P-ISSN 2586-9000
E-ISSN 2586-9027
Homepage : https://tci-thaijo.org/index.php/SciTechAsia
Science & Technology Asia
Vol. 26 No.1 January - March 2021
Page: [216-223]
Original research article

Transforming Growth Factor Beta1 in Porcine Seminal Plasma on Characteristic of Sperm and Reproductive Efficiency in Sows

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Received 20 November 2019; Received in revised form 21 October 2020
Accepted 26 October 2020; Available online 16 March 2021

ABSTRACT

Transforming growth factor-β1(TGF-β1) in porcine seminal plasma (SP) and TGF-β1 concentrations under different storage periods at 16°C were investigated. The effects of adding TGF-β1, to boar semen, on sperm characteristics and reproductive performance in sows were also examined. TGF-β1 in boar SP was measured by enzyme-linked immunosorbent assay (ELISA) technique. Mean concentrations of TGF-β1 in individual ejaculated SP samples demonstrated a wide variation at 8.94-588.24 pg/mL with a decrease in concentration of 46.10±5.52% after 72 hours. The supplementation of TGF-β1 to fresh semen at 0, 1000, and 2000 pg/100 mL was performed in order to evaluate its effect on sperm characteristics. Results showed a significant difference at 24 hours in the following characteristics: viability, progressive, and acrosome integrity, viability, velocity average path (VAP), and velocity curvilinear (VCL) at 72 hours ($P<0.05$). Acrosome integrity, mitochondrial function, motility, velocity average path (VAP), velocity straight line (VSL), and velocity curvilinear (VCL) were not significantly difference at 24 hours ($P>0.05$). Mitochondrial function, progressive motility, and VSL were not significantly different at 72 hours ($P>0.05$). In addition, the supplementation of TGF-β1 at 0, 500, and 1000 pg/100 mL of fresh boar semen for artificial insemination in sows did not result in a significant difference in conception rate, farrowing rate, litter size, or total born alive ($P>0.05$).

Keywords: Transforming growth factor beta1 (TGF-β1); Sperm characteristics; Semen quality; Reproductive efficiency.

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doi: 10.14456/scitechasia.2021.20

1. Introduction

Manipulation of the female reproductive tract to improve reproductive efficiency in terms of conception rate, farrowing rate, and total born is a major process in the swine industry. Embryo mortality is an important factor affecting the swine production industry. It is found that the embryo mortality rate is up to 40%. In addition, embryo loss is found between 0-30 days after matting [1]. The embryo survival rate depends on many important factors, such as maternal and seminal plasma (SP).

Previous studies have demonstrated that SP contains many substances, including proteins, hormones, growth factors, and others that are important for the reproductive processes [2, 3]. These protect and maintain sperm traveling through the reproductive tract. Moreover, SP components stimulate myometrium layer contractions which transports sperm to the uterotubal junction to begin the cascade of capacitation and embryo development [4]. In addition, molecular SP components associate in key events related to sperm and female reproductive functions to promote a healthy outcome of potential offspring [5].

Currently, researchers are interested in studying the balance of immune function in the reproductive tract; the importance of SP in relation to the mechanisms of immune function was studied. It was discovered that cytokines, an SP component, stimulate a cascade of leucocyte events. It contributes to preparation of the endometrium for embryonic implantation. According to previous reports, it was found that the key component which stimulates this process is transforming growth factor beta (TGF-β) proteins.

SP is a source of TGF-β's and it has been suggested that the TGF-β protein family plays an important role in embryo survival. This molecular cascade stimulates an immune response reducing the resistance of antigens as well as triggering the release of embryonic cytokines, which improves embryo survival rate.

The TGF-β family of cytokines is produced by the accessory gland and is an important component of SP [6]. In mice, TGF-β's act as a stimulating factor and transmit signals stimulating the immune system to respond to the antigen (sperm), which causes the endometrium to be prepared for embryo development [7]. In addition to SP signaling, TGF-β's impact reproductive fertility both in males and females [8,9]. In mammals, there are 3 TGF-β isoforms: TGF-β1, β2, and β3 [10]. In pigs, TGF-β1 has been implicated in playing a key role in the maternal immune response in the endometrium, and the remodeling of reproductive tissue for embryo development [9]. However, TGF-β1 has many other roles such as regulating cell proliferation, apoptosis, and differentiation in the reproductive tract [10]. Previous studies demonstrated that concentrations of TGF-β1 in boar SP were found to be less than 20% of the mean value of each boar. In addition, the concentrations of TGF-β1 and TGF-β2 were different in fractions of boar ejaculate [11].

