

## Sperm Fertility of Transgenic Boar Harboring hEPO Gene is Decreased

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### ABSTRACT

This study was conducted to compare the reproduction ability of the wild type boar and recombinant human erythropoietin (hEPO) transgenic boar semen. Ejaculated boar semen was analyzed by flow cytometry, Elisa and IVF methods. In experiment 1, flow cytometric analysis showed that the live sperm ratio of transgenic boar sperm significantly lower ( $P<0.05$ ) than that of wild type boar after incubation at 20, 22, 24 and 26 hr. In experiment 2, the presence and levels of various cytokines (IL-6, IL-10 and TNF- $\alpha$ ) to related animal reproduction in the seminal and blood plasma were examined using specific enzyme immunoassay. There was no significant difference between both groups. In experiment 3, the fertilizing capacity and developmental ability of both boar sperm were compared. The transgenic boar sperm had a significantly low capacity of penetration, sperm-zona binding, embryo development, and blastocyst formation compared to wild type sperm ( $P<0.05$ ). These results suggest that transgenic boar sperm harboring hEPO gene has low sperm viability than wild type boar, and it is a reason to decrease of fertility and litter size.

(Key words : hEPO transgenic pig, Semen, Flow cytometry, Cytokine, IVF)

### INTRODUCTION

Human erythropoietin (hEPO), a 166 amino acid mature form of a glycoprotein hormone, is related with the differentiation and proliferation of erythroid cells (Erslev, 1953; Jelkmann, 1992). It could be synthesized as a secreted form in the liver during the fetal period and in the kidney after birth (Lacombe *et al.*, 1988). National Livestock Research Institute reported that transgenic pig producing erythropoietin mammary gland by microinjection technique. Presently there are already sixty pigs produced from the transgenic pig (Park *et al.*, 2006). However, they have problems in their reproductive function. The wild type female Landrace had an average litter size of 11.4 mated to wild type boar, but hEPO transgenic boar crossed with wild type sows had an average litter size of only 6.33.

Garner and colleagues (1995) developed a method for combination of flow cytometry and fluorescence microscopy. This method was commercialized and available as LIVE/DEAD® Sperm Viability Kit (molecular probes) that has yielded reliable results with both bovine and goat sperm and has also been tested on human sperm. This kit provides a fluorescence-based

assay to analyzing the viability and fertilizing potential of sperm. The membrane-permeate nucleic acid stain included in this kit also provide researchers with a valuable tool for labeling and staining live sperm, facilitating analysis of their motility and number in semen samples. The adaptation of flow cytometry to sperm analysis began when it was used for measuring their DNA content (Evenson *et al.*, 1980) and its application to semen analysis has gradually increased over the last 10~15 years.

The concentration of cytokines interleukins 6, 10 (IL-6 and IL-10) and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) were investigated using species specific enzyme immunoassay in the seminal plasma of various species. Concentrations of cytokines in semen showed their interactions with spermatozoa. For example, Yamauchi-Takahara *et al.* (1995) reported that IL-6 could cause membrane damage of spermatozoa that might decrease the sperm motility. And IL-10 in seminal plasma also related to male reproduction. An increase of IL-10 reflects the state of immunity and infection of the reproductive system, and influences sperm functions (Zhang *et al.*, 2004). TNF- $\alpha$  also decreased the motility of human spermatozoa when the spermatozoa was exposed to high concentration of TNF- $\alpha$  *in vitro* (Ei-

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serman *et al.*, 1989) and increased the production of reactive oxygen species (Rajasekaran *et al.*, 1995). Gruschwitz *et al.* (1996) reported that variation in the concentrations of seminal cytokines and their soluble receptors in pathological conditions, especially when urological infections or genital inflammatory responses are processed.

IVF tests are the most suitable for assessing overall sperm function during fertilization. Harrison (1997) reported that the binding and penetration of the zona pellucida were one of the most important barriers the spermatozoa must overcome in the fertilization process. Various researchers reported that developmental competence and viability of IVM-IVF oocytes after IVC have been confirmed (Mattioli *et al.*, 1989, Yoshida *et al.*, 1990).

