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Original Article

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Abstract

One of the causes of infertility in boars is nutritional deficiency. Interestingly, zinc has been used for improving semen quality in boars. Thus, the objective of this study was to investigate the effect of zinc as feed supplementation in order to improve semen quality in infertile boars. Three purebred Pietrain boars with a poor semen quality history were selected for the experiment. The semen samples were collected once a week for 4 weeks before the treatment period and the semen evaluated immediately. Fifteen grams (a total concentration of 150 ppm) of zinc were supplemented daily in the normal feed. The testing period was 16 consecutive weeks. The semen samples were evaluated for volume, concentration, motility (by computer-assisted sperm assay), sperm viability (by SYBR-green staining), acrosome integrity (by FITC-PNA staining), mitochondrial activity (by JC-1 staining) and sperm morphology (by Giemsa's staining for sperm head and formal saline fixation for sperm tail). The semen samples were also preserved in commercial semen extender and stored at 16 °C for three days after ejaculation. The results showed that zinc supplementation significantly improved acrosome integrity (40.38 ± 7.13% and 70.56 ± 21.36%, respectively) and sperm viability (66.38 ± 5.46% and 79.66 ± 4.11%, respectively) at the ejaculation date and also significantly enhanced acrosome integrity after three days preservation (27.84 ± 7.78% and 67.36 ± 22.43%, respectively). In addition, we also found an increasing trend in acrosome integrity, sperm viability and progressive motility after 4 to 8 weeks of zinc supplementation. This study revealed that daily zinc addition can improve semen quality in infertile boars and can be used on farms to improve semen quality.

Keywords: zinc, supplementation, poor semen quality, boar

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Introduction

Livestock farming is an important sector of agriculture in Thailand. The pig industry has been developed and represents a high level of national income every year. In 2018, the Office of Agricultural Economics reported an interesting statistical value of pig production that the total number of pigs bred increased from 19.25 million head in 2017 to 19.88 million head in 2018, while the total estimated number for internal consumption was 19.34 million head or 1.45 million tons of pork production; 768,211 head were exported to other countries, worth 2917.08 million Baht. In addition, the trend in value of pig production increased around 9% every year (Office of Agricultural Economics, 2018).

Boar stations play a key role in farm reproductive management to produce the high quality semen used for artificial insemination (AI) (Tretipskul *et al*.,2012). However, infertility problems in boars, from either infectious or non-infectious causes, have effects on farm production. Infectious diseases such as leptospirosis, brucellosis, chlamydia, swine influenza virus, mycoplasma, classical swine fever (CSF) virus, Aujeszky'S disease (AD) virus, porcine parvovirus (PPV), porcine reproductive and respiratory syndrome (PRRS) virus, porcine coronavirus type 2 (PCV2) etc., affect semen quality. These diseases affect the male reproductive organs, contaminate semen and are able to be shed during insemination (Guérin and Pozzi, 2005; Althouse, 2007; Maes *et al*., 2016). Non-infectious diseases also affect infertility. It has been found that trace minerals are needed for spermatogenesis, spermiogenesis and the function of the epididymis. Zinc has been used in boars to improve semen quality and protect semen from heat stress effects (Li *et al*., 2017) which damage the sperm membrane lipid peroxidation (Am-in *et al*., 2010). It is interesting to see whether infertility in boars with low semen picture (especially morphology) can be improved after treatment by supplementation with zinc.

Zinc has been found in high concentrations within the semen of many species. During zinc deficiency, retarded development of testicular growth involving marked atrophy of the tubular epithelium, reduced deoxyribonucleic acid, ribonucleic acid and protein levels, as well as reduced zinc content in the testis, epididymis and dorsolateral prostate, are seen. In addition, zinc deficiency in animals results in decreased output of pituitary gonadotrophins and androgen production and zinc turnover involves testosterone as well as pituitary hormones. Metabolic regulation of sperm appears to be mediated through zinc as a regulator of enzyme activity in the semen. Within spermatozoa, zinc is closely associated with sulfhydryl groups and disulfide linkages and is concentrated in the tail. Control of the motility of sperm by zinc apparently involves control of energy utilization through adenosine triphosphate systems involved in contraction and through regulation of phospholipid energy reserves (Hidiroglou and Knipfel, 1984).

The objective of this study was to investigate the effect of zinc as a feed additive in order to improve semen quality in infertile boars.

Materials and Methods

Animals and experimental plan: Three purebred Pietrain boars with a history of poor semen quality exhibited at least one of the following criteria that were sperm motility <70% (Rozeboom, 2009), sperm viability <64.5% (Tsakmakidis *et al*., 2012) and acrosome integrity <51% (Rozeboom, 2009). The boars were brought from an AI station (Evaporative cooling system housing) and transported to the Farm Animal Hospital of the Faculty of Veterinary Science, Nakhon Pathom (Non-evaporative cooling system housing), one month before the experiment started. The experiment was done during the period of April to September (summer and rainy season). Moreover, the animals were fed with 2.0 kg of commercial feed at 14.0% protein twice a day. Semen was collected once a week for four consecutive weeks. The semen was evaluated and the data described as "Before treatment". Fifteen grams (a total concentration of 150 ppm of zinc (Zinpro, USA)) were supplemented into each individual feed (Liao *et al*., 1985), starting to feed the zinc by topping up the normal feed daily. The testing period was 16 weeks. In addition, semen was collected from the boars for four weeks after the addition of zinc was stopped and the semen also evaluated, as showed in Table 1. All experimental procedures were approved by Institutional Animal Care and Use Committee, Prince of Songkla University (MHESI 68014/808).