Currently, artificial insemination is increasing in the pig industry. Throughout this process, there are many factors that may contribute to a decrease in swine fertility. Artificial insemination programs in commercial piggeries collect semen and then dilute it 10-50 fold before use. The extensive dilution of semen decreases concentrations of important seminal components, such as TGF-β's, and can lead to poorer reproductive outcomes [12]. In addition, previous studies have demonstrated that concentrations of TGF-β1 in ejaculate have high variability between individual boars, and that there is a decrease in the concentration of these proteins over time while in storage. The aim of the experiment was to observe the effect of TGF-β1 on semen quality and reproductive efficiency in sows. Our study investigated first, the concentration of TGF-β1 in boar SP and its correlations with semen

characteristics; second, the effect of supplemental TGF-β1, in fresh semen, on semen quality; and third, the effect of supplemental TGF-β1, in fresh semen, on reproductive performance in sows.

2. Materials and Methods

2.1 Animals

The semen samples were collected at Swine Commercial Artificial Insemination Center (Khon Kaen province, Thailand). Forty boars (Duroc, 1.6-2 years old) were placed in individual pens, using randomized assignment, held in housing using an evaporative cooling system, were provided clean water, and were fed ad libitum with automatic feeding. Feed volumes were adjusted to semen production requirements. The semen samples included in the experiments were required to have a sufficient motility (≥ 80%), normal morphology (≥80%), and sufficient sperm viability (≥80%), with a volume of ≥100 ml per ejaculate. The boars were used for routine boar collection for artificial insemination. The experiment protocol was approved by the Animal Ethics Committee of Khon Kaen University (Record No. IACUC- KKU-80/62).

Thirty sows were progenies of a two-way cross between Large White x Landrace. The sows were treated in 3 groups, separated by age, parity, and weight. The sows in the experiment were approximately 2-3 years old, 150 kg., and had a parity of 2-4. The sows were weaning to return to estrous intervals approximately from 3 to 7 days after weaning. A standing reflex was detected in weaned sows twice daily (06.00 a.m. and 18.00 p.m.).

2.2 Semen diluter

All chemicals in the study were obtained from Sigma Aldrich Chemical (St. Louis, MO, USA). The semen diluter used Beltsville Thawing Solution (BTS) composed of glucose (37g; 3.7%), sodium citrate (6g; 0.6%), EDTA (1.25g; 0.125%), sodium bicarbonate (1.25g; 0.125%), and potassium chloride (0.75g; 0.075%), in deionized water (1000 mL).

2.3 Semen collection and preparation

Semen collection was performed using the glove in hand technique; after collection, semen evaluation was carried out under a microscope with computer software (CASA; Dynamic swine sperm; Optika). Only samples with a total sperm motility and sperm normal morphology at more than 80% were used in experiments.

TGF-β1 immunoassay (experiment 1: Semen samples from 16 boars were collected and preparation of the SP was done by centrifugation at 1200 g at 4°C for 20 minutes. Concentrations of TGF-β1 were determined by enzyme-linked immunosorbent assay (ELISA) (ELISA-Kits, MB100B, TGF-β1; Quantikine porcine immunoassays, RαD System Europe, Abingdon, UK). The optical density reading was 450 nm to 540 nm. The SP samples were stored at 16°C for the measurement of TGF-β1 concentrations at day 1 and 3.

Experiment 2 and artificial insemination in experiment 3: 24 boar semen samples were collected. The fresh semen was divided into 100 mL centrifuge tubes and diluted 1:1 with BTS and then supplemented with different concentrations of TGF-β1 (0, 1000, 2000 pg/dose in experiment 2) at 35°C. In experiment 3, semen was also supplemented with different concentrations of TGF-β1 (90, 500, 1000 pg/dose). The semen samples were then kept at 25-28°C and immediately transported to the laboratory 30 minutes after collection for further processing. At the laboratory, the boar semen was further diluted with TGF-β1 supplemented in BTS for a final spermatozoa concentration of $3x10^6$ cells in a total volume of 100 mL. Then, the samples of each experimental group were placed in a refrigerator at 16°C and stored for 24 and 72 hours. The temperature was monitored constantly by a digital thermometer.

