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Antioxidant Supplementation Enhances the Porcine Semen Preservation Capacity

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ABSTRACT

Preservation of liquid semen is an important factor for breeding management in swine industry. Oxidative stress of spermatozoa during liquid preservation has a detrimental effect on sperm quality and decreases fertility. Objective of this study was to determine the effect of antioxidant, Quercetin, on capability of porcine liquid semen preservation. Freshly collected porcine semen from boars (n=3), having proven fertility was counted, diluted to 3×10^7/mL and divided into 5 different semen extenders. Aliquots of diluted semen with different extenders were subjected to measure the pH, motility, viability and sperm DNA structure status on elapse time after preservation for 10 days. For the first 3 days, semen preserved in all 5 different extenders maintained their initial pH and either gradually decreased or increased thereafter, indicating lipid peroxidation has started. Sperm motility (r=0.52, p=0.01) and viability (r=0.55, p=0.03) had positive correlation with semen pH. Sperm motility was maintained well (p<0.05) in especially 2 extenders containing Tris and antioxidant compared to other extenders, suggesting both Tris and antioxidant worked as pH regulator and had beneficial effects on sperm characteristic during preservation. Sperm DNA structure status accessed by sperm chromatin structure assay on elapsed time after preservation, tended to be higher in semen preserved without antioxidant. Taken together, addition of antioxidant to extender prevents the sperm from oxidative stress during storage in mechanism by which antioxidant slows the lipid peroxidation, and thus reduced the reactive oxygen species in preserved porcine semen resulted in maintaining semen pH, sperm motility and viability for 7~10 days.

(Key words : Boar spermatozoa, Porcine semen extender, Anti oxidant, Sperm preservation)

INTRODUCTION

As artificial insemination has been widely practiced in swine industry, necessity of boar semen extender's preserving capacity has been increased. For higher fertility rate following AI, maintaining of sperm motility, viability, and sperm DNA status during preservation period are important factors for successful swine breeding management. Since the first report on lipid peroxidation in mammalian spermatozoa (Lardy and Phillips. 1941), it has been reported that lipid peroxidation by reactive oxygen species (ROS) could be a detrimental factor due to a high concentration of unsaturated fatty acids and low level of antioxidants in spermatozoa (Alvarez & Storey, 1983; Storey 1997, Cerolini *et al*., 2000; 2001). Oxidative damage to mitochondrial membrane structure may be a major important factor to explain the impaired fertility and motility of preserved human spermatozoa (Cummings *et al*., 1994). During liquid preservation, boar semen undergoes several changes such as motility, viability, DNA structure, and semen pH. Among those changes, lipid peroxidation is most vulnerable stress for boar spermatozoa due to little or no protective enzymes and relatively low antioxidant activity in boar seminal plasma (Brezezinska-Slevdozinska *et al*., 1995). Chatterjee and Gagnon (2001) reported excessive reactive oxygen species during boar semen preservation has been associated with sperm quality. The formation and membrane lipid peroxidation have been recognized as problems for sperm survival and fertility (Guthrie & Welch, 2012).

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Although, in earlier studies, there were several attempts to protect the sperm from oxidation stress in adding antioxidants such as vitamin C, vitamin E, glutathione, cysteine, or hypotaurine to extender and ROS level on sperm cryopreservation (Yeste *et al*., 2013; 2014), the effect was not clear and ambiguous.

In this study, we conducted the experiment to determine the effect of antioxidant, Quercetin, on porcine liquid semen preservation capacity. During preservation period, sperm viability, sperm motility, sperm DNA structure were accessed by sperm chromatin assay (SCSA), and semen pH was monitored to see if beneficial effect of antioxidant on boar liquid semen storage at 17℃.

MATERIALS AND METHODS

Reagent

Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich Co (St . Louis MO, USA).

Animals

Three Duroc boras raised in "D" AI center in Cheonan were assigned randomly. Animals were housed individually to solid steel rod flooring pen and fed according to the guide line of NRC.

Semen Collection

Sperm-rich fraction of ejaculates collected by gloved-hand technique from three mature boars of proven fertility were extended in equal volumes of 5 different extenders (Androhep™, and BTS, Minitüb, Tiefenbach Germany; Ace, Beijing China; Modena, SGI, USA; Seminark™, Noah Biotech, Korea) and stored at 17℃ throughout the experimental period.