Table 1 Time period of zinc treatment

Plan	weeks																							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Before treatment (Zn-)	■	■	■	■																				
Treatment (Zn+)					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■				
After treatment (Zn-)																					■	■	■	■

Semen collection: Semen was collected by the gloved-hand method weekly in the morning. The semen was sent immediately to the laboratory for evaluation and preserved with Bio Pig semen extender (Magapor®, Spain). The semen samples were evaluated for the following parameters.

1.) *Semen volume (ml)*: the semen samples of each boar were kept in sterile plastic bags and were weighed by weighting machine for semen volume evaluation.

2.) *Sperm concentration (x106 sperm cells/ml)*: the parameter was calculated by photometer SpermaCue (Minitube®); A drop of fresh semen was transferred by

micropipette tip to microcuvette for insertion in the testing chamber of SpermaCue, and a concentration (million sperm cells per ml) was digitally reported from this machine.

3.) *Sperm motility (%) was evaluated by computer-assisted sperm assay (CASA system)*; semen mixed with extender samples was diluted with 37 °C warmed phosphate buffer solution (PBS) to proper portions for the CASA system (SCA® CASA system, MICROPTIC S.L, Barcelona, Spain). The diluted semen was placed in a chamber and investigated on a warmed stage (TOKAI HIT, Shizuoka-ken, Japan) at 37 °C under a phase-contrast microscope (BX41, Olympus, Shinjuku, Japan) coupled to a video test sperm system (Wang *et al*., 2018). Before observation of each analysis, a waiting period of 1 minute took place (Iguer-ouada and Verstegen, 2001; Tretipskul *et al*., 2010). A minimum of five fields and 1,000 spermatozoa were analyzed for each sample. The user-defined parameter settings for the program were as follows: frame rate (fps), 50; static (µm/s), <10; slow-medium (µm/s); 25; rapid (µm/s), >45; progressive (STR>), 45; connectivity (pixels), 11; velocity average path (VAP) points (pixels), 5. The evaluation by CASA also detected many parameters, including progressive motility (%), rapid motility (%), rapid progressive motility (%), curvilinear velocity (VCL, µm/s), straight line velocity (VSL, µm/s), average path velocity (VAP, µm/s), linear coefficient (VSL/VCL, LIN (%)), straightness (VSL/VAP, STR (%)), wobble (VAP/VCL, WOB, (%)), amplitude of lateral head displacement (ALH, µm), beat cross frequency (BCF, beats/s) and hyperactive spermatozoa (%).

4.) *Sperm viability (%)*: The diluted semen was assessed using SYBR-14/Ethidium homodimer-1 staining technique (Fertilight®, Sperm Viability Kit, Molecular Probes Europe, Leiden, The Netherlands); the diluted semen was mixed with those stains and incubated at 37 °C for 15 minutes. The viable sperm were bright green stained while the dead sperm were bright orange stained. Moreover, two hundred stained sperm plasma membranes were counted under fluorescent microscope for the percentage of sperm viability (Axnér *et al*., 2004).

5.) *Acrosome integrity (%)*: The diluted semen was assessed using fluorescein isothiocyanate–labeled peanut agglutinin (FITC-PNA) staining. Briefly, the diluted semen was mixed with Ethidium homodimer-1 and incubated at 37 °C for 15 minutes. Then, the mixtures were smeared on a glass slide and air dried. After that, 50 µl of FITC-PNA stain were spread on the slide and incubated in the moist chamber at 4 °C for 30 minutes. Lastly, the slides were rinsed with cold PBS, air dried and investigated for acrosome under fluorescent microscope. The acrosome integrity was done by evaluating intact acrosome sperm which contained a fine and smooth of acrosomal cap surface with bright green color staining. In addition, acrosome-reacting sperm displayed patchy disrupted fluorescence over the acrosome and acrosome-unreacted sperm showed no FITC-PNA staining of acrosomal cap. The positive results were presented as the percentage of spermatozoa with intact acrosome by counting 200 stained sperm. (Chanapiwat *et al*., 2009).

6.) *Mitochondrial activity (%)*: Sperm mitochondrial membrane potential was determined using the fluorochrome 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1; Molecular Probe Inc.). Briefly, the diluted semen was mixed with staining solution, containing JC-1, SYBR-14, and propidium iodide (PI), and incubated at 37 °C for 30 minutes. The intact mitochondrial membrane integrity exhibited yellow-orange fluorescence over the midpiece while sperm with a damaged plasma membrane of mitochondria revealed less green or no green fluorescence over the midpiece. Moreover, two hundred sperm were evaluated under fluorescent microscope for the percentage of mitochondrial activity (Huo *et al*., 2002).

7.) *Sperm morphology (%)*: Diluted semen were thinly smeared on the slide and air dried for the next step of staining. Giemsa's staining was done for sperm head observation by counting 500 sperm heads under the phase contrast microscope (1,000X) (Mansur *et al*., 2018). The abnormalities of the head were classified into narrow, narrow at the base, pear shaped, variable size, abnormal contour, undeveloped, abaxial and looseness of the head. On the other hand, the diluted semen were also fixed in formal saline for tail morphologic evaluation. Two hundred sperm tails were observed under a phase contrast microscope (400X). The abnormalities of the tail included cytoplasmic droplets, simple bent tail, coil tail, abnormal midpieces, acrosome defects and looseness of the tail.

After that, the semen preserved with extender samples were kept at 16 °C. The evaluation for all parameters was carried out again three days after ejaculation and the data summarized at the end of the experiment.

Data analysis: Data was analyzed using the SPSS 22 version. All semen parameters were tested for nomal distribution using Explore command and the data which was not of normal distribution was tranformed by Arc-Sine. The data was analyzed statistically between and within groups using repeated measurement ANOVA. Linear Mixed-Effects Modeling was used for the effect of individual boar variation on semen parameter. Differences were considered statistically significant when $P \leq 0.05$.