2.4 Semen evaluation

Motility: fresh semen total motility was evaluated using a microscope at 400x magnification (Halminton Thorne Biosciences IVOS, Version 12 TOX IVOS, Beverly, USA). The samples were pre-warmed in a water bath at 37°C and placed on an analyzer with a stage warmer to prepare for evaluation. The semen samples were measured in 5 fields and were evaluated per sample and 2000 cells per field were evaluated. The parameters measured were: total, progressive motility, curvilinear velocity (VCL; μm/s), linear velocity (VSL; μm/s), and velocity average path (VAP; μm/s).

Acrosome integrity, viability, and mitochondrial function: acrosome normality, viability, and sperm mitochondrial membranes were evaluated by fluorescent multiple staining (FMS). Briefly, 150 μl sperm suspension was mixed with 2 μl propidium iodide (PI; 0.5 mg/mL; Live/dead® sperm viability kit L7011 Invitrogen USA), 5 μl fluorescein isothiocyanate labeled peanut (Arachis hypogaea), agglutinin (FITCPNA), and 2 μl 5,5',6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolyl-carbocyanine iodide (JC-1). Then, the fresh semen samples were incubated in a no light chamber at room temperature for 10 minutes. For fluorimetric assessment, small drops of suspension were placed on a clean slide, covered with a cover slip, and evaluated immediately by fluorescence microscope with a triple filter, following a set of UV-2E/C (excitation 340-380 nm and emission 435-485 nm), and G-2E/C (excitation 540-525 nm and emission 605-655 nm) and B-25/C (excitation 465-495 nm and emission 515-555 nm) at 400x magnification. Sperm that showed a colorless head with red mitochondria were classified as living and undamaged, with high membrane potential.

2.5 Artificial insemination

Experiment 3 used 30 sows (parity 2-4) and all groups had sows of parity 2 (3 sows), parity 3 (3 sows) and parity 3 (4 sows). Five boars were used for artificial insemination (Duroc,1.6-2 years old). The fresh boar semen was diluted using BTS and was separated into 3 groups and supplemented with TGF-β1 (0, 500 and 1000 pg/ dose) for artificial insemination in the sows. The detection of estrus was accomplished by mature boars following nose- to- nose contact with the sows and applying back pressure. After detection of estrus, and at the planned artificial insemination, the sows standing in heat were followed by a boar to contract while being artificially inseminated using a catheter. The AI used $3x10^9$ spermatozoa/100 mL and then 12 hours later, a second AI was performed and the detection of their return to estrus at day 35 after AI was done by ultrasound.

2.6 Statistical analysis

The data were analyzed by general linear model (GLM) procedure of SAS (SAS Institute, Inc., Cary, NC, USA). The Pearson correlation test was used to investigate the concentrations of TGF-β1 and its effects on boar semen characteristics. The semen evaluation of motility, viability, acrosome integrity, and mitochondrial function were analyzed by repeated measurement in completely randomized design. The proportions of sows with an appearance of different conception rates and farrowing rates were analyzed using chi-square analysis. The differences of total born and total born alive were analyzed using Duncan's multiple range test to determine significance in all the parameters among groups. A probability level of $p \leq 0.05$ was required. The data were presented as least-squares means ± standard errors.

3. Results and Discussion

Semen samples of spermatozoa in SP from 16 boars were analyzed to detect the concentration of TGF-β1. The average of TGF-β1 concentrations in individual SP for the 16 boars are shown in Table 1. Results showed a wide variation (range 8.94-588.24 pg/mL) on the first day with a mean concentration of TGF-β1 as 140.64±37.98 pg/mL. Concentration of TGF-β1 after storage at 16°C decreased by an average of 46.10% (range 12.50-83.47%) on the third day. Retrospective breeding data for the 16 boars showed no correlation between TGF-β1 concentration and semen volume (0.070), concentration (0.626), sperm motility (0.068), progressive (0.074), and viability (0.054) (Table 2). A previous study demonstrated that the mean concentration of cytokine TGF-β1 in SP presented at total concentrations of 20.4-766.5 pg/mL [3]. Previous research demonstrated the differences in storage temperatures (-20°C or -80°C) did not affect TGF-β1 concentrations after one year of storage. The concentrations of TGF-β1 were highly variable between individual boars and the highest TGF-β1 concentrations were found in the sperm rich fraction of the ejaculate. In addition, frequency of semen collection affected the concentration of TGF-β1. According to [1], concentrations of TGF-β1 were detected in all SP, and TGF-β1 concentrations varied between individual boars. Experiments showed that TGF-β1 concentrations decreased between 1 and 3 days after storage. The decrease in TGF-β1 concentration resulted from many factors, and low concentrations caused by the dilution procedure before use in artificial insemination can have poor reproductive outcomes.