Flowcytometry

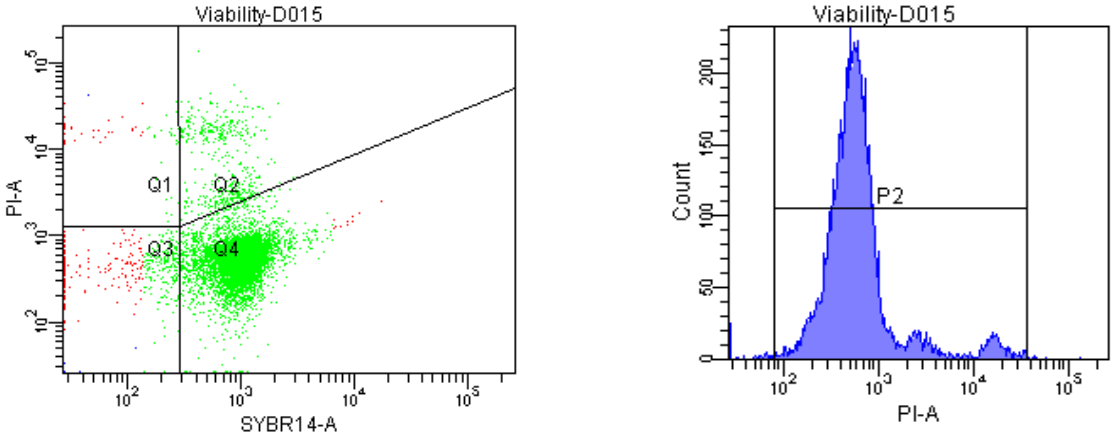
For the viability test, cells were stained with two dyes, SYBR-14 (green fluorescence emitted and collected through a 530/30 BP filter) and propidium iodide (PI, red fluorescence emitted and collected through a 610/20 BP filter). SYBR-14 is a membrane-permeant DNA dye and tends to be accumulated in live cells. In contrast, PI is 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. In the second experiments, mitochondrial function was analyzed to estimate the motility of sperm which is energized by mitochondria. The quality of mitochondria can be accessed using the difference of potential across mitochondrial membrane. In cells with healthy mitochondria, the lipophilic cationic dye tends to be accumulated at the inside of mitochondria. To estimate mitochondria 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. The percentage of sperms containing fragmented DNA was determined by sperm chromatin structure assay (SCSA; Boe-Hansen *et al.,* 2005). Acridine Orange (AO) dye was used to stain both normal double stranded DNA (green fluorescence emitted and collected by 530/30 BP filter) and damaged single stranded DNA (red fluorescence emitted and collected by 610/20 BP filter). Small particles and debris from semen sample were eliminated using threshold function to minimize the spill-over of fluorescence signal.

Statistical Analysis

One way analysis of Variance (ANOVA) was performed and mean of three replicated experiment's data was compared by Duncan's new multiple range test for each preservation time point. Correlation coefficient was also carried out among parameters. In all studies, statistical significance was determined at $p<0.05$.