Results

The average of the first week's sperm production was 77.78 billion sperm cells per ejaculate (251.67 ml of semen volume and 309 million sperm/ml of concentration) and this decreased to 55.42 billion at week 4 (161.67 ml of semen volume and 342.78 million sperm/ml of concentration) and the average of sperm production for the period between 4 to 11 weeks was defined as before treatment and the period between 12 to 24 weeks was stated as the treatment period. Data showed that the treatment with zinc cloud improved sperm production significantly; 55.52 and 47.54 billion sperm cells per ejaculate respectively ($P < 0.05$). In addition, the data in Table 2 shows that the sperm quality was not significantly improved after treatment. Although the sperm motility and progressive motility

during the treatment period was not significantly higher than the control, there seemed to be an increasing trend in those parameters after one to two months (4 to 8 weeks) of Improves Semen Quality. At month 3 (12 weeks after supplementation), we found a slight decrease in the sperm values; however, the data measured was still greater than at the beginning or in the control (Figs. 1A-1B). In addition, we also found improvement in sperm viability and acrosome integrity ($P < 0.05$) at the ejaculation date and Figs. 1C-1D show a dramatic increase in those parameters. The percentage of sperm viability revealed an increasing level after 1 to 2 months (4 to 8 weeks) of zinc supplementation. However, sperm mitochondrial activity was not improved after treatment (Table 2).

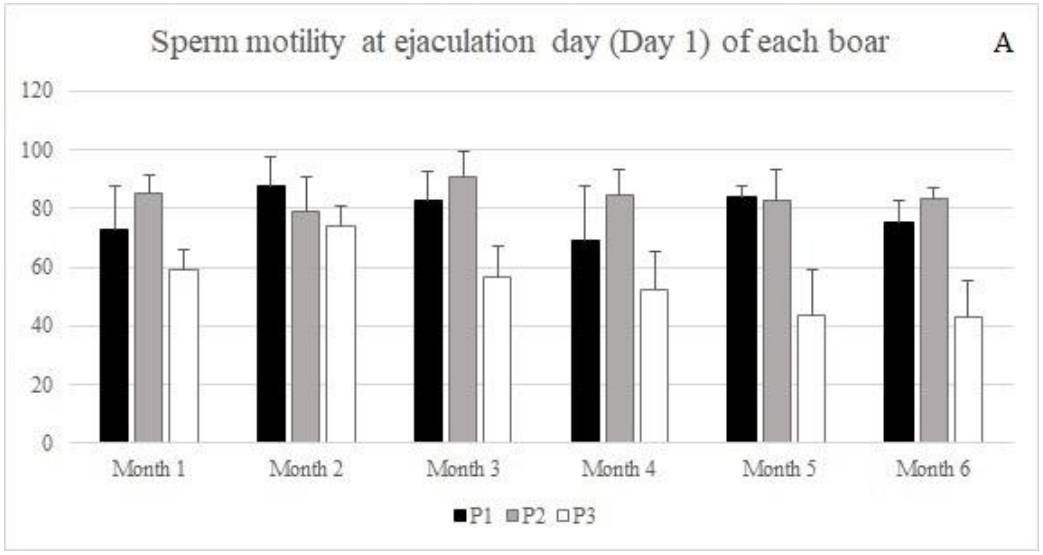
The semen mixed with commercial extender was preserved at 16 °C in a refrigerator, producing chilled semen for AI. Normally, the quality of chilled semen should be similar or slightly lower compared to fresh semen. The evaluation results of chilled semen are shown in Table 3. Fig. 2 reveals that there were increasing trends in sperm motility and progressive motility (Figs. 2A-2B) of chilled semen after treatment at month 2 (8 weeks after supplementation). Moreover, zinc administration (Figs. 2C-2D) seemingly improved sperm acrosome function compared to the control ($P<0.05$) but we did not find a significant change in the sperm viability of preserved semen. The percentage of acrosome integrity was dramatically higher than the control after treatment.

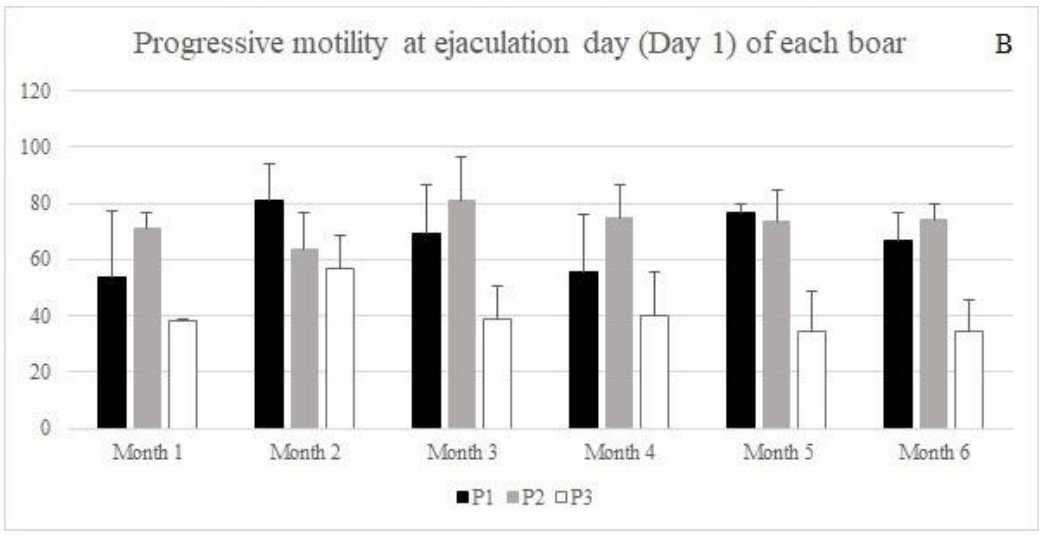
The sperm morphology results are shown in Table 4. In the first four weeks more than 90% of sperm heads were normal. The defects found in sperm tails were 41.79 ± 15.35% proximal droplets, 3.17 ± 2.88% distal droplets, 2.50 ± 1.44% simple bent tail, and 6.21 ± 5.18% coiled tail. The tail characteristics of the sperm showed non-significant changes compared to treatment.