This study evaluated the fertilizing capacity from hEPO transgenic pig and its developmental ability in subsequent embryos via sperm-ZP binding assay after *in vitro* culture. The number of apoptotic cells of embryo that produced by IVF with hEPO transgenic pig sperm was counted. Therefore, this study also was conducted to investigate that the causes of the low fertility by the hEPO transgenic boar.

## MATERIALS AND METHODS

### Preparation of Semen Samples

Semen was obtained from three transgenic and three non-transgenic boars by gloved hand technique and filtered to remove the gel fractions with gauze. The samples were diluted with Beltsville TS (BTS) (Johnson *et al.*, 1988); semen extender, to a sperm concentration of  $10 \times 10^6$  sperm/ml. Thereafter each diluted sample containing  $5 \times 10^6$  sperm were pipetted into 5 ml round bottom tubes fixed at 36°C.

### Sperm Viability Analysis

An SYBR®-14 capable of staining only live sperm nuclei by fluorescing bright green color when excited at 488 nm, was used in combination with propidium iodide (PI). The PI, membrane-impermeable dye detected stained membrane damaged sperm when excited at 543 nm. These dyes were provided by LIVE/DEAD® Sperm Viability Kit (Molecular Probes, Eugene, OR). Both dyes were dissolved and diluted according to the manufacturer's instruction. Both SYBR-14 dye and PI can be excited with visible wavelength light. When bound to DNA, the fluorescence emission maxima of these dyes are 516 nm and 617 nm, respectively. Quantitative data on the fluorescently stained sperm populations were analyzed through fluorescent-activated cell sorting on a BD FACS Vantage SETM System (Becton

Dickinson Canada, Inc., ON). A total of 10,000 sperm per sample were analyzed for the log of their fluorescence and analysis of flow data was performed using the Cell Quest Pro software of the FacScan unit (Becton Dickinson Canada, Inc., ON).

### Analysis of Cytokine Concentration

Cytokine concentrations (IL-6, IL-10 and TNF- $\alpha$ ) were determined in semen samples from six boar by specific porcine enzyme immunoassay (R&D Systems, Inc., Minneapolis, MN, USA) that was using the immunometric sandwich technique. The semen samples were centrifuged at 360  $\times$ g for 10 min and blood samples were centrifuged at 2,000  $\times$ g for 20 min to obtain the seminal plasma and blood plasma incubated with microtiter strips coated with mouse monoclonal antibody specific for porcine IL-6, IL-10 or TNF- $\alpha$ . After a washing procedure to remove the unbound antibody, the bound IL-6, IL-10 or TNF- $\alpha$  were subjected to a second incubation step with specific polyclonal antibodies against porcine IL-6, 10 or TNF- $\alpha$  conjugated horseradish peroxidase (HRP). Thereafter excess IL-6, IL-10 or TNF- $\alpha$  conjugated HRP were detected in a third reaction step by the addition of two color reagents. A color reaction was developed in each case by incubation with a substrate solution containing stabilized hydrogen peroxidase and a stabilized chromogen (tetramethylbenzidine). The enzymatic reaction was terminated by the addition of a diluted hydrochloric acid solution and the absorbance was measured at 450 nm after addition of terminal solution at 40 min. The samples were analyzed in duplicate and the standard curve was run with each assay with Microplate Manager™ (Bio-Rad Laboratories, Inc., CA). The standard dilutions of IL-6 were from 0 to 2,500 pg/ml, IL-10 from 0 to 2,000 pg/ml and TNF- $\alpha$  from 0 to 1,500 pg/ml.