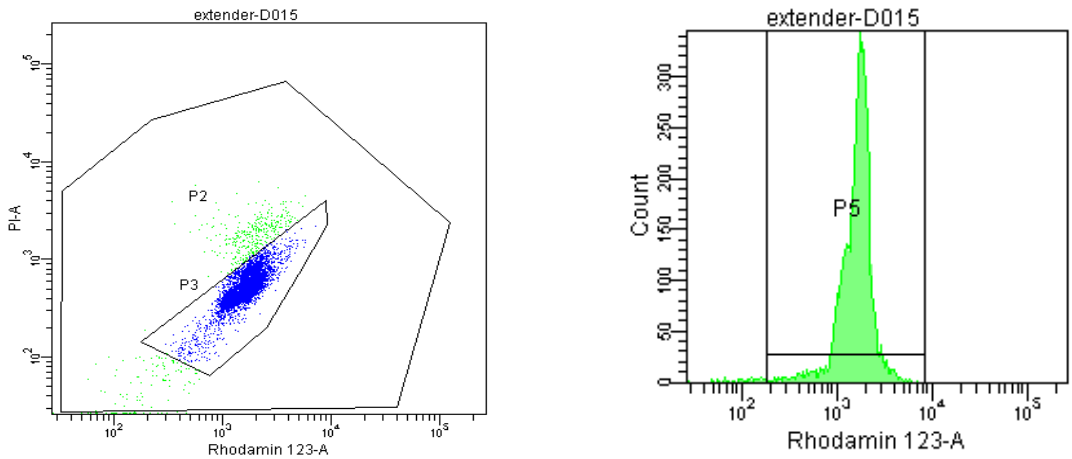
RESULTS

In sperm viability test, no significant difference was detected among groups for the first three days (ranged 76.4 ±0.94～84.9±1.67, $p>0.05$, Fig. 1) of preservation. However, beginning of 4 days after preservation, sperm from extenders without antioxidant showed a tendency of lower viability and there was a significant difference among groups from 5 to 10 days ($p<0.05$, Fig. 1) after preservation, indicating antioxidant slowed the lipid peroxidation and reduced oxidative stress during extended period of preservation. In mitochondrial function test as a sperm motility measurement (Fig. 2), it showed a similar pattern as sperm viability and there was a positive correlation between two parameters (motility, $r=0.52$, $p=0.01$; viability, $r=0.55$, $p=0.03$). Changes of pH in semen from 4 days after preservation showed either increased or decreased ($p<0.05$, Fig. 3) depending on the extenders containing either Tris (Modena) and antioxidants (Seminark ™), suggesting Tris works as pH stabilizer and antioxidant helps the semen maintain the pH from harmful reactive oxygen species in oxidation process while sperm preservation. Although, DNA fragmentation was not statistically different on first 3 days after preservation, there was a clear tendency of DNA damage being increased in semen preserved without antioxidant as preservation time extended ($p<0.05$, Fig. 4).



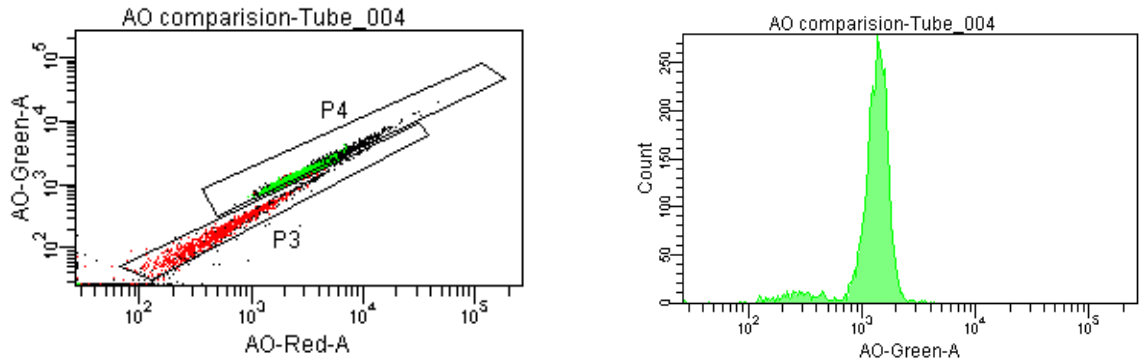
A. Percentage of sperm viability

Dot plot shows dead and live sperm populations that were identified by dual staining. Q1 population represents the dead sperm; Q2 population represents moribund sperm; Q3 population represents debris; Q4 rep population represents live sperm.



B. Percentage of sperm mitochondrial function

Mitochondrial function was analyzed to estimate the motility of sperm.
Dot plot shows the relative portion of sperm mitochondria that were identified by dual staining. Fluorescence intensity for the amount of mitochondria in live sperm is indicated on histogram.



C. Percentage of sperm DNA fragmentation

Histogram shows shifting of green florescence to red florescence

Fig. 1. Flowcytometry for the measurements of sperm viability, motility and DNA fragmentation.

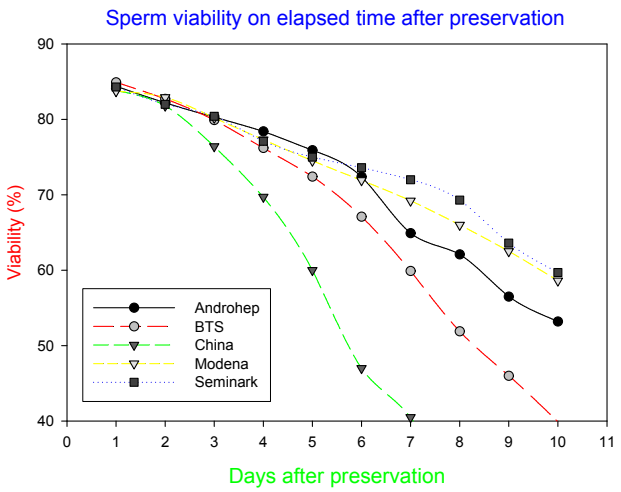


Fig. 2. Sperm viability on elapsed time after liquid preservation (n=3) in the five extenders during a period of 10 days.

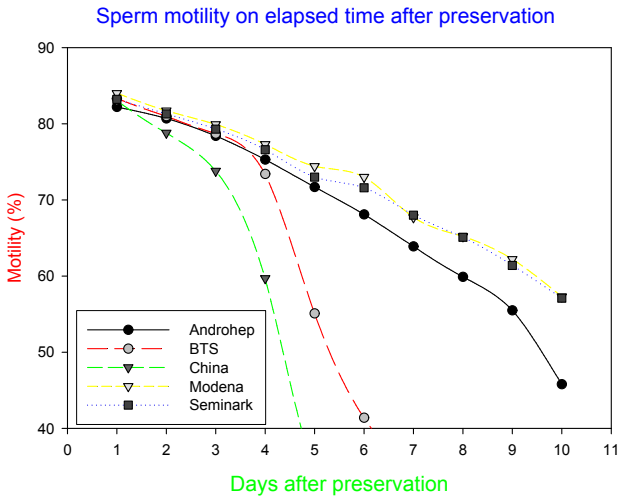


Fig. 3. Sperm motility on elapsed time after liquid preservation (n=3) in the five extenders during a period of 10 days.

