in Duroc, Landrace and Yorkshire boars, respectively.

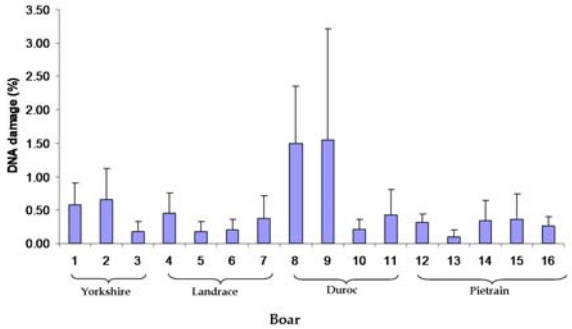

Figure 2 Percentage (Mean±SEM) of the sperm DNA damage for each individual 16 boars (3 Yorkshire, 4 Landrace, 4 Duroc and 5 Pietrain)

Effect of L-cysteine supplementation: Subjective motility, viability, acrosome integrity and the sperm DNA damage were compared among groups of semen with different concentrations of *L*-cysteine supplementation. Subjective motility, viability and

acrosome integrity in the FT boar semen supplemented with 10 mM were higher than those supplemented with 0 and 15 mM (Table 2). The sperm DNA damage in the extenders supplemented with 0, 5, 10 and 15 mM were 0.6%, 0.4%, 0.5% and 0.9%, respectively (Table 2). The sperm DNA damage in the FT boar semen supplemented with 15 mM was significantly higher than those supplemented with 5 mM (p<0.05) (Table 2).

Sperm DNA damage in good and poor freezability sperm: DNA damage in good and poor freezabilty sperm is demonstrated in Table 3. As can be seen from the table, the sperm DNA damage was not significantly different between good and poor freezability semen (p=0.23).

Table 2 Means±standard deviation of progressive motility (%), sperm viability (%), acrosome integrity (%) and sperm DNA damage (%) of FT boar semen between 4 groups (0, 5, 10, 15 mM *L*-cysteine supplementation)

Sperm parameters	*L*-cysteine supplementation (mM)			
	0 (n=41)	5 (n=41)	10 (n=11)	15 (n=11)
Subjective motility	17.6±9.1[a]	28.8±13.9[b]	34.4±13.5[b]	15.8±8.2[a]
Sperm viability	28.9±6.8[a]	44.5±5.2[b]	50.5±5.9[b]	32.5±8.3[a]
Acrosome integrity	18.7±6.5[a]	32.8±10.3[b,c]	41.3±10.2[c]	26.3±11.1[a,b]
Sperm DNA damage	0.6±0.6[a,b]	0.4±0.3 [b]	0.5±0.4[a,b]	0.9±1.2[a]

[a,b,c] values with different superscripts within row differ significantly (p<0.05)

Table 3 Means±standard deviation of subjective motility (%), sperm viability (%), acrosome integrity (%) and sperm DNA damage (%) of good and poor FT boar semen

Sperm parameters	**Good freezability (n=29)**	**Poor freezabilty (n=75)**	***P*-value**
Subjective motility	42.5±7.9	20.4±8.2	<0.001
Sperm viability	53.1±13.9	41.3±15.6	<0.001
Acrosome integrity	48.7±16.2	32.4±17.0	<0.001
Sperm DNA damage	0.34±0.33	0.44±0.59	0.23

Discussion

In the present study, the sperm DNA damage of FT boar semen was relatively low and varied considerably among the boars. The low sperm DNA damage as determined by AO staining in the present study was in agreement with earlier studies in pigs, where other methods, e.g., SCSA and sperm chromatin dispersion test, were used (Rybar et al., 2004; Enciso et al., 2006; Perez-Llano et al., 2006). On the other hand, Evenson et al. (1994) demonstrated that the sperm DNA damage of the boar FT semen as determined by SCSA was as high as 4.5%. In addition, Rybar et al. (2004) found that the sperm DNA damage of boar spermatozoa after the FT process measured by SCSA varied between 1.6-17.6% among individual boars. These findings indicate that the sperm DNA damage of the FT boar semen was relatively low and varied considerably among the boars. The difference on the sperm DNA damage between the present study and earlier studies might be due to the difference in populations of boars, freezing technique and evaluation technique were used.