### Culture Media

All chemicals used in this study were purchased from Sigma (St. Louis, MO) unless otherwise stated. Cumulus oocyte complexes were washed three times with TL-HEPES supplemented 0.1% polyvinyl alcohol (PVA) (Abeydeera *et al.*, 2000) and the medium used for oocyte maturation was tissue culture medium (TCM) 199 (Gibco, Grand Island, NY) supplemented with 0.57 mM cysteine, 0.1% PVA, 10 ng/ml epidermal growth factor (EGF), 75  $\mu$ g/ml potassium penicillin G, 50  $\mu$ g/ml streptomycin sulfate, 0.5  $\mu$ g/ml LH and 0.5  $\mu$ g/ml FSH. The basic medium used for IVF was essentially the same as that used by Abeydeera and Day *et al.* (1998). This medium designated as mTBM, consists of 113.1 mM sodium chloride, 3 mM potassium chloride, 7.5 mM calcium chloride dihydrate, 20 mM Tris (crystallized free base; Fisher Scientific, Fairlawn, NJ), 11 mM glucose, 5 mM sodium pyruvate,

and no antibiotics. The embryo culture medium for IVF embryos was NCSU 23 containing 0.4% BSA (fraction V; A 8022).

#### ***In Vitro* Maturation and Fertilization of Porcine Oocytes**

Ovaries were obtained from prepubertal gilts at a local slaughterhouse and brought to the laboratory in 0.9% NaCl containing 75 µg/ml potassium penicillin G and 50 µg/ml streptomycin sulfate maintained at 25–30 °C. Oocytes were extracted from follicular fluid with oocytes of different medium size of follicles (3–6 mm in diameter) with an gauge 18 needle attached to a 10 ml disposable syringe. Oocytes surrounded by a compact cumulus mass and having evenly granulated cytoplasm were washed with PVA-TL-HEPES. Thereafter 50 oocytes were washed three times in maturation medium and transferred into each well of a Nunc 4-well multidish (Nunc, Roskilde, Denmark) containing 0.5 ml of pre-equilibrated maturation medium covered with warm mineral oil and cultured for 44 hr with 5% aerial CO<sub>2</sub> at 39°C. After the completion of IVM culture, cumulus cells were removed with 0.1% hyaluronidase in Medium 199 (GibcoTM, Gland island, NY) containing 25 mM HEPES buffer and washed three times with pre-equilibrated IVF medium containing 2.5 mM caffeine and 0.4% BSA (fraction V; A 7888). The sperm from each group was washed three times by centrifugation at 1,900 g for 3 min in Dulbecco's PBS (Gibco, Grand Island, NY) supplemented with 0.1% BSA, 75 µg/ml potassium penicillin G, and 50 µg/ml streptomycin sulfate. Concentrations of sperm were adjusted to 1–2×10<sup>4</sup> motile washed sperm that were quantified by Markler counting chamber (Sefi-medical Instrument Ltd) and were co-incubated with 20–25 oocytes in a 100 µl droplet of mTBM medium. After 6 hr incubation with sperm, fertilized oocytes were transferred into embryo culture medium.

#### ***In Vitro* Sperm-ZP Binding Test**

Sperm quality of hEPO were evaluated for their binding ability to cumulus-free, ZP-intact mature oocytes using the procedure described by Hogan *et al.* (1994) known as *in vitro* sperm-ZP binding assay (Tanphaichitr *et al.*, 1994). To isolate loosely bound sperm from oocytes after 30 min IVF, it was pipetted through a drawn glass micro capillary with a bore diameter of 300 µm. The number of bound spermatozoa per oocyte was counted after Hoechst 33342 staining and the cytoplasm was ruptured with cover glass under an inverted microscope at ×200 magnification. Statistical differences were analyzed in the numbers of sperm bound per oocyte in both treatments. The three replicates of each treatment were also statistically determined.

