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Improvement of Boar Semen Quality by Sperm Selection Using Magnetic Nano-particles

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The objective of this study was to see if fairly simple magnetic nano-particle treatment enhances boar semen qualities. Boar semen samples were prepared from the swine AI center and samples were divided by 4 different motility groups (1, >90%; 2. 80~90%; 3. 70~80%; 4. <70%) using computer assisted sperm analysis (CASA) evaluation. Boar semen was extended using BTS extender and same number of magnetic nano-particles as total number of spermatozoa in each sample was treated for 20 min and collected for 5 min at room temperature. Sperm qualities such as motility and viability were evaluated by the CASA before and after treatment. Sperm abnormality and degree of agglutination were also evaluated under the microscopic examination before and after treatment. There were significant changes (p<0.05) on sperm motility from all 4 different groups in the average of 7.11% after treatment. The enhancement of sperm motility changes was more clear in the groups of lower sperm motile groups (<70% and 70~80%; 19.12±1.08% and 5.67±0.71%, p<0.05). The sperm motility character in terms of curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP) and linearity (LIN, %) showed also similar pattern but motility enhancement wear more clear in below 70% motile group. Average sperm viability was increased to 4% by magnetic nano-particles (p<0.05). The percentage of sperm abnormality was also reduced significantly (p<0.05) to the range of 3.7~4.5% before after treatment. The degree of sperm agglutination was also reduced in lower motility groups by the magnetic nano-particle purification.

Key words : Artificial insemination, boar semen, magnetic nano-particles, motility, viability

Introduction

Assisted reproductive techniques have become the treatment of choice in many cases of infertility [; however the current success rates of these procedures in animal especially in pig remain suboptimal due to higher number of spermatozoa and volume. The ideal protocol for enrichment/selection of sperm cells with high fertilizing ability should be: a) non-toxic for spermatozoa, b) easy to perform and inexpensive, c) able to support high-throughput sample processing, d) capable of selecting the best sperm subpopulation for ART s, leaving behind, seminal plasma, extender (in case of frozen semen) and bioactive substances and cells (leukocytes) that could damage sperm cells [23]. Despite the efforts invested in developing an ideal sperm selection technique (i.e swim-up; density gradient; electrophoresis, fluorescence cell sorting; glass wool filtration) by laboratories around the word, to date no single sperm selection protocol meets all desirable characteristics mentioned above [1] especially, this is true in boar semen.

Magnetic cell separation offers advantages of simplicity of operation, low cost and specificity and sensitivity afforded by use of immunospecific reagents. Typically, this technique employs the use of magnetic particles conjugated to proteins or antibodies to tag cells of interest. To label and to separate cells, many types of magnetic micro beads and nano beads have been developed. The addition of iron oxide compounds (ferromagnetic magnetite γ-Fe_2O_3 and magnetite Fe_3O_4) to monodisperse polystyrene spheres or to a sugar based skeleton is a typical technique of bead production [17].

The consistent production of high quality spermatozoa is crucial for male fertility. Numerous factors associated with the male itself (i.e., age, health, genetic line, nutrition status [5, 12, 13] and the environment (i.e., seasonal changes of temperatures, cryopreservation [2, 5, 21] are known to affect semen quality. Most importantly, the semen itself contains a heterogeneous population of spermatozoa with attributes

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that have significant impacts on male fertility potential [3, 20]. For example, semen collected from high fertile males usually result in significant low proportions of abnormal spermatozoa and high proportions of motile spermatozoa, with better viability (i.e., intact acrosome, plasma and mitochondrial membranes, low DNA fragmentation index,) than those collected from low fertile males [9, 14].

In many agricultural and clinical laboratories, routine practices of semen quality analyses for artificial insemination purpose are often limited to the evaluation of sperm concentration and proportion of motile spermatozoa due to the rapidity of the tests, but these parameters are still relatively poor predictors of semen fertility [5, 9]. The aforementioned viability factors have tremendous impact on the fertility of spermatozoa during their progression within the female genital tract and their interactions with eggs at the site of fertilization [6, 11, 14], but their evaluation can be laborious and results are often available after the preparation of insemination doses. Although current available tests can be effective at quantifying the proportions of viable spermatozoa within semen ejaculates or doses for artificial insemination [8], the removal of damaged spermatozoa would be of great add-on. This procedure may contribute to the elimination of the needless competition between viable and non-viable spermatozoa, leading to higher number of viable spermatozoa reaching the fertilization site for improved fertility. Recently, many progresses on nanotechnology contribute the excellent opportunity for a damaged or defective sperm selection. These nano-particles have unique properties (optical, high photo-stability, and magnetic) and their possible attachment to various biomolecules offers a great potential for non-invasive and ultra-sensitive targeting and/or imaging of molecules in living cells and organisms [7, 16].

