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Effects of Different Concentrations of *Escherichia coli* and Days of Preservation on Boar Sperm Quality

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ABSTRACT

The objective of this study was to investigate the effect of bacterial contamination on elapsed time after preservation on boar semen. Known numbers of *Escherichia coli* (*E. coli*) were inoculated to freshly ejaculated semen and sperm parameters such as viability, motility, agglutination, acrosome integrity and hypo-osmotic swelling test were performed during 7 days of liquid preservation. Semen samples were prepared using antibiotic free BTS extender and 4 different levels of *E. coli* were treated to semen with following concentrations; 3,000, 5,000, 7,000, 10,000 CFU/ml of sperms. Semen samples were preserved at 17℃ for 7 days in semen storage until analyzed. Aliquots were subjected to measure the sperm viability, motility and agglutination using computer assisted sperm analysis (CASA) system, acrosome integrity was performed using chlortetracycline (CTC) staining method and hypo-osmotic swelling test was performed using hypotonic solution from day 1 (day of semen collection) to 7. Detrimental effects on sperm motility and viability were observed 3 days after preservation at the level of 5,000 CFU/ml ($p<0.05$). Percentage of sperm abnormality was higher ($p<0.05$) in over 5,000 CFU/ml groups. Sperm agglutination rate was also significantly higher ($p<0.05$) in groups of 5,000 and 7,000 CFU/ml. The rate of acrosome reacted sperm was higher as preservation time goes in all the samples but the pattern was clearly higher among *E. coli* contaminated groups ($p<0.05$). The sperm membrane integrity in terms of hypo-osmotic test, *E. coli* affects little compared to other sperm parameters. The deleterious effects observed due to the bacterial contamination in semen suggest that importance of hygiene protocol to minimize the bacterial contamination during semen collection and processing.

(Key words : Bacterial contamination, *E. coli*, Semen quality, Extended boar semen)

INTRODUCTION

The quality of the semen is fundamental for successful swine artificial insemination (AI). AI practice rate is 90% and 1.8 million doses of semen were used annually in Korean swine industry (Kim *et al*., 2011). As AI applications take a huge part in swine industry worldwide, prior studies have been performed to enhance production efficiency (Diemer *et al*., 2003; Althouse *et al*., 2008), farrowing rate and litter size (Maroto Martí *et al*., 2010) by improving semen quality. Poor quality of semen by bacterial contamination during processing from semen collection, preparation, and providing to the breeding farm has been continuously brought the concern.

According to the field investigation in Korea, 94.8% of fresh ejaculates and 19.0% extended semen from AI centers were contaminated and isolation result showed that it belonging to 10 genera of gram negative and 5 genera of gram-positive were isolated (Jung *et al*., 2011). In North America, 13 bacteria isolated from extended boar semen and the majority of contaminants identified in extend semen are gram negative bacteria, with a majority of the contaminants bacteria belonging to the family Enterobacteriaceae (Sone, 1989). In previous study from our lab, number of bacteria were not difference until 3 days of preservation but increased significantly 5 days after preservation (Lee, 2007). One of the most frequently isolated bacteria in porcine semen was *E. coli* (Tamuli *et al*., 1984; Dagnall, 1986; Sone *et al*., 1989; Arredondo *et al*., 2001; Althouse & Lu, 2005). Over-

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growth by contaminant bacteria such as *E. coli* has a deleterious effect on semen quality and longevity (Auroux *et al*., 1991; Kuster and Althouse, 1997; Althouse *et al*., 2000; Althouse and Lu, 2005).

Therefore, the objective of this study was to suggest the proper preservation days of liquid boar semen by investigating the relationship between the bacterial contamination and preservation period on semen quality.

MATERIALS AND METHODS

Semen Collection and Processing

Semen samples from Duroc boars raised in local AI centers were collected. Before each semen collection, boars are thoroughly cleaned and only phases two and three of the ejaculations were collected because they are the sperm rich fraction and to avoid bacterial contamination as well.