#### **TUNEL Assay**

Blastocysts were fixed in 3.7% paraformaldehyde in PBS for 1 hr at room temperature. The fixed blastocysts were washed twice in PBS containing 0.3% polyvinylpyrrolidone (PBS-PVP). The fixed blastocysts were permeabilized by 0.5% Triton X-100 in PBS-PVP for 30 min at room temperature. Fixed embryos were incubated in terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL *in situ* cell death detection kit, TMR red; Roche, Mannheim, Germany) reaction medium for 1 hr at 39°C in dark, and mounted on slides with anti-fading gel mount (Molecular Probes). DNA strand breaks identification and labelled with free 3'-OH termini containing modified nucleotides assessed DNA fragmentation in blastomere. Whole-mount embryos were examined with a fluorescent microscope (Olympus, Tokyo, Japan) by a detection to determine the number of apoptotic nuclei.

#### **Statistical Analysis**

The statistical significance among each group in each experiment was determined by using General Linear Models Procedure, *t*-test and ANOVA analysis.

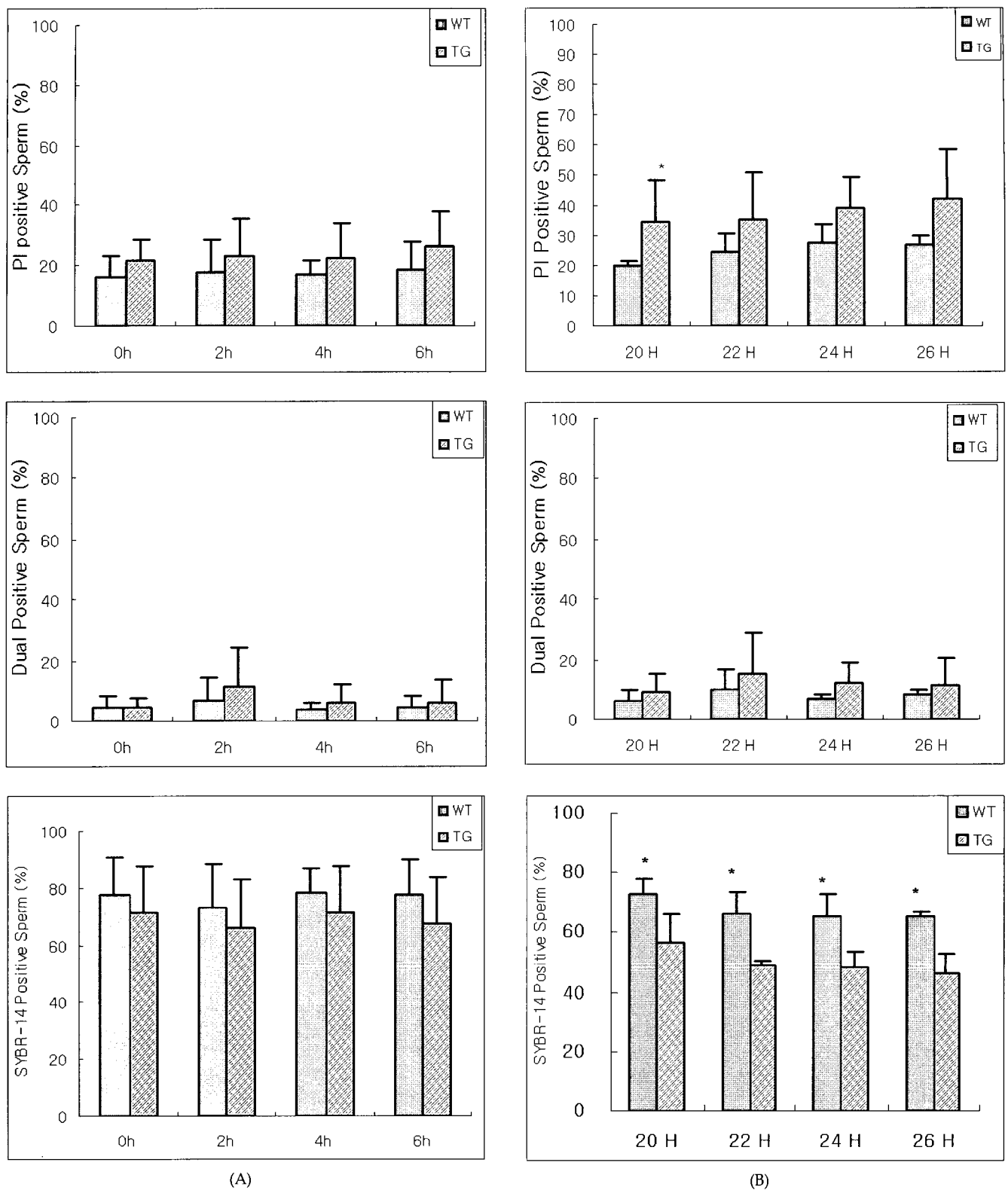
## **RESULTS**

#### **Sperm Viability Test of Both Boar Sperm during Various Times after Ejaculation**

The sperm were stained with SYBR-14 and PI to assess the differences in sperm viability among three boars of each group using flow cytometry. The relative proportions of sperm staining