Numerous studies have reported the potential applications of nano-particles in agricultural and biomedical research [7, 16]. In livestock, boar spermatozoa have been shown to harmlessly incorporate self-illuminating quantum dot nano-particles without impairment to their motility and fertilizing potentials [4]. In addition, the use of magnetic iron oxide nanoparticles to target and remove moribund and abnormal spermatozoa from AI doses has led to improved fertility of bulls [18]. In order to see the effect of magnetic iron oxide nano-particles coated with silica on sperm selection, we applied these nano-particles on freshly ejaculated boar semen.

Material and Methods

Semen collection and processing

Semen samples from Duroc boars raised in local AI centers were collected. Before each semen collection, boars are thoroughly cleaned and only sperm rich fractions were collected by gloved-hand technique. Beltsville Thawing Solution (BTS) extender was used to dilute the semen and semen samples were transferred to the laboratory immediately using 17℃ semen storage container.

Magnetic nano-bead treatment

Iron oxide (Fe_2O_3) nano-particles were coated with silica (Noah Biotech Inc., S. Korea) to selectively bind to the outer membrane of dead and moribund spermatozoa. Prepared magnetic nano-particles were obtained in stock of 15.9 mg/ml of PBS buffer and stored at 4°C until use for sperm labeling. Semen samples were mixed with magnetic nano-particles for 20 min and collected for 5 min using 12,000 gauss neodymium magnet at room temperature. Free and sperm-bound magnetic nano-particles were pulled down to the wall and nano-particle-free spermatozoa were collected into 50-mL Falcon centrifuge tubes. Numbers of magnetic beads used for the purification of sperm are the same as total number of spermatozoa in each sample.

Analysis of sperm motility

The sperm movement characteristics were measured using CASA system determined as Zeng et al., [23]. In brief, 1.5 ml semen sample was incubated for 30 min at 37℃ water bath and 10 μl of semen were applied to pre-warmed Makler counting chamber (Sefi-Medical, Israel). Semen samples were analyzed by SAIS II system (Medical Supply Co. Ltd., S. Korea) connected with CCD camera (Veltek, S. Korea) on microscope (Olympus, Japan) connected with a warm plate (37℃).

Analysis of sperm viability and abnormality

For sperm viability and abnormality test, fast green FCF (2% v/v) and Eosin B (0.8%, v/v) were solved in PBS and filtered before staining the sperm. Ten micro liters of sperm were applied to the slide and equal volume of dye were added, smeared with cover glass, and dried quickly to avoid live sperm stain as dead. If sperm head stains with dye, it determines as dead. Total of 100 sperm from 25 each of 4 different compartments were counted for both viability and

Table 1. Change of boar sperm motility after treatment of magnetic nano-particle

Treatment (%)	Sperm motility (%)		
	Before (%)	After (%)	Δ (%)
> 90	91.3 ± 1.1^{Ba}	92.8 ± 1.1^{Aa}	1.67
80~90	84.7 ± 0.7^{Bb}	88.6 ± 0.5^{Ab}	4.63
70~80	76.8 ± 1.0^{Bc}	81.2 ± 0.8^{Ac}	5.67
< 70	63.0 ± 0.9^{Bd}	75.0 ± 0.8^{Ad}	19.12
Total/Mean	79.1 ± 1.3^{B}	84.7 ± 1.0^{A}	7.11

^{a,b}With the same columns, values with different superscripts differ significantly (p<0.05).
^{A,B}With the same row, values with different superscripts differ significantly (p<0.05).
Data are expressed as Mean ± SEM.

abnormality test.

Analysis of sperm agglutination

Under microscopic examination, sperm should be freely swimming and not sticking to one another. Agglutination of sperm occurs when the head or tail of one sperm sticks to another sperm restricting the motility of the sperm. The score of agglutination is graded 1-3 as outlined as below.

