Effects of *Escherichia coli* Contamination on Extended Porcine Semen Parameters

Kyoung Min So[1], Soo Jin Sa[1], Hyo Jin Kim[1], Ki Hwa Chung[2], Byeong Yeal Jung[3], Jung Ho Son[4] and In Cheul Kim[1,†]

[1]*National Institute of Animal Science, RDA, Seonghwan 330-801, Korea*

[2]*Gyeongnam National University of Science and Technology, Jinju 660-758, Korea*

[3]*Animal Disease Diagnostic Division, Animal, Plant and Fisheries Quarantine & Inspection Agency, Anyang 430-757, Korea*

[4]*Noah Biotech. Inc., Cheonan 331-858, Korea*

ABSTRACT

The objective of this study was to determine the effects of *E. coli* isolated from porcine semen on sperm viability, motility, and semen pH. Semen samples were prepared using commercial extender, Seminark^{Pro} (Noahbio Tech, Korea) that did not contain antibiotics. And 4 different levels of *E. coli* were artificially innoculated to semen with following concentrations; 4,000 of sperms with 1 of *E. coli* (T1), 400 with 1 (T2), 40 with 1 (T3), and 4 with 1 (T4). Semen samples were preserved at 17℃ for 5 days in semen storage box until analyzed by flowcytometer. Aliquots were subjected to measure the sperm viability (Live/Dead® stain), motility (mitochondrial function), and semen acidity (pH) from day 0 (day of semen collection) to day 5. Sperm motility and viability were significantly decreased (p<0.05) on day 0 (4 hrs after preservation at 17℃) in T3 and T4 compared to control groups and were significantly decreased (p<0.05) in all groups from day 3. Sample pH was acidic in T3 (6.90～6.86) and T4 (6.86～6.65) from day 3 to day 5 (p<0.05). On the other hand, sample pH was maintained 7.0～7.1 in control, T1, and T2 during the experimental period. Sperm motility and viability were significantly decreased from day 0 to day 5 compared to control in samples contaminated with *E. coli* above a value of 40:1 (20×10^6 sperm cells/ml : 5×10^5 cfu/ml). Even on day 1 in T4 and on day 3 in T3, semen pH was acidic probably due to the acidification of dead spermatozoa. These results suggest that *E. coli* contamination has a concentration-dependent detrimental effect on extended porcine semen quality.

(Key words : Extended porcine semen, *E. coli*, Semen quality, Flowcytometery, Semen parameters)

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

Therefore, this study was carried out to determine the effects of *E. coli* in extended porcine semen

MATERIALS AND METHODS

Animals

Two Large Yorkshire and one Landrace boars raised and selected in National Institute of Animal Science were used. Boars were average of 18 months of age and about 170 kg body weight. Boars were housed individually to a combination of concrete and plastic flooring pen and fed according to the guideline for breeder boars of National Institute of Animal Science.

Semen Preparation

Sperm-rich fraction of ejaculates, collected by gloved-

† Corresponding author : Phone: +82-41-580-3440, E-mail: kickic@korea.kr

hand technique from three mature boars of proven fertility were extended in equal volumes of commercial extender, Seminark^{Pro} (Noahbio Tech, Korea) that did not contain antibiotics.

To standardize the test and to establish exact spermatozoa/bacteia ratios, the sperm suspension was diluted to a concentration of 20 million ml^{-1} with extender. The prepared suspension was split into five fractions. Four samples were inoculated with *E. coli* isolated from a fresh porcine semen and one sample used as a control that did not contain any bacterial contamination. All samples were stored at 17℃ for 5 days.

Bacterial Isolation

E. coli was isolated from a fresh porcine semen collected at dedicated boar studs. It was hemolytic and enteroaggregative *E. coli* heat-stable enterotoxin 1(EAST-1)-positive.

Bacterial suspensions of the following initial concentrations were used in the experiment with *E. coli* : 200 million, 20 million, 2 million, 200,000 bacteria ml^{-1}, and a control with extender. The initial spermatozoa/bacteria ratios were 4:1 (T4), 40:1 (T3), 400:1 (T2) and 4,000:1 (T1).

Analysis of Sperm Parameters

For the viability measurement, cells were stained with two dyes (dual staining), SYBR-14 (green fluorescence emitted and collected through a 530/30 BP filter) and propidium iodide (PI, red fluorescence emitted and collected through a 610/20 BP filter). SYBR-14 was a membrane-permeant DNA dye and tended to be accumulated in live cells. In contrast, Propidium Iodide (PI) was a dye specifically accumulated in the nucleus of dead cell. Thus, cells emitting green fluorescence were regarded as live cells and cells emitting red fluorescence were regarded as dead cells.