Antibiotics free BTS extender was used to dilute the semen. Extended semen samples were transferred to the laboratory using 17℃ semen storage container. Unless otherwise stated, all the chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Bacterial Inoculation

Bacteria used in this study was hemolytic and enteroaggregative *E. coli* heat-sTable enterotoxin 1 (EAST-1)-positive. *E. coli* was cultured on Tryptic Soy Broth (Bacto™) plate until the numbers reached to $\times 10^8$ CFU/mL and then *E. coli* was diluted using 10-step method. Bacterial suspensions of the following initial concentrations were used in the experiment with *E. coli*: 3,000, 5,000, 7,000 and 10,000 CFU ml^{-1}, and a control with extender.

Numbers of Bacteria Measurement

Semen samples from designated AI centers were allocated to 50ml conical tube and centrifuged at 3,000 ×g for 15 min. Supernatant was removed, sediments were diluted using 10-step method and bacterial suspensions were inoculated on plate counting agar (Difco™) and cultured at 37℃ in air for 24 hr and total numbers of bacteria were counted.

Analysis of Sperm Motility

Semen samples were stored at 17℃ for 7 days. The sperm movement characteristics were measured using CASA system determined as Zeng *et al*. (2001). In brief, 1.5 ml semen sample was incubated for 30 min at 37℃ water bath and 10 μl of semen were applied to pre-warmed Makler counting chamber (Sefi-Medical, Israel). Semen samples were analyzed by SAIS II system (Medical Supply Co. Ltd., Korea) connected with CCD camera (Veltek, Korea) on microscope (Olympus, Japan) connected with a warm plate (37℃).

Analysis of Sperm Viability and Abnormality

For sperm viability and abnormality test, fast green FCF (2% v/v) and Eosin B (0.8%, v/v) were solved in PBS and filtered before staining the sperm. Ten micro liters of sperm were applied to the slide and equal volume of dye were added, smeared with cover glass, and dried quickly to avoid live sperm stain as dead. If sperm head stains with dye, it determines as dead. Total of 100 sperm from 25 each of 4 different compartments were counted for both viability and abnormality test.

Analysis of Sperm Acrosome Integrity

Sperm suspension (100 μl) was stained with 2 μl of Hoechst 33258 at dark chamber for 3 min and followed by 3% polyvinyl-pyrolidone(PVP-40) for fixation for 3 min. Stained and fixed spermatozoa were centrifuged at 400×g for 5min and sperm suspension was mixed with chlortetracycline(CTC) at 1:1 ratio for 30 sec. Chlortetracycline (CTC) solution was prepared the day before use by adding 750 μM CTC and 5 mM cysteine to a buffer containing 130 mM NaCl and 20 mM Tris (Trizma base; Sigma) and passed through a 0.22 micron filter (Millex-GV, low solute binding, Millipore SLGV 02565); the pH was adjusted to 7.8. The solution was protected from light by foil and held at 5℃ until time of use when it was brought to room temperature before addition to the spermatozoa. This CTC-Ca21 complex preferentially binds to hydrophobic regions of the cell membrane, resulting in a pattern of membrane staining characteristic of the various transitional phases that the spermatozoa display: F (uncapacitated, acrosome intact), B (capacitated, acrosome intact), and AR (acrosome reacted), as described by Storey (1997).

Hypo-Osmotic Sperm Swelling Test

The hypo-osmotic swelling (HOS) test was performed by mixing semen of centrifuged at 500 ×g for 5 min with BTS solution (75 mOsml/kg hypo-osmotic solution prepared by mixing BTS with distilled water 1: 3 v/v). The mixture was incubated for 30 min at 37℃ in water bath. Then 10 μl of the mixture was placed on a slide and mounted with a cover slip and immediately examined at a magnification of ×400 under a phase contrast microscope. The percentage of reacted sperm (curled tails) and non-reacted sperm (non-curled tails) were assessed by counting a minimum of 100 sperm.

Statistical Analyses

Data were analyzed using the Generalized Linear Model procedure (PROC-GLM) of the Statistical Analysis System (SAS Institute, Cary, NC, USA, 2000). For statistical analyses, however, percentage values were subjected to arcsin transformation before applying unpaired Students *t*-tests to means and standard deviations for each data point. Differences among treatment means were determined by using the Duncan's multiple range tests. A probability of $p<0.05$ was considered statistically significant.