Grade 1: Isolated with < 10 sperm per agglutinate
Grade 2: Moderate with 10-50 sperm per agglutinate
Grade 3: > 50 sperm per agglutinate

Statistical analysis

Data were analyzed using the Generalized Linear Model procedure (PROC-GLM) of the Statistical Analysis System (SAS Institute, Cary, NC, USA, 2000). For statistical analyses, however, percentage values were subjected to arcsine transformation before applying Students t-tests to means and standard deviations for each data point. 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 and Discussion

Effect on sperm motility

Data were analyzed (at least n =4) and summarized. There were significant changes (p<0.05) on sperm motility from all 4 different groups in the average of 7.11% after treatment (Table 1). The enhancement of sperm motility changes was more clear from the groups of lower sperm motile groups (< 70% and 70~80%; 19.12±1.08% and 5.67±0.71%, p<0.05, Table 1). After magnetic nano-particles treatment, it is clear that the proportion of motile spermatozoa moving straightforward (VCL, progressive) and fast (VSL, rapid) were showed increasing tendency (Table 2, p<0.05). It suggested that the nano-purification procedure using the designed magnetic nano-particles resulted in the selection of spermatozoa with higher motility (p<0.05).

The motility character in terms of VCL, VSL, VAP and LIN showed (Table 2) improved more in below 70% motile group indicating moribund sperm were removed by magnetic nano-particles and it contributed to sperm motility. However, no significant effects of the nano-purification were found on other motility parameters such as the Average Path velocities (VAP) and Linearity (LIN). Average sperm viability was increased to 4% by magnetic nano-particles (Table 3, p<0.05) indicating dead spermatozoa also removed by magnetic nano-particles. The percentage of sperm abnormality was also reduced significantly (Table 3, p<0.05) to the range of 3.7~4.5% before after treatment. The degree of sperm agglutination was also reduced in lower motility groups by the magnetic nano-particle purification (Table 4) and it is clear that lower motile group with higher agglutination semen needed more number of treatment for the re-

Table 2. Effects of magnetic nano-particle purification on boar sperm motility parameters*

Treatment (%)	VCL (%)		VSL (%)		VAP (%)		LIN (%)	
	Before	After	Before	After	Before	After	Before	After
> 90	31.5±1.1^{a}	30.7±1.1^{a}	13.0±1.2	13.1±1.2^{a}	24.9±0.8^{a}	24.6±0.8^{a}	45.8±1.8	47.3±1.8
80~90	27.2±0.7^{b}	26.4±0.5^{b}	12.3±0.8	11.9±0.6^{ab}	21.8±0.5^{b}	21.3±0.4^{b}	46.5±1.1	46.6±0.8
70~80	24.1±1.1^{bc}	24.9±0.8^{b}	10.7±1.1	10.7±0.8^{b}	19.2±0.7^{c}	19.7±0.5^{c}	48.8±1.6	47.0±1.3
< 70	21.5±1.0^{c}	22.1±0.8^{c}	11.9±1.0	10.3±0.9^{b}	16.6±0.6^{d}	17.1±0.6^{d}	46.1±1.5	49.1±1.3
Total/Mean	25.9±0.6	25.5±0.5	12.0±0.5	11.4±0.4	20.6±0.5	20.5±0.4	46.7±0.7	47.3±0.6

*Curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP) and linearity (LIN).
^{a,b} With the same columns, values with different superscripts differ significantly (p<0.05).
Data are expressed as Mean ± SEM.

Table 3. Change of boar sperm viability and abnormality after treatment of magnetic nano-particle

Treatment (%)	Viability (%)		Abnormality (%)	
	Before	After	Before	After
> 90	91.3±1.1^{Bd}	93.0±1.2^{Aa}	9.8±0.5^{Ab}	6.1±0.5^{Bb}
80~90	85.5±0.8^{Bc}	89.9±0.8^{Aa}	10.4±0.3^{Aab}	6.6±0.3^{Bab}
70~80	77.0±1.1^{Bb}	81.5±1.1^{Ab}	11.9±0.5^{Aa}	7.4±0.5^{Bab}
< 70	65.2±1.7^{a}	71.1±1.7^{c}	11.8±0.7^{Aa}	7.9±0.7^{Ba}
Total/Mean	82.7±1.4^{B}	86.9±1.4^{A}	10.8±0.3^{A}	6.8±0.3^{B}