Mitochondrial function was analyzed to estimate the motility of sperm which was energized by mitochondria. The quality of mitochondria could be accessed using the difference of potential across mitochondrial membrane. In cells with healthy mitochondria, the lipophilic cationic dye tended to be accumulated at the inside of mitochondria. To estimate mitochondrial function, cells were stained with a lipophilic cationic dye, Rhodamine-123 and a dead cell-detecting PI stain to enhance the contrast. Subsequently, stained cells were characterized using a second flowcytometry. Green fluorescence of Rhodamine-123 that passed through a 575/26 BP filter and red fluorescence of PI that passed through 610/20 BP were measured and compared. Small particles and debris from semen sample were eliminated using threshold function to minimize the spill over of fluorescence signal.

The pH of each sample was measured using a pH meter (Orion, Thermo Scientific, Beverly, MA). To avoid the cross contamination, pH electrode was thoroughly washed with distilled water before and after each measurement.

Table 1. Concentration of spermatozoa and *E. coli* in the each group

Group	Concentration of spermatozoa (cells/ml)	Concentration of *E. coli* (cfu/ml)
C	20×10^6	0
T1	20×10^6	5×10^3
T2	20×10^6	5×10^4
T3	20×10^6	5×10^5
T4	20×10^6	5×10^6

Statistical analysis

Data were analyzed using the Generalized Linear Model procedure (PROC-GLM) of the Statistical Analysis System(SAS Institute, Cary, NC, USA). 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

Sperm Motility, Viability and Evaluation of Sperm Agglutination

Sperm motility was significantly decreased ($p<0.05$) even on day 0 (approximately 4 hrs after preservation at 17℃) in T3 and T4 groups compared to control group (Table 2). In all groups, sperm motility was gradually decreased as preservation time increased but the decline pattern was more drastic in T3 and T4 groups from day 3 ($p<0.05$) compared to control group.

Sperm viability also showed similar pattern as motility (Table 3). Sperm viability was below 60% in T4 group on day 5, indicating *E. coli* contamination as many as 5×10^6 cfu/ml affected semen quality and was not suitable for AI after 3 days of preservation. Both sperm motility and viability were significantly decreased from day 0 to day 5 compared to control in samples contaminated with *E. coli* above a range of 5×10^5～5×10^6 cfu/ml (Table 2, 3). Mostly, sperm agglutination in T3 and T4 groups was observed during the experimental period (Fig. 1), suggesting strong relationship with *E. coli* contamination.

Semen pH

The pH of semen samples was acidic in T3 (6.90～6.86) from day 3 to day 5 and T4 (6.86～6.65) from day 1 to day 5 (Table 4, $p<0.05$). On the other hand,

Table 2. Effect of *E. coli* contamination on motility (mitochondrial function) of porcine spermatozoa

Elapsed time (day)	C	T1	T2	T3	T4
0	83.4±0.8^{a,A}	81.8±3.1^{a,A}	80.4±2.2^{a,A}	75.1±2.4^{b,A}	69.5±2.8^{c,A}
1	80.3±1.2^{a,AB}	79.1±2.3^{a,A}	76.6±2.2^{a,AB}	70.1±3.5^{b,AB}	63.9±5.5^{c,A}
3	77.1±1.9^{a,BC}	73.7±3.2^{ab,AB}	70.3±3.7^{ab,BC}	65.0±5.0^{bc,BC}	58.3±8.1^{c,AB}
5	73.2±4.0^{a,C}	68.1±7.4^{ab,B}	65.2±6.5^{ab,C}	58.1±7.8^{bc,C}	48.6±8.1^{c,B}

$^{a\sim c}$ Means with different superscripts in the same row differ significantly (p<0.05).
$^{A\sim C}$ Means with different superscripts in the same column differ significantly (p<0.05).
Values are expressed as Mean±SD.

Table 3. Effect of *E. coli* contamination on viability of porcine spermatozoa

Elapsed time (day)	C	T1	T2	T3	T4
0	86.3±2.1^{a,A}	86.0±3.0^{ab,A}	84.0±3.2^{ab,A}	80.5±3.8^{b,A}	74.5±2.7^{c,A}
1	83.7±1.9^{a,AB}	83.8±2.7^{a,A}	81.4±1.4^{a,A}	75.9±1.6^{b,AB}	70.1±3.2^{c,AB}
3	80.8±1.4^{a,BC}	78.5±2.1^{a,B}	76.2±2.2^{ab,B}	70.9±2.5^{bc,B}	65.7±6.4^{c,BC}
5	78.6±1.2^{a,C}	75.7±2.3^{ab,B}	71.8±1.9^{b,C}	65.0±2.9^{c,C}	57.8±4.5^{d,C}

$^{a\sim d}$ Means with different superscripts in the same row differ significantly (p<0.05).
$^{A\sim C}$ Means with different superscripts in the same column differ significantly (p<0.05).
Values are expressed as Mean±SD.