In addition, the effect of TGF-β1 in boar semen on sperm motility was examined as 3 treatments (0, 1000 and 2000 pg/dose). Significant levels influencing days of storage and treatments of progressive motility, viability, acrosome integrity, and mitochondrial function are shown in Table 3. A change in viability was observed within the first 24 hours of storage, except for the 2000 pg/dose ($p<0.05$). Average progressive motility for treatment 3 was significantly greater than treatment 1 within 24 hours of storage ($p<0.05$), while VAP and VCL of treatment 3 were significantly greater than those of treatment 1 after 72 hours ($p<0.05$). Motility, VAP, VCL, and VSL at 24 hours showed no significant difference ($p>0.05$). Motility, progressive, and VSL at 72 hours were also not significantly different ($p>0.05$).

Table 1. The variations of TGF-β1 concentrations in individual boar SP (mean±SE).

	TGF-β1 (pg/mL)		
Boar No.	Day 1	Day 3	Decrease (%)
1	8.94	7.83	12.50
2	31.30	17.89	42.86
3	31.30	8.94	71.43
4	40.24	27.39	31.94
5	40.24	35.21	12.50
6	44.71	31.30	30.00
7	44.71	31.91	28.63
8	48.61	27.39	43.66
9	115.63	55.89	51.66
10	140.63	46.88	66.67
11	140.63	97.22	30.86
12	166.67	62.50	62.50
13	225.00	77.13	65.72
14	250.00	171.88	31.25
15	333.33	93.75	71.88
16	588.24	97.22	83.47
mean ±SE	140.64±37.98	55.65±10.81	46.10±5.52

Table 2. The correlations of TGF-β1 concentrations in porcine SP on semen quality characteristics (mean±SE).

	Concentration of TGF-β1		
	mean±SE	Correlation (r)	P value
Volume (mL)	327.63±37.98	0.070	0.827
Concentration (10^6/mL)	396.75±20.98	0.626	0.290
Motility (%)	94.69±0.60	0.068	0.833
Progressive (%)	50.38±0.88	0.074	0.817
Viability (%)	10.94±61	0.054	0.066

Finally, supplementation of TGF-β1 to fresh semen (0, 500, and 1000 pg/dose) for artificial insemination on reproductive performance in sows was evaluated. Average conception rates of 80, 80, and 90% and farrowing rates of 70, 80, and 90%, respectively were not significantly different ($p>0.05$). For litter size and total born alive, results were not significant ($p>0.05$) (Table 4) [13, 14]. The results from this study demonstrated support for the main hypothesis that porcine TGF-β1 is a special determinant of semen characteristics and improves fertility in sows. The specific functional proteins in SP promoted sperm survival compared with proteins in SP from the remainder of the collection in individual boars [15]. The results demonstrated that sperm removed from SP have lower motility than sperm not removed from SP [3]. However, concentration of TGF-β1 did not relate to the influence of TGF-β1 on semen quality.

The concentration of TGF-β1 in artificial insemination used for commercial swine was probably very low due to dilution before using, and decreased concentrations of TGF-β1 led to low reproductive efficiency [6]. It is possible that the positive conception rate and litter size in the TGF-β1 treatment group is due to sperm characteristics and uterine environment. Concentrations of TGF-β1 supplemented in the experiment (1000-2000 pg/dose) was within the reproductive function of levels found in boar SP (Experiment 1), and calculated from artificial insemination usage in commercial farms. The possible effect of the TGF-β family is an increase in reproductive performance, including: conception rate, farrowing rate, litter size, and total born. Previous research has extensively studied the TGF-β family signal transduction pathway. As was shown in mice, TGF-β super family members are essential to various reproductive processes [16].