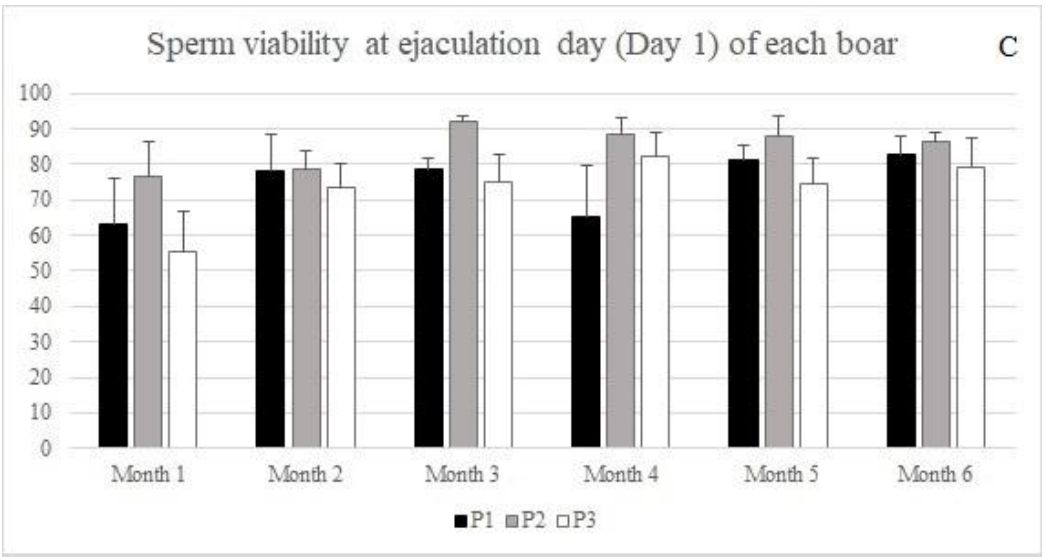
The fluorescence microscopy tests of sperm viability, acrosome integrity and mitochondrial activity are shown in Fig. 3. The viable spermatozoa were stained green and also the mitochondria located at the neck of each sperm (under fluorescence microscope) (Figs. 3A, 3C). Fig. 3B shows the acrosome activity; the intact acrosome of the spermatozoa was observed as a fine and smooth acrosomal surface (red arrows), while the reacted acrosome of the spermatozoa showed a rough or non-smooth acrosomal surface (blue arrows). In this study, we found an increased percentage of intact acrosomal surfaces after zinc supplementation. This result confirms the semen data shown in Tables 2 and 3.

Table 2 The average semen quality 4 weeks before zinc administration and during the 16 weeks of zinc treatment at ejaculation day

Parameters	Before treatment	Treatment period
	Mean ± SD	Mean ± SD
CASA evaluation		
Motility (%)	72.40 ± 6.93 [a]	73.97 ± 8.16 [a]
Progressive motility (%)	54.34 ± 7.25 [a]	62.12 ± 9.80 [a]
Rapid motility (%)	50.56 ± 8.06 [a]	56.16 ± 10.28 [a]
Rapid progressive motility (%)	23.64 ± 4.85 [a]	25.50 ± 6.91 [a]
Curvilinear velocity (VCL, µm/s)	84.44 ± 10.48 [a]	87.87 ± 13.68 [a]
Straight line velocity (VSL, µm/s)	20.60 ± 1.74 [a]	18.36 ± 4.27 [a]
Average path velocity (VAP, µm/s)	45.36 ± 4.16 [a]	39.83 ± 7.36 [a]
Linear coefficient (VSL/VCL, LIN (%))	19.11 ± 0.46 [a]	18.95 ± 2.13 [a]
Straightness (VSL/VAP, STR (%))	39.93 ± 1.42 [a]	41.33 ± 4.01 [a]
Wobble (VAP/VCL, WOB, (%))	45.07 ± 0.60 [a]	43.24 ± 1.83 [a]
Amplitude of lateral head displacement (ALH, µm)	1.92 ± 0.16 [a]	1.94 ± 0.23 [a]
Beat cross frequency (beats/s)	8.45 ± 0.75 [a]	8.38 ± 1.47 [a]
Hyperactive spermatozoa (%)	8.77 ± 2.02 [a]	7.22 ± 3.11 [a]
Sperm viability (%)	65.25 ± 6.10 [a]	79.66 ± 4.11 [b]
Acrosome integrity (%)	40.38 ± 7.13 [a]	70.56 ± 21.36 [b]
Mitochondrial activity (%)	75.00 ± 7.02 [a]	72.90 ± 6.49 [a]


Sperm motility at ejaculation day (Day 1) of each boar
A
120
100
80
60
40
20
0
Month 1
Month 2
Month 3
Month 4
Month 5
Month 6
P1 P2 P3


Progressive motility at ejaculation day (Day 1) of each boar
B
120
100
80
60
40
20
0
Month 1
Month 2
Month 3
Month 4
Month 5
Month 6
P1 P2 P3