with PI, SYBR-14, or both PI and SYBR-14 (dual stained) for the three pigs from both groups is presented in Fig. 1. The sperm viability after ejaculation during 2 hr interval using flow cytometry showed individual differences of each boar in both groups. However, sperm viability was unaffected in both groups that used different staining dyes (Fig. 3a). Fig. 3b displays both sperm viability after 20, 22, 24 and 26 hr incubation at 38.5°C, 5% CO<sub>2</sub> and 100% humidity. At 20 hr incubation, a lower sperm viability count was observed in TG compared to WT using PI positive stain (*P*<0.05). And SYBR-14 positive, a significant difference was observed in the TG compared to WT at each 2 hr interval (*P*<0.05).

#### **Quantitative Analysis of Cytokines Concentrations of Seminal and Blood Plasma from Both Boar Types**

The levels of interleukin 6, interleukin 10, and tumor necrosis factor-α were analyzed with ELISA Kits in seminal and blood plasma from WT and TG boar (Fig.



**Fig. 1.** Histograms showing the three major populations of sperm that were identified by dual DNA staining with SYBR-14 and PI. The relative proportions of PI-, dual-, and SYBR-14-positive sperms at 3 replicates from 3 individual boars; (a) sperm viability at different post-ejaculation periods. (b) sperm viability after incubation at 38.5°C, 5% CO<sub>2</sub> and 100% humidity (\* $P < 0.05$ ).