RESULTS

Detrimental effects on sperm motility and viability were observed 5 days after preservation as dose dependent manner at the level of > 5,000 CFU/ml (Table 1 & 2, $p< 0.05$). Percentage of sperm abnormality was higher (Table 3, $p< 0.05$) in over 5,000 CFU/ml groups even d1 of preservation. Sperm agglutination rate was also significantly higher (Table 4, $p< 0.05$) in all groups 5 days after preservation but bacterial contamination groups (> 5,000 CFU/ml) showed higher tendency.

The rate of acrosome reacted sperm were higher as

Table 1. Effect of different concentrations of *E. coli* and days of preservation on sperm motility

Days of preservation	Sperm motility (%) (CFU/ml)				
	Con	3,000	5,000	7,000	10,000
1	75.5^{A}	78.6^{A}	71.9^{A}	77.8^{A}	73.8^{A}
3	73.5^{ABa}	73.3^{ABa}	68.2^{Aab}	71.2^{Ba}	64.6^{Bb}
5	69.1^{Ba}	68.7^{Ba}	62.4^{Bb}	62.1^{Cb}	55.3^{Cc}
7	61.2^{Ca}	59.9^{Cab}	53.1^{Ccd}	54.4^{Dbc}	47.8^{Dd}

$^{a\sim c}$ Means with different superscripts in the same column differ significantly ($p<0.05$).
$^{A\sim D}$ Means with different superscripts in the same row differ significantly ($p<0.05$).

Table 2. Effect of different concentrations of *E. coli* and days of preservation on sperm viability

Days of preservation	Sperm viability (%) (CFU/ml)				
	Con	3,000	5,000	7,000	10,000
1	77.8	72.3^{A}	61.8^{A}	65.0	63.5
3	73.8	70.2^{A}	62.0^{A}	62.0	58.8
5	62.3	56.3^{B}	53.8^{B}	55.2	51.5
7	58.5	53.8^{B}	49.5^{B}	51.2	48.0

^{A,B} Means with different superscripts in the same row differ significantly ($p<0.05$).

Table 3. Effect of different concentrations of *E. coli* and days of preservation on sperm abnormality

Days of preservation	Sperm abnormality (%) (CFU/ml)				
	C	3,000	5,000	7,000	10,000
1	15.3^{b}	16.3^{b}	26.5^{a}	24.0^{a}	27.3^{aC}
3	19.0	20.3	27.5	27.0	27.3^{C}
5	21.8	28.5	31.3	35.7	35.8^{B}
7	27.3	28.3	36.3	39.5	44.8^{A}

$^{a\sim c}$ Means with different superscripts in the same column differ significantly ($p<0.05$).
$^{A\sim C}$ Means with different superscripts in the same row differ significantly ($p<0.05$).

Table 4. Effect of different concentrations of *E. coli* and days of preservation on sperm agglutination

Days of preservation	Sperm agglutination score[1] (CFU/ml)				
	C	3,000	5,000	7,000	10,000
1	0.0^{B}	0.0^{B}	0.0^{C}	0.0^{D}	0.0^{D}
3	0.0^{Bc}	0.0^{Bc}	0.5^{bC}	1.0^{aC}	1.0^{aC}
5	1.0^{Ac}	1.0^{Ac}	$1.5A^{Bb}$	2.0^{aB}	2.0^{aB}
7	1.3^{Ab}	1.3^{Ab}	2.0^{Ab}	3.0^{Aa}	3.0^{Aa}

Agglutination score[1] : No agglutination:0, 5~10% Agglutination: 1, 10~15% Agglutination:2, 15~12% Agglutination:3
$^{a\sim c}$ Means with different superscripts in the same column differ significantly ($p<0.05$).
$^{A\sim C}$ Means with different superscripts in the same row differ significantly ($p<0.05$).