Sperm viability at ejaculation day (Day 1) of each boar
C
100
90
80
70
60
50
40
30
20
10
0
Month 1
Month 2
Month 3
Month 4
Month 5
Month 6
P1 P2 P3

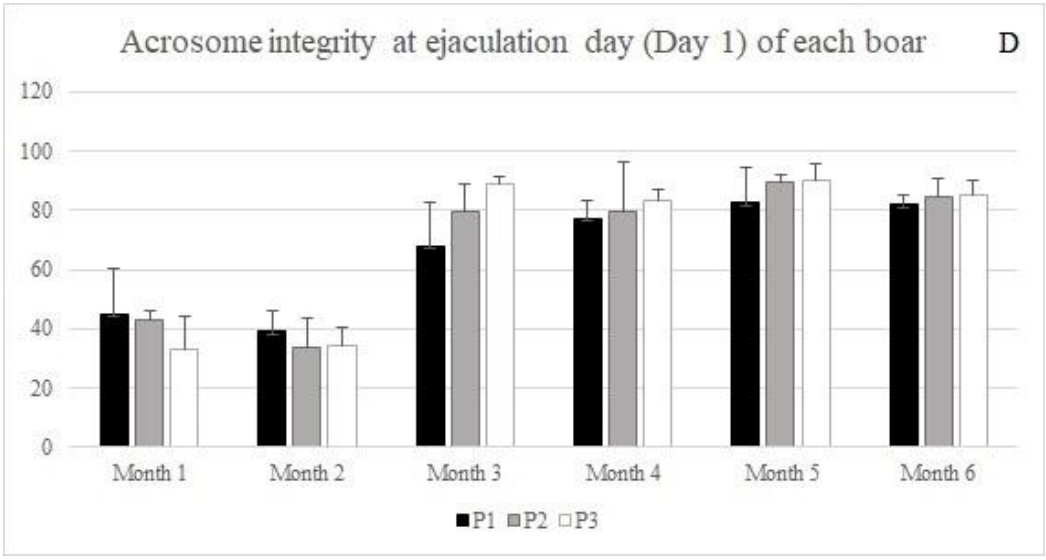
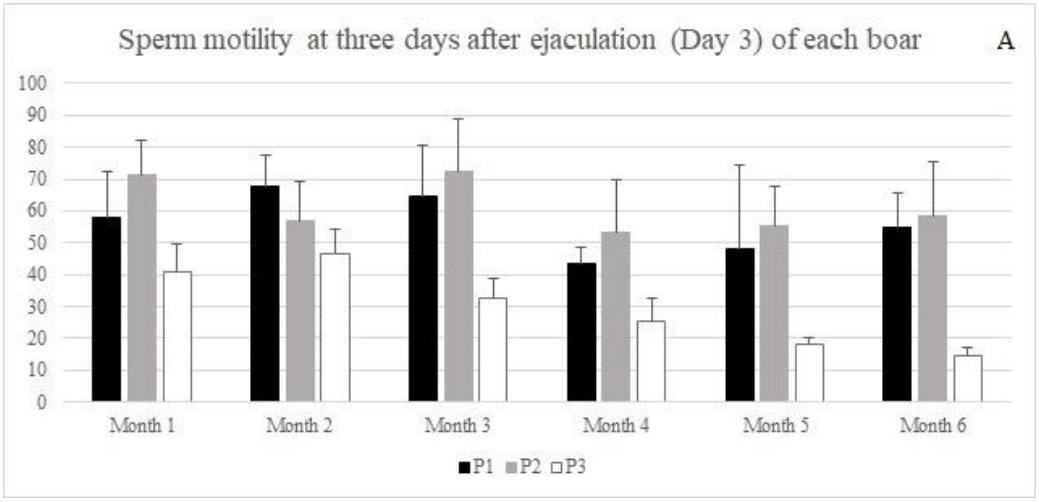
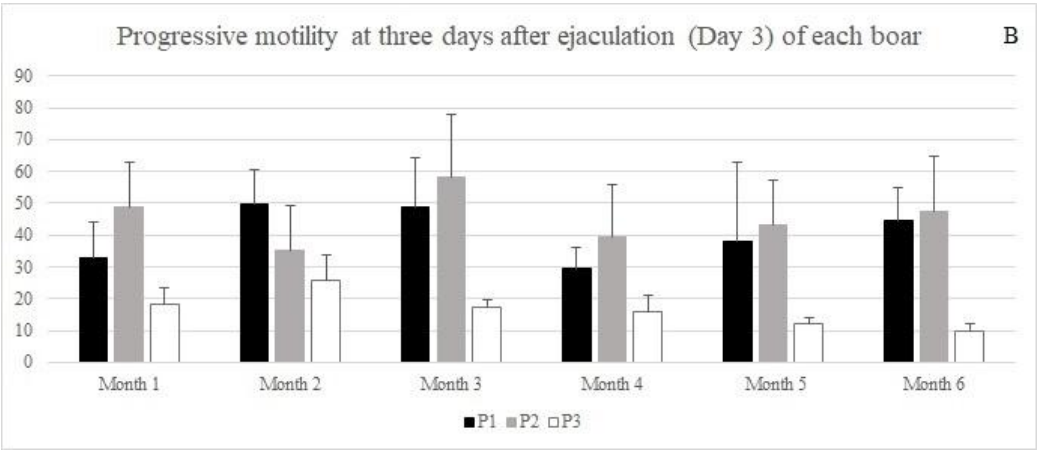