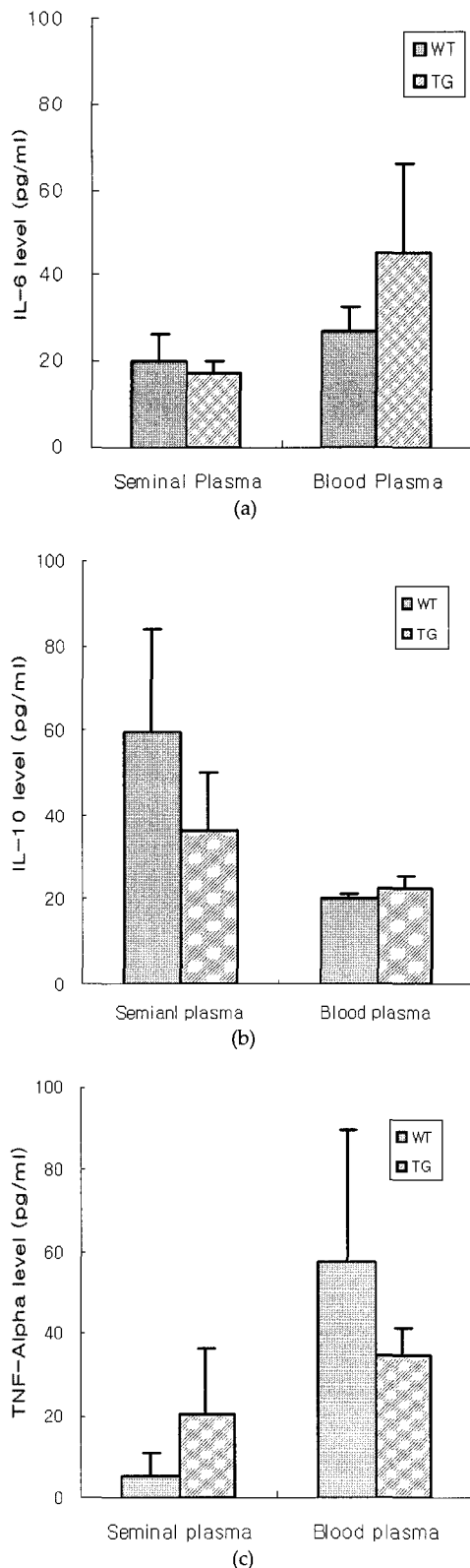


Fig. 2. The levels of IL-6, IL-10, and TNF- $\alpha$  in seminal and blood plasma on both boar. Each bar represents the average value of 3 boars. (a) IL-6, (b) IL-10, (c) TNF- $\alpha$  levels in seminal and blood plasma.

2). The levels of IL-6 in the WT boar seminal and blood plasma were  $20.1 \pm 6.5$  and  $27.3 \pm 5.5$  pg/ml, respectively while in TG boar were  $17.1 \pm 3.0$  and  $45.1 \pm 21.0$  pg/ml respectively. The levels of IL-10 in the WT boar seminal and blood plasma were  $59.4 \pm 24.8$  and  $20.1 \pm 1.25$  pg/ml and TG boar were  $36.2 \pm 14.1$  and  $22.8 \pm 2.64$  pg/ml. WT boar had a higher level of IL-6, IL-10 in seminal plasma than TG boar. Conversely, WT boar had lower level of IL-6, IL-10 in blood plasma than TG. The levels of TNF- $\alpha$  in the WT boar seminal and blood plasma were  $5.50 \pm 5.53$  and  $57.51 \pm 32.2$  pg/ml respectively, while that in the TG boar were  $20.65 \pm 16.04$  and  $34.74 \pm 6.32$  pg/ml, respectively. The levels of TNF- $\alpha$  in WT boar seminal plasma were lower than TG boar, but the levels of TG boar in blood plasma was higher than WT boar. Finally, no significant differences were obtained in all the above parameters.

#### Fertilizing and Developmental Ability of Both Boar Sperm

Following the procedures of flow cytometry and ELISA, sperm-ZP binding assay and IVF were carried out to compare fertilizing and developmental abilities of both types of boar semen. There were more spermatozoa bound to the zona pellucida of the oocyte with  $72.86 \pm 14.72$  in WT sperm as compared to the TG sperm which were only  $42.98 \pm 4.77$  ( $P < 0.01$ ) (Fig. 3). The result of *in vitro* fertilization using both types of sperm is summarized in Table 1. The WT sperm had lower ability to cleave the oocyte than the TG boar (59.6 vs. 44.5%,  $P < 0.01$ ). There was a significant number of blastocyst fertilized by WT sperm at 22.5% than that of the TG sperm that was only 13.4% ( $P < 0.01$ ).