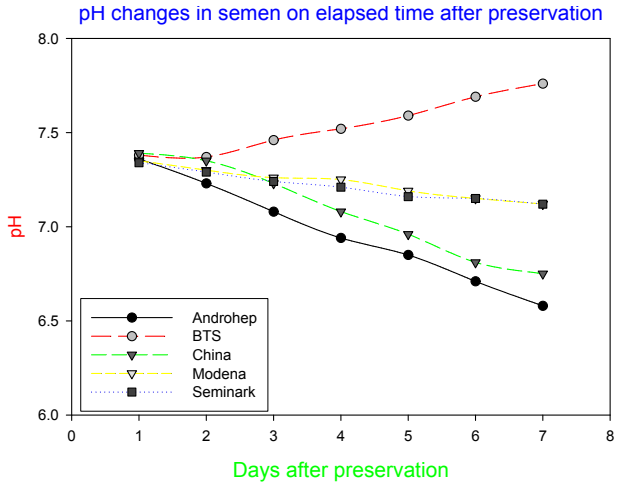


Fig. 4. pH changes in semen on elapsed time after liquid preservation (n=3) in the five extenders during a period of 7 days.

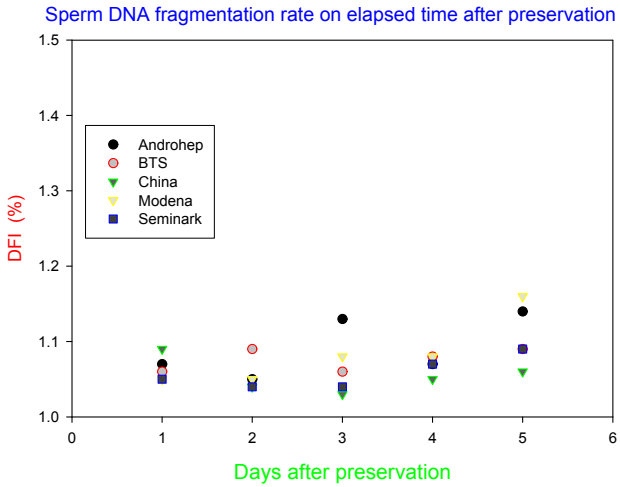


Fig. 5. Sperm DNA structure status on elapsed time after liquid preservation (n=3) in the five extenders during a period of 5 days.

DISCUSSION

Quercetin used in this study for antioxidative agent is a flavonoid widely distributed in nature (Fischer, 1997) and they scavenge damaging particles in the body known as free radicals, which damage cell membranes, tamper with DNA, and even cause cell death. Antioxidants can neutralize free radicals and may reduce or even help prevent some of the damage they cause (Boots *et al*., 2008). Result of the present study, revealed that decrease in sperm functions as preservation time goes more than 4 days associated with an increase in oxidative stress. Decreased sperm functions with an increase in lipid peroxidation level have been reported in cattle (O'Flaherty *et al*., 1999) and buffalo (Kumaresan *et al*., 2006). In the present study, addition of 5 mM of Quercetin as an antioxidant into commercially available extender, improved the sperm functions.

In this study we also evaluated the extenders in commonly utilized in the field. Huo *et al*. (2002) performed an interesting study on the quality of semen diluted in different media (Androhep, Zorlesco, BTS and Kiev) and stored for up to 15 days. Their findings indicated that sperm viability and mitochondrial activity exceeded 50% after 13 days of storage in long-term diluents (Androhep and Zorlesco). In subsequent evaluations of short-term diluents, BTS was found to be more efficient than Modena in terms of fertility (Aalbers *et al*., 1983; Blichfeldt *et al*., 1988). When we compared short-term diluents no significant differences in viability and motility and sperm DNA integrity

were generally noted in the first 3～4 days of storage, although the long-term extenders could be used for up to 7 days (Fig. 1 & 2). Similar findings were described by Johnson *et al*. (1988), when they compared BTS to MR-A and Modena. The choice of diluents should depend on its proposed use. When it is planned that the time from semen collection to use will be less than 3～4 days, the most rational choice would be to use short-term diluents. When the semen dose needs to be preserved for more than 5 days before insemination for specific reasons, a long-term semen extender would be to use.

In conclusion, based on the findings from this study revealed that antioxidant, Quercetin, prevents the sperm from oxidative stress during liquid preservation and would help in maintaining the sperm quality of preserved boar semen for up to 7 days.

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