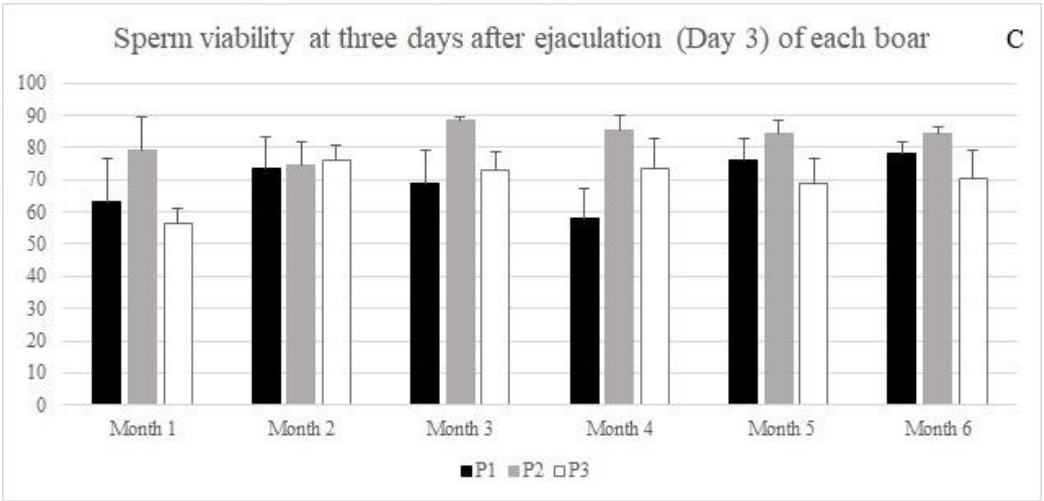
Figure 1 Sperm evaluation at ejaculation day (Day 1) of each boar
A. Sperm motility
B. Progressive motility
C. Sperm viability
D. Acrosome integrity

Table 3 The average semen quality 4 weeks before zinc administration and during the 16 weeks of zinc treatment at 3 days after ejaculation

Parameters	Before treatment	Treatment period
	Mean ± SD	Mean ± SD
CASA evaluation		
Motility (%)	56.51 ± 10.66 [a]	48.61 ± 12.03 [a]
Progressive motility (%)	33.22 ± 9.02 [a]	34.46 ± 10.26 [a]
Rapid motility (%)	28.04 ± 8.89 [a]	30.07 ± 10.07 [a]
Rapid Progressive motility (%)	10.97 ± 4.72 [a]	11.23 ± 5.27 [a]
Curvilinear velocity (VCL, µm/s)	69.23 ± 10.09 [a]	71.22 ± 8.42 [a]
Straight line velocity (VSL, µm/s)	13.04 ± 3.75 [a]	12.55 ± 2.51 [a]
Average path velocity (VAP, µm/s)	32.32 ± 6.11 [a]	32.32 ± 4.71 [a]
Linear coefficient (LIN, µm/s)	16.41 ± 3.23 [a]	15.69 ± 1.59 [a]
Straightness (VSL/VAP, STR (%))	35.99 ± 3.46 [a]	34.44 ± 2.87 [a]
Wobble (VAP/VCL, WOB (%))	42.79 ± 3.50 [a]	42.72 ± 2.16 [a]
Amplitude of lateral head displacement (ALH, µm)	1.47 ± 0.17 [a]	1.68 ± 0.25 [a]
Beat cross frequency (beats/s)	5.02 ± 1.04 [a]	5.53 ± 1.00 [a]
Hyperactive spermatozoa (%)	3.33 ± 2.50 [a]	3.05 ± 2.05 [a]
Sperm viability (%)	66.38 ± 5.46 [a]	75.05 ± 5.16 [a]
Acrosome integrity (%)	27.84 ± 7.78 [a]	67.36 ± 22.43 [b]
Mitochondrial activity (%)	68.50 ± 8.96 [a]	67.90 ± 5.77 [a]


Sperm motility at three days after ejaculation (Day 3) of each boar
A
100
90
80
70
60
50
40
30
20
10
0
Month 1
Month 2
Month 3
Month 4
Month 5
Month 6
P1 P2 P3


Progressive motility at three days after ejaculation (Day 3) of each boar
B
90
80
70
60
50
40
30
20
10
0
Month 1
Month 2
Month 3
Month 4
Month 5
Month 6
P1 P2 P3


Sperm viability at three days after ejaculation (Day 3) of each boar
C
100
90
80
70
60
50
40
30
20
10
0
Month 1
Month 2
Month 3
Month 4
Month 5
Month 6
P1 P2 P3