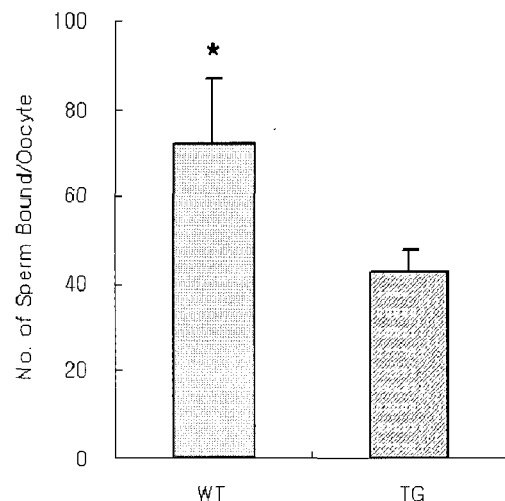


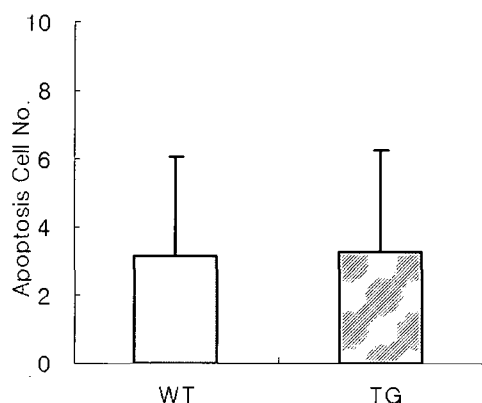
Fig. 3. Sperm binding assay. Data from each experiment were expressed as the mean  $\pm$  SD of sperm bound per oocyte from three replicates of gamete co-incubates on each experimental day (\* $P < 0.01$ ).

**Table 1.** Developmental competence of oocytes fertilized with WT and TG boar sperm

Sperm	No. of oocyte	Cleaved (%) <sup>*</sup>	Blastocyst (%) <sup>*</sup>
WT	258	158 (59.6) <sup>a</sup>	57 (22.5) <sup>a</sup>
TG	251	95 (44.5) <sup>b</sup>	29 (13.4) <sup>b</sup>

<sup>\*</sup> The rates of cleavage and blastocyst were determined at 48 and 168 hr after IVC.

<sup>a,b</sup> Values with different superscripts in the same column significantly differ ( $P < 0.01$ ).

**Fig. 4.** TUNEL assay on *in vitro* fertilized blastocysts from WT and TG spermatozoa.

#### TUNEL Assay on Blastocysts following IVF with WT and TG Boar Spermatozoa

Apoptotic cell numbers of *in vitro* fertilized blastocysts were counted. The number of apoptotic cells in WT sperm IVF blastocyst was  $3.18 \pm 2.86$  and that of TG was  $3.29 \pm 2.94$  (Fig. 4). There was no difference between WT and TG sperms.

## DISCUSSION

This study was conducted to compare semen quality of WT and TG using flow cytometry, ELISA and IVF procedures. There have many studies conducted already to produce transgenic animal such as mice and rabbit harboring erythropoietin using WAP promoter (Burdon *et al.*, 1991; Li and Rosen, 1994; Paleyanda *et al.*, 1994; Limonta *et al.*, 1995; Wall *et al.*, 1996). However negative physiological symptoms such as hyperemia, blood hyperviscosity and polydipsia were found in hEPO-secreted rabbits with pathological abnormalities such as heart hypertrophy, increased hematopoiesis in the bone marrow, and enlarged kidney and bladder. These symptoms were also found in the erythrocytemia in humans.

Sperm viability test by staining of nucleic acid has fewer variables than enzyme-based stain and sperm

DNA is believed to be more suitable for cellular target due to its stainability and uniformity (Garner *et al.*, 1996). Many researchers developed the staining techniques for characterization of spermatozoa using combination of fluorescent dyes and flow cytometry (Gillan *et al.*, 2005). Garner (1995) reported that a combination of fluorescent dyes (SYBR-14 and PI) and flow cytometry was a useful method to assess sperm viability. This method was adapted for analysis of viability of hEPO TG spermatozoa in this study. There was no significant difference in 0, 2, 4 and 6 hr on PI-, dual-, SYBR-14-stained sperm between spermatozoa of TG and WT boar. The results of staining after 20 hr showed that SYBR-14 and PI positively stained sperm were significantly different between WT and TG sperms. Although, for examine of the viability of WT and TG spermatozoa, the sperm was incubated at 38°C for 8, 10, 12, 14, 16 and 18 hr, the differences in both boar sperm viability could be expected based on time factor.