In the present study, most of the sperm parameters significantly decreased after the FT process, but the sperm DNA damage was not affected. This finding is in agreement with an earlier study in boars that boar sperm DNA was highly tolerant to cryopreservation. The reason is that the boar sperm DNA is highly condensed with nuclear proteins especially protamine-1 in the sperm nucleus (Evenson et al., 1994). In other species, e.g., bulls (Martins et al., 2007; Van der Schans et al., 2000) and dogs (Rota et al., 2005), similar findings have also been demonstrated. On the other hand, Fraser and Strzezek (2007) demonstrated that the sperm DNA damage of the FT boar semen was significantly increased. Likewise, Bochenek et al. (2001) demonstrated that the cryopreservation of the bull semen significantly affected the percentage of sperm DNA damage. In bucks, Peris et al. (2004) found that the cryopreservation also affected the DNA damage of the sperm cells. The reasons behind these contradictory results are unknown, but might be related to the different freezability among different populations. In the present study, ejaculates with a poor freezability had a relatively higher percentage of sperm DNA damage than ejaculates with a good freezability.

The present study demonstrated that the supplement of *L*-cysteine in the freezing extenders up to a concentration of 10 mM did not influence the sperm DNA damage of FT boar semen. It has been demonstrated that oxidative stress is one mechanism which contributes to sperm DNA damage (Bilodeau et al., 2000). The FT process produced a high level of ROS which may induce sperm DNA damage (Barroso et al., 2000; Bilodeau et al., 2000). The finding in those studies indicated that, although *L*-cysteine was known to enhance membrane integrity and motility of the FT spermatozoa, but did not alter the sperm DNA damage of the boar sperm (Kaeoket et al., 2008; Thuwanut et al., 2008). However, in the present study,

the FT boar semen supplemented with *L*-cysteine up to 15 mM resulted in a higher percentage of sperm DNA damage compared to those supplemented with of 5 mM of *L*-cysteine. The reason for this is unknown, but awareness of the too-high concentration of antioxidant supplementation should be addressed. The advantage of the *L*-cysteine supplementation included an improvement of post-thawed motility and membrane integrity of the boar semen (Chanapiwat et al., 2009), while supplementation of L-cysteine with too high concentration may increase DNA damage and subsequently cause infertility.

In the present study, the negative correlations between the sperm DNA damage and other sperm parameters are observed. This is in accordance with the previous studies in boars (Hernandez et al., 2006), bulls (Januskauskas et al., 2001), and bucks (Peris et al., 2004). This indicated that when the sperm motility and viability decreased, the sperm DNA damage increased. The reasons might be due to that the DNA fragmentation might disrupt the vitality of spermatozoa during the FT process. In addition, a high level of sperm DNA damage indicates that the spermatozoa may have a low fertilizing capacity. This could not be determined by using others routine semen evaluation techniques. Therefore, both routine semen evaluation and sperm DNA damage assessment should be carried out in order to effectively determine the fertilizing capacity of the FT boar semen.

In conclusion, the sperm DNA damage of the FT boar spermatozoa was relatively low. No adverse effect of *L*-cysteine supplementation up to 10 mM was found on the sperm DNA damage. Boar characteristic is the most important factor affecting the damage of sperm DNA.

Acknowledgement

A research grant was provided by National Research Council of Thailand (NRCT) and the Thailand Research Fund (IUG5080002). P. Chanapiwat is a grantee of the Ph.D. Program scholarships of the Commission on Higher Education. Language editing of the manuscript has been coordinated by Chula Unisearch, Chulalongkorn University.

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