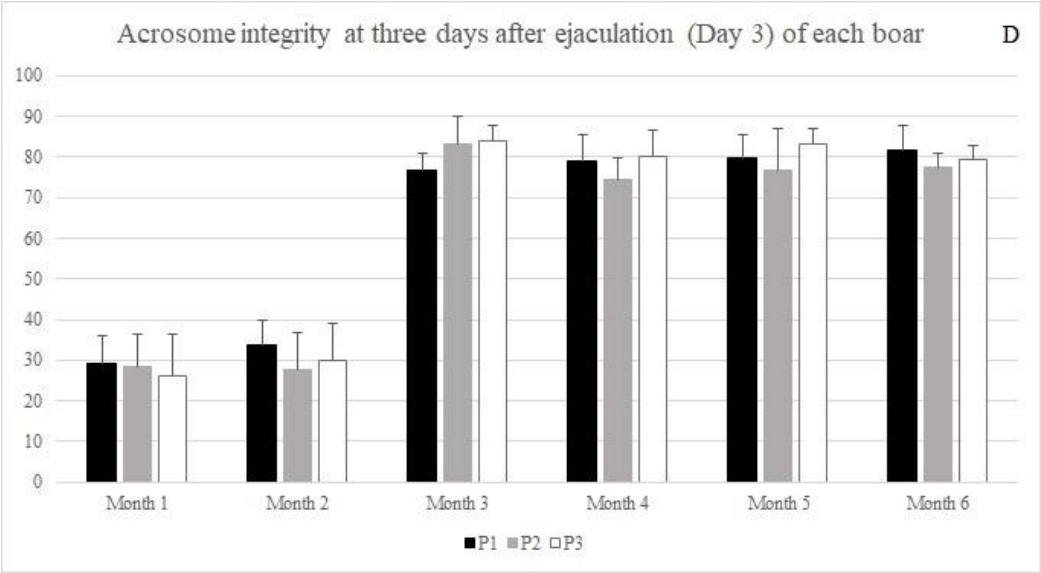


Figure 2 Sperm evaluation three days after ejaculation (Day 3) of each boar
A. Sperm motility
B. Progressive motility
C. Sperm viability
D. Acrosome integrity

Table 4 Comparison of the average sperm morphology 4 weeks of before administration and during the 16 weeks of treatment on day 1 and day 3 after ejaculation

Sperm morphology	**Before treatment**	**Treatment period**
	Mean ± SD	**Mean ± SD**
Sperm head (%)		
Normal	92.88 ± 3.97	95.85 ± 1.43
Narrow	1.00 ± 0.30	0.84 ± 0.21
Narrow at the base	3.02 ± 1.55	1.74 ± 1.26
Pear shape	0.77 ± 0.55	0.38 ± 0.23
Variable size	1.33 ± 1.18	0.45 ± 0.23
Abnormal contour	0.10 ± 0.10	0.01 ± 0.02
Undeveloped	0	0.01 ± 0.02
Abaxial	0.02 ± 0.03	0
Loose	0.80 ± 1.00	0.70 ± 0.49
Sperm tail (%)		
Normal	45.96 ± 16.44	46.63 ± 15.09
Proximal droplet	41.79 ± 15.35	41.30 ± 15.90
Distal droplet	3.17 ± 2.88	5.73 ± 5.66
Simple bent tail	2.50 ± 1.44	2.78 ± 0.41
Coil tail	6.21 ± 5.18	2.98 ± 1.82
Abnormal midpieces	0	0
Acrosome defect	0	0
Loose	0.38 ± 0.45	0.18 ± 0.10

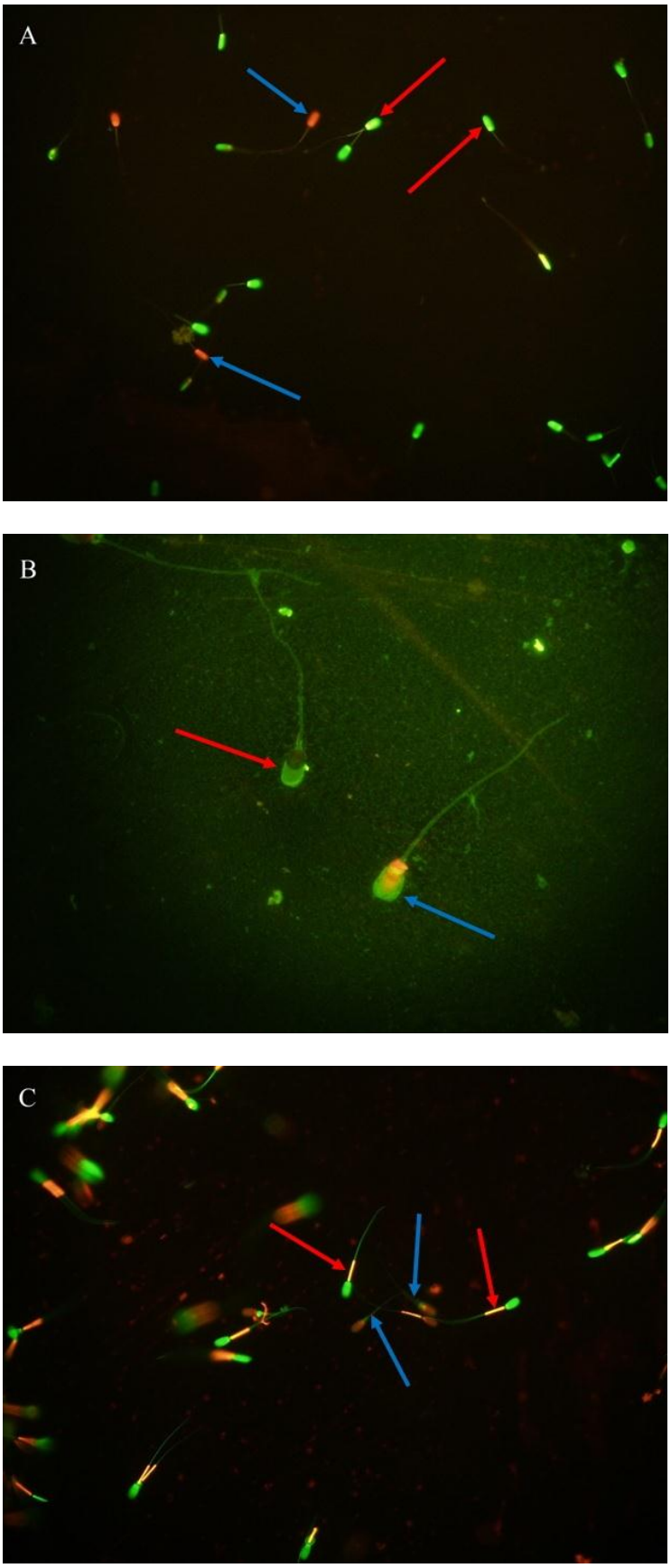


Figure 3 Sperm evaluation of infertility boars
A. Sperm viability test
B. Acrosome integrity test
C. Mitochondrial activity test
Red arrows are positive results of each technique
Blue arrows are negative results of each technique