Various cytokines were known to be involved in the regulation of the reproductive system (Bookfor *et al.*, 1991; Skinner *et al.*, 1991). One of the cytokines, IL-6, was one of the pre-inflammatory cytokines that was secreted by various cell types such as macrophages, leukocytes, endothelial cells and fibroblasts. Previous reports suggest that higher level of IL-6 was observed to have negative correlation with sperm motility in humans and bulls; high levels of IL-6 in semen resulted to lower sperm motility (Kelly *et al.*, 1995; Vera *et al.*, 2003). Moreover IL-10 is known as an anti-inflammatory cytokine and tends to suppress inflammation and promote tissue repair and regeneration. The levels of IL-6 and IL-10 were reversely correlated to each other. Furthermore, reverse correlation between the level of IL-6 and TNF- $\alpha$  was observed in seminal plasma of bulls (Vera *et al.*, 2003). The results of this study of hEPO boar seminal plasma, the levels of the cytokines concentration of IL-6 and TNF- $\alpha$  in seminal and blood plasma showed similar patterns of negative correlation in Vera's report (2003). The close relationship was reported among the levels of TNF- $\alpha$  and of IL-10 in the semen of bulls (Vera *et al.*, 2003). In a previous study, Zhu *et al.*, (2004) reported that IL-6 can induce inflammation and its level in blood plasma had

a range of 20 to 40 pg/ml. However, at the present study had a higher level at  $45.1 \pm 21.0$  pg/ml in the TG blood plasma while that of the WT blood plasma was  $27.3 \pm 5.5$  pg/ml. This results postulated that hEPO was over expressed in TG boar induced more inflammatory reaction in tissues than WT boar. It has been postulated that due to the elevated concentration of IL-10 present in the semen of healthy individuals (Vera *et al.*, 2003), its function in the male genital tract is to maintain immunological balance and avoid rejection of the spermatozoa. Mechanical circulatory assist devices (MCADs) support has been correlated with elevated plasma levels of inflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$ , and IL-6, which were independently found to inhibit erythropoietin-induced erythrocyte (RBC) maturation (Pierce *et al.*, 2005). Majority of hEPO TG pigs has symptoms such as polycythemia vera, accompanied with high concentration of erythrocytes indicating low TNF- $\alpha$  levels in blood plasma of hEPO TG boar. Therefore the relation of *in vivo* TNF- $\alpha$  levels and spermatogenesis should be investigated in the future.

Cytokines levels did not showed significant differences in terms of sperm binding ability in the oocyte using IVF, TUNEL assay for both the WT and TG groups. Both boar spermatozoa were evaluated for their ability to bind to cumulus-free, ZP-intact *in vitro* matured oocytes using the *in vitro* sperm-ZP binding assay. This method depicts the fertilizing ability of spermatozoa. The result of this experiment showed that higher number of WT boar spermatozoa (30%) were bounded to the oocyte than TG spermatozoa ( $P < 0.01$ ), indicating that TG boar spermatozoa has lesser number of binding receptors to oocyte. Based on the above result, the TG spermatozoa have a weaker capacity to fertilize an oocyte than the WT. These results suggest that TG sperm have lower developmental ability up to blastocyst stage. To evaluate the embryo developmental competence, the TUNEL assay was experimented. Apoptosis occurs during pre-implantation embryo development in both *in-vivo* and *in-vitro* produced species. Also it may contribute to embryonic loss (Levy *et al.*, 2001, Feugang *et al.*, 2002, Gjorret *et al.*, 2002). In this study, the TUNEL assay resulted in no significant difference in number of apoptotic cells for both types of boar sperm based on blastocyst formation through IVF procedure ( $3.18 \pm 2.86$  vs.  $3.29 \pm 2.94$ ), suggest that both types of boar sperm did not affect the developmental capacity of oocyte for 7 days.

In conclusion, wild type and transgenic boar sperms are comparable in terms of sperm viability after a day of incubation. Cytokines in seminal and blood plasma levels did not show significant differences in both semen, but TG sperm has a significantly low zona-binding ability. Finally, the low fertility coming from sows mated by TG boar can be attributed to hEPO gene of the transgenic sperm that affected the repro-

ductive disorder as polycythemia vera phenomenon according to red blood cell increased.

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