Table 5. Effect of different concentrations of *E. coli* and days of preservation on sperm acrosome integrity

Days of preservation	F pattern rate[1] (CFU/ml)				
	C	3,000	5,000	7,000	10,000
1	83.4^{A}	79.7^{A}	76.8	71.4^{A}	68.5
3	80.4^{AB}	77.0^{A}	74.5	67.9^{A}	64.9
5	74.0^{BC}	70.7^{A}	67.3	60.7^{AB}	57.4
7	67.5^{aC}	59.5^{Bb}	52.8^{bc}	48.0^{Bcd}	43.2^{d}

[1] (F) uncapacitated, acrosome intact
$^{a\sim d}$ Means with different superscripts in the same column differ significantly ($p<0.05$).
$^{A\sim C}$ Means with different superscripts in the same row differ significantly ($p<0.05$).

Table 6. Effect of different concentrations of *E. coli* and days of preservation on hypo-osmotic sperm swelling test

Days of preservation	Sperm swollen (%) (CFU/ml)				
	C	3,000	5,000	7,000	10,000
1	98.4^{A}	95.9^{A}	97.0^{A}	95.0^{A}	91.5
3	97.0^{Aa}	94.0^{Aab}	94.0^{Aab}	91.4^{ABbc}	88.9^{c}
5	95.4^{Ba}	90.9^{Bab}	93.5^{Aa}	90.4^{Bab}	86.0^{b}
7	92.0^{Ca}	87.7^{Cab}	88.0^{Bab}	85.0^{Cb}	78.7^{c}

$^{a\sim c}$ Means with different superscripts in the same column differ significantly ($p<0.05$).
$^{A\sim C}$ Means with different superscripts in the same row differ significantly ($p<0.05$).

preservation time goes in all the samples but the pattern was more clear among *E. coli* contaminated groups (Table 5, $p<0.05$). The sperm membrane integrity in terms of hypo-osmotic test, *E. coli* affects little compared to other sperm parameters (Table 6). The deleterious effects observed due to the bacterial contamination in semen suggest that importance of hygiene protocol to minimize the bacterial contamination during semen collection and processing.

DISCUSSION

For successful swine AI, sperm motility and viability were important parameters. Flowers (1996) reported that motility was considered as optimal when at least 60% of sperm cells were motile. In our study, motility in 3×10^3 CFU/ml group and in 5×10^3 CFU/ml group on day 5 of preservation were declined, indicating *E. coli* contamination affected sperm motility and resulted in suboptimal sperm performance. Not only motility but also sperm agglutination was affected by *E. coli* contamination (Kaur *et al*., 1986; Arredondo *et al*., 2001; Kozdrowski *et al*., 2005). Our data showed that sperm motility was decreased (Table 1) and sperm agglutination was increased (Table 4) in *E. coli* contamination group, therefore, we speculated that *E. coli* adhered to sperm surface reduced the sperm motility. The spermicidal effects of *E. coli* were also confirmed. Office of International Epizooties published the standard that 5.013×10^3 CFU/ml as a limit for the bacterial contamination of semen samples (OIE, 2001) and Maroto Martí *et al*. (2010) reported that when semen used for AI with *E. coli* contamination above a threshold value of 3.5×10^3 CFU/ml, there is a significant reduction in litter size. According to Bussalleu *et al*. (2011), significant adverse effects on porcine sperm quality were observed from 10^3 CFU/ml *E. coli* in the experiment performed at 37℃. In our work, significant adverse effects were observed from 5×10^3 CFU/ml *E. coli* in the experiment done at 17℃. Since incubation temperature influences bacterial growth, incubation at 37℃ could have a greater influence on poor quality of semen by bacterial contamination than incubation at 17℃. Our data showed that *E. coli* contamination had concentration-dependent detrimental effects on extended porcine semen quality especially in sperm viability, motility, abnormality and agglutination. Sperm acrosome integrity (Table 5) and sperm cell membrane integrity (HOST test, Table 6) were little affected by *E. coli* contamination compared to other semen parameters. It was recommended that periodic evaluation of bacteria in semen, hygiene and sanitation efforts should be considered during semen collection and processing.

In conclusion, based on data obtained from this study, hygiene protocol should be applied to minimize the bacterial contamination (> 5×10^3 CFU/ml) during semen collection and processing and it is recommended that semen should be used before 5 days of liquid preservation to avoid detrimental effects of bacterial contamination on semen quality.

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