Discussion

In this work, the main problems of boars were lower acrosome integrity, caused by environmental and/or genetic (Chenoweth, 2005) and sperm tail defects; and cytoplasmic droplets, which referred to the sperm maturation in epididymis (Cooper, 2011). The presence of sperm cytoplasmic droplets at the proximal or distal part of the spermatozoa in an ejaculate has been considered to be injurious to semen storage (Waberski *et al*., 1994) or indicative of reduced fertility potential (Thundathil *et al*., 2001; Peña *et al*., 2007), sperm plasma membrane alteration (Althouse, 1998) or a reduced ability to interact with porcine oviductal epithelial explants (Petrunkina *et al*., 2001). The factors influencing cytoplasmic droplet retention of ejaculate are nutritional deficiency, i.e., zinc (Li *et al*., 2017), frequency of collection (Pruneda *et al*., 2005), environment, i.e., temperature-induced heat stress (Althouse, 2007), photoperiod (Knecht *et al*., 2013), management (Kunavongkrit *et al*., 2005), the age of boars (Banaszewska *et al*., 2015) and disease (Guérin and Pozzi, 2005; Althouse, 2007; Maes *et al*., 2016). Hence, in this study, we considered using zinc to improve poor semen quality in infertility boars.

In the first week, sperm production was highest and continuously decreased in the following weeks because of low semen volume. This may have resulted from changing of the housing from evaporative cooling system to non-evaporative cooling system housing for the experiment. This may have induced environmental heat stress in the boars. The study of Smital (2009) and Knecht *et al*., (2014) revealed that high temperature in summer caused low semen volume and also semen output. In this study, the experiment started in the summer season of Thailand. For this reason, we considered classifying the before treatment period for sperm production as the period of week 4 to 11 and the treatment period as week 12 to 24 because we started the supplementation at week 5. The significant increase of sperm production after zinc supplementation occurred because zinc support sperm maturation and the survival of germ cells (Foresta *et al*., 2014) and also regulate testosterone synthesized from Leydig cells and promote spermatogenesis in the testes (You *et al*., 2017), which may have increased sperm production after zinc supplementation in this study.

The zinc concentration in the male reproductive tract has been reported by Hidiroglou and Knipfel (1984) who found the concentration of zinc to be high in the testes, epididymis and dorsolateral prostate glands. The level was highest in the testes and diminished continuously in the reproductive tracts (epididymis, vas deferens, and at ejaculation). Zinc has many advantageous effects on sperm differentiation and maturation by improving sperm membrane and chromatin stability, which may enhance sperm viability and sperm tail function, control the maturation of the sperm tail within the epididymis and improve sperm tail morphology and motility (Lehti and Sironen, 2017). In addition, zinc may involve the metabolism of androgen at the cellular level, which may concomitantly support the maturation mechanisms. In this study we found that the addition of zinc can improve acrosome integrity and sperm viability significantly at day one and three of ejaculation, which accords with previous studies. Sutovsky *et al*., (2019) revealed the interesting information that zinc may be important in capacitation of the sperm by promoting the zinc ion (Zn2+) after in vitro capacitation (IVC). This may be the mechanism by which zinc supports the sperm acrosome.

The effect of zinc on sperm motility and sperm tail morphology has been reported to enhance the sperm membrane and chromatin stability (Lehti and Sironen, 2017) and to promote oxygen uptake, improving motility (Huacuja *et al*., 1973). Our experiment found a non-significant change in sperm motility and also in tail morphology in the treatment group but we found an increasing trend in progressive motility (Fig. 1-2) which may explain that there are multiple factors involving the sperm tail (reviewed above) and zinc is just one factor affecting these phenomena. Thus, zinc addition did not improve sperm tail morphological impairment. Moreover, the trend of sperm motility and progressive motility found in our study decreased after 8 weeks until 16 weeks after supplementation. This may have been caused by the change in the temperature and humidity in the summer and rainy season. Heat stress in boars has been confirmed can have a deleterious effects on sperm quality especially lower sperm motility and rates of abnormal of sperm morphology (Barranco *et al*., 2013). In addition, the study of Liao *et al*., (1985) recommended a high level of zinc concentration (150 ppm) addition daily in boars from age 11 to 24 months of age (13 months) which could improve semen quality in boars without adverse effects. Furthermore, the concentration of 150 ppm of zinc in feed has been recommended to improve growing in starter, nursery and Grow-finish pigs (Gaudré, 2016). Hence, the concentration of zinc supplementation performed in this study may have affected sperm motility and progressive motility.

We considered evaluating the semen quality after cold storage for 3 days in 16 °C refrigeration to estimate the longevity of semen quality after zinc supplementation. Normally, the sperm motility of chilled semen should be higher than 70% (Rozeboom, 2009) but, in this study, sperm motility and progressive motility was lower than normal which may have been the effect of multi-deleterious factors on sperm. However, we found sperm viability and acrosome integrity still to be above the standard after treatment (Rozeboom, 2009; Tsakmakidis *et al*., 2012; Gong *et al*., 2017). Interestingly, this may have been from the effect of zinc on the sperm membrane (Lehti and Sironen, 2017) improving the ability to resist cold damage.

In conclusion, the addition of zinc by topping up the normal feed at 150 ppm concentration daily for 16 weeks can improve sperm quality by enhancing acrosome integrity and sperm viability in infertile boars and can be used generally on farms. In addition, the effect of zinc on sperm quality improvement may occur after 4 weeks of supplementation.

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