^{*a,b}With the same columns, values with different superscripts differ significantly (p<0.05).
^{A,B}With the same row, values with different superscripts differ significantly (p<0.05).
Data are expressed as Mean ± SEM.

moval of aggregated spermatozoa (Table 4). Because the currently used magnetic nano-particles were designed (Silica-coated) to interact outer membrane of damaged and/or dead spermatozoa, the current findings suggest that such defective spermatozoa were present in the semen doses and their removal allowed the enrichment of semen doses with high motile (motile and progressive) and viable spermatozoa. These observations are in agreement with the findings using fresh and frozen-thawed bovine semen [18]. The application of magnetic-activated cell sorting (MACS) allows for sperm selection based on sperm apoptosis in addition to routine parameters such as motility and morphology. Data generated from previous studies serve as a basis for projects that evaluate magnetic cell sorting safety and efficiency in clinical settings for patients undergoing IVF in various species [10, 15]. This novel strategy appears as a viable and non-invasive tool for the enrichment of semen doses with high quality spermatozoa to enhance fertility performance. With this improved motility characteristics and together with the potential viability of spermatozoa as previously described in bovine [18], it becomes reasonable to expect extended effects on the sow fertility potential of nano-purified spermatozoa in pigs.

Table 4. Effects of magnetic nano-particle purification on sperm agglutination

No. of magnetic nano-particle treatment*	Agglutination grade			
	> 90	80~90	70~80	< 70
0	1	2	3	3
1	1	2	3	3
2	1	1	2	2
3	1	1	1	1

*Grade 1: Isolated with < 10 sperm per agglutinate
Grade 2: Moderate with 10-50 sperm per agglutinate
Grade 3: > 50 sperm per agglutinate

Results obtained from this study suggested that sperm selection (purification) by magnetic beads would be a beneficial for swine reproductive performance and considered to be an inexpensive method to improve boar sperm quality for AI studs as well as self on-farm collection. Magnetic beads sperm selection procedure takes only 30 min, so it can be done while extending the law semen.

Altogether, this data suggested the possibility of use of magnetic nano-particles for removing the dead, moribund and aggregated boar sperm in fairy short time period.

Further investigations are needed for the sow fertility inseminated with magnetic nano-particle purified sperm to confirm and expand the present findings.

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초록 : 마그네틱 나노비드를 이용한 돼지 정자 품질의 향상

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본 연구는 간단히 활용할 수 있는 나노 크기의 마그네틱 비드를 이용하여 정자의 품질을 향상시킬 수 있는지 여부를 규명하기 위하여 실시하였다. 돼지 정자 시료는 인공수정 센터에서 공급받아 실험실로 2시간 이내로 이송한 후 CASA 측정을 통하여 4개의 활력을 나타내는 그룹으로(1, > 90%; 2. 80~90%; 3. 70~80%; 4. < 70%) 분류하였다. 정액은 BTS 희석제를 사용하여 보존하였고, 총 정자수와 동일한 농도의 마그네틱 비드를 정액에 20분간 처리한 후, 5분간 실온에서 마그네틱 비드에 반응한 정자를 자석을 이용하여 분리하였다. 마그네틱 비드 처리 전 과 후 정자의 생존율 및 활력은 CASA를 이용하여 측정하였고, 기형율과 정자응집의 정도는 현미경으로 검사하였다. 처리 후의 정자 활력은 4개 그룹 모두에서 유의하게(p<0.0.5) 높은 차이를 보였으며 처리 전에 비하여 평균 7.11% 향상되었다. 정자 활력의 변화는 처리 전 낮은 활력을 보인 그룹에서 보다 현저한 차이를 보였다(< 70% and 70~80%; 19.12±1.08% and 5.67±0.71%, p<0.0.5). 정자 활력을 VCL, VSL, VAP 및 LIN (%)로 구분한 성적에서도 유사한 패턴을 나타냈고, 이러한 현상은 활력 70% 이하를 나타낸 그룹에서 개선 효과가 더욱 뚜렷하였다. 마그네틱 비드 처리 후 정자 생존율은 처리 전에 비하여 평균 4%가 향상 되었고(p<0.0.5), 정자 기형율 또한 3.7~4.5% (p<0.0.5) 정도 감소하였다. 정자 응집의 정도 또한 마그네틱 비드 처리를 통하여 처리 전 낮은 활력을 나타낸 그룹에서 감소됨을 알 수 있었다.