The TGF-β super family regulates pro-inflammatory mechanisms of responsiveness, migrates to the anti-inflammatory regulating cascade. For example, TGF-β's secreted by activated T-cells inhibit cytolytic helper-T cell proliferation and differentiation to the effector functions of T-cells. This controls immune response and maintains homeostasis [17], suggesting that cytokine factors may be one of the factors that are an active moiety in boar semen [1]. Experiment 2 showed that addition of TGF-β improved and did not affect sperm motility. Therefore, addition of TGF-β1 to semen was beneficial for artificial insemination of sows. TGF-β1 in porcine SP was a key factor to activate the immune function in reproductive organs in females. TGF-β1 was the key molecule to activate cytokines as it was secreted from the accessory gland in a substrate, TGF-β1 appeared to be activated in the female reproductive tract after mating or artificial insemination [18]. It was demonstrated that in rodent species and swine, the mechanism of SP component in activating the embryonic cytokines and preparing the uterine function for the embryo development and survival [19]. Therefore, the regulatory molecules in semen preparation play an important role in manipulating the reproductive environment to promote maternal immune tolerance with regard to improved fertility in swine.

The results showed no statistically significant differences between the control and experimental groups. However, it was experimental animals and the ways to find that the experimental group tended to have higher efficiency and a higher productive efficiency than the control group. The economic growth of pigs and the number of pigs born is important for profit. Therefore, it would be interesting to conduct a study in the future using a higher number of experimental animals, and look for the ways to improve efficiency.

Table 3. Effect of TGF-β1 addition to fresh boar semen, and storage time on semen quality (mean±SE).

Time (hour)		TGF-β1 (pg/100 mL)		
		0	1000	2000
24	Acrosome integrity	78.00±0.33	78.13±0.44	78.88±0.35
	Mitochondrial function	80.63±0.55	80.75±0.31	81.25±0.41
	Viability	83.12^{b}±0.35	84.12^{ab}±0.40	84.5^{a}±0.46
	Motility (%)	79.32±1.55	81.91±1.39	82.40±0.93
	Progressive (%)	50.60^{b}±1.69	53.22^{ab}±2.32	56.78^{a}±1.78
	VAP (μm/s)	78.28±3.91	80.31±2.33	82.97±3.01
	VSL (μm /s)	53.81±2.22	55.49±5.1.75	57.37±1.50
	VCL (μm/s)	160.68±8.31	159.80±5.68	168.33±5.58
72	Acrosome integrity	76.12^{b}±0.40	77^{ab}±0.42	77.625^{a}±0.22
	Mitochondrial function	78.50±0.27	79.00±0.27	79.00±0.50
	Viability	81.12^{b}±0.40	81.5^{a}±0.27	82.75^{a}±0.25
	Motility (%)	79.11±1.15	81.56±0.88	81.30±0.86
	Progressive (%)	50.51±2.01	52.98±1.04	53.81±1.46
	VAP (μm/s)	68.21^{b}±0.99	71.91^{b}±2.59	81.33^{a}±1.93
	VSL (μm /s)	47.88±1.02	49.45±1.43	55.46±1.45
	VCL (μm/s)	142.44^{b}±2.78	152.59^{ab}±5.67	162.78^{a}±4.91

Velocity average path (VAP; μm/s), Velocity straight line (VSL; μm/s), Velocity curvilinear (VCL; μm /s) mean with difference (^{a,b,c}) in the same low are significantly difference ($p<0.05$).

Table 4. Effects of TGF-β1 on reproductive performance in sows.

	TGF-β1 (pg/dose)		
	0	500	1000
No. of sow	10	10	10
Conception rate (%)	80 (8/10)	80 (8/10)	90 (9/10)
Farrowing rate (%)	70 (7/10)	80 (8/10)	90 (9/10)
Litter size (mean±SE)	10.5±0.89	11.12±0.72	12.37±0.38
Total born alive (mean±SE)	9.75±0.73	10.00±0.65	10.87±0.72

4. Conclusion

Boar SP showed high variation in concentration between individual boars, while concentration of TGF-β1 decreased as storage time increased. Addition of TGF-β1 to fresh semen improved motility, conception rate, farrowing rate, litter size, and total born alive in sows which all showed improvement over the control. Further research is required to determine the importance of TGF-β1 concerning reproductive function and applications for enhanced reproductive performance in swine.

Acknowledgements

This research was financially supported by the National Research Council of Thailand (NRCT) under the program research scholarship for graduate students 2019. National Science and Technology Development Agency (NSTDA) under the program STEM Workforce for graduate students 2018 and Progressive Co., Ltd. We also thank Betagro Co., Ltd. and the Khon Kaen regional office for supporting the collection of boar semen and North Eastern Bull Frozen Semen Production and Research Center for semen evaluation by using the computer software.

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