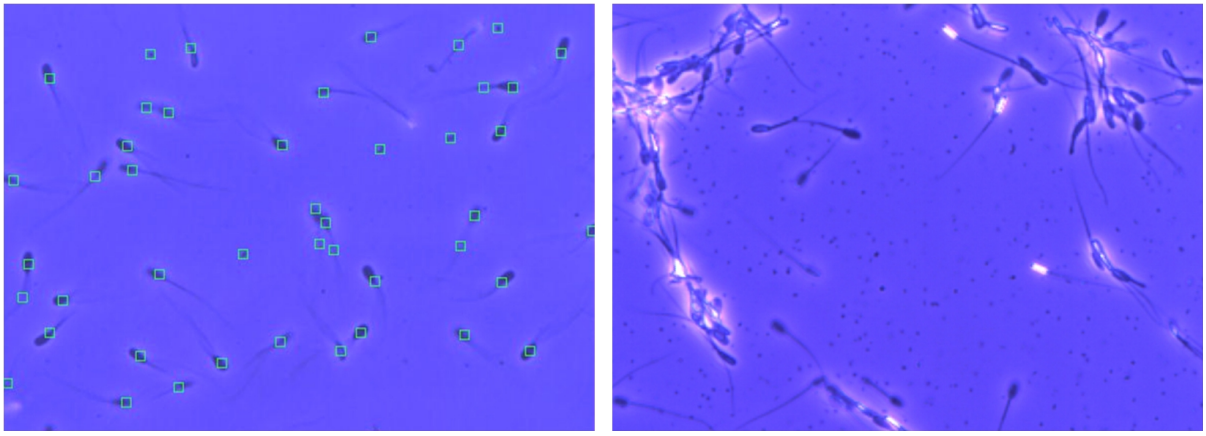

Fig. 1. Normal (left, Control) and agglutinated (right, T3) images in semen samples captured from a CASA(Computer-Assisted Semen Analysis, Medical Supply, Korea) video monitor.

Table 4. Effect of *E. coli* contamination on pH of extended porcine semen

Elapsed time (day)	C	T1	T2	T3	T4
0	7.01±0.02^{A}	7.00±0.01^{A}	6.99±0.01^{A}	7.00±0.03^{A}	6.97±0.02^{A}
1	7.02±0.03^{a,A}	7.02±0.01^{a,A}	7.04±0.01^{a,B}	7.03±0.01^{a,A}	6.86±0.03^{b,B}
3	7.05±0.02^{a,A}	7.02±0.02^{a,A}	7.04±0.02^{a,B}	6.90±0.02^{b,B}	6.63±0.04^{c,C}
5	7.13±0.04^{a,B}	7.12±0.08^{a,B}	7.10±0.04^{a,C}	6.86±0.07^{b,B}	6.65±0.08^{c,C}

$^{a\sim c}$ Means with different superscripts in the same row differ significantly (p<0.05).
$^{A\sim C}$ Means with different superscripts in the same column differ significantly p<0.05).
Values are expressed as Mean±SD.

the pH was maintained 7.0~7.1 in control, T1, and T2 during the experimental period. Even on day 1 in T4 and on day 3 in T3, semen pH was acidic probably due to the acidification of dead spermatozoa and lipid peroxidation by reactive oxygen species during preservation in the presence of *E. coli*.

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 T4 group (5×10^6 cfu/ml) on day 3 and in T3 group (5×10^5 cfu/ml) on day 5 of preservation were declined to 58%, indicating *E. coli* contamination affected sperm motility and resulted in sub-optimal 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 confirmed that *E. coli* adhered to sperm surface reduced the sperm motility (Table 2) and agglutinated sperm (Fig. 1). The spermicidal effects of *E. coli* were also confirmed (Table 3).

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 Martín *et al*. (2010) reported that when semen used for AI is contaminated with *E. coli* 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 5×10^5 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 and it was recommended that periodic evaluation of bacteria in semen, hygiene and sanitation efforts should be considered during semen collection. Also, selection of effective antibiotics in extender was considered for providing high quality porcine semen.

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