Brief communication

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In vitro fertility of cryopreserved spermatozoa from boars fed diets supplemented with selenium

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Summary

In vitro fertilization rates were determined for cryopreserved spermatozoa from control boars and boars supplemented with selenium from organic or inorganic sources. Percentages of embryos cleaved and becoming blastocysts were greatest ($P < .01$) for boars fed 0.3 ppm organic selenium. Dietary selenium may improve fertility of cryopreserved boar spermatozoa.

Keywords: swine, spermatozoa, cryopreservation, selenium

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Resumen - Fertilidad in vitro de esperma crioconservado de machos alimentados con una dieta suplementada con selenio

Se determinaron los índices de fertilización in vitro del esperma crioconservado de machos control y machos suplementados con selenio de fuentes inorgánicas y orgánicas. Los porcentajes de embriones divididos y que se convierten en blastocitos fue mayor ($P < .01$) en los machos alimentados con 0.3 ppm de selenio orgánico. El selenio dietético puede mejorar la fertilidad del esperma de macho crioconservado.

Résumé - Fertilité in vitro de spermatozoïdes cryopréservés provenant de verrats nourris avec une diète supplémentée avec du sélénium

Les taux de fertilisation in vitro ont été déterminés pour des spermatozoïdes cryopréservés provenant de verrats témoins et de verrats nourris avec un supplément de sélénium de sources organiques et inorganiques. Les pourcentages d'embryons clivés et devenant des blastocystes étaient plus élevés ($P < 0,01$) pour les verrats nourris avec 0,3 ppm de sélénium organique. Le sélénium dans la diète pourrait améliorer la fertilité des spermatozoïdes cryopréservés de verrats.

Beneficial effects of supplemental selenium on reproductive characteristics in boars are well-documented.[1-5] For example, improvements in sperm production and morphology and fertility were reported for boars fed diets supplemented with inorganic selenium in the form of sodium selenite at a concentration of 0.5 ppm.[1-3] Mahan and Kim[6] suggested that selenite may not be as biologically effective as the selenium indigenous to grains, which is incorporated in an organic form (selenomethionine). This concept is supported by work in which boars fed an organic source of selenium containing 63% selenomethionine (Sel-Plex; Alltech, Inc, Nicholasville, Kentucky) tended to have greater in vitro fertilization (IVF) rates than did boars fed 0.3 ppm selenium from sodium selenite or unsupplemented control boars.[7] Moreover, enhanced IVF rates displayed by selenomethionine-fed boars were maintained during storage at 18°C for 8 days.[7] The objective of this experiment was to extend these findings by determining the effect of dietary supplementation with 0.3 ppm selenium from an organic source on IVF rates for cryopreserved boar spermatozoa.

Materials and methods

The protocol was reviewed and approved by the Virginia Tech-Institutional Animal Care and Use Committee.

Animals and housing

At weaning, crossbred boars were each randomly assigned, using a random number table, to one of three dietary treatments fed during a six-phase feeding program, ie, Nursery 1, 2, and 3; Grower 1 and 2; and Finisher. The dietary treatments were basal corn and soybean meal-based diets that met the nutrient recommendations for growing boars[8] with the exception of selenium; basal diets supplemented with 0.3 ppm selenium from an organic source (selenomethionine); and basal diets supplemented with 0.3 ppm selenium from sodium selenite (Premium Selenium 270; North American Nutrition Co, Inc, Lewisburg, Ohio). The US Food and Drug Administration (FDA) allows a maximum of 0.3 ppm supplemental selenium in swine diets.[9] Boars had ad libitum access to feed and water.

Following completion of the finisher phase, boars were individually penned and were trained to mount an artificial sow to allow semen collection. During the training period and throughout the remainder of the study, boars received approximately 2.73 kg of a basal, breeder-boar diet[8] or the basal diet supplemented with 0.3 ppm selenium from either selenomethionine or sodium selenite. Selenium concentrations in the basal diets, determined at the Virginia-Maryland College of Veterinary Medicine Toxicology Laboratory in Blacksburg using previously reported procedures,[7] were 0.03 ppm.

Semen collection and processing

Boars were maintained on a once weekly semen collection frequency, and the experiment reported here was conducted when animals were approximately 1.5 years of age.

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Semen was collected using the gloved-hand technique from boars receiving the control diet (n = 4), the diet supplemented with 0.3 ppm selenomethionine (n = 5), or the diet supplemented with 0.3 ppm sodium selenite (n = 7). The sperm-rich fraction of semen was filtered (US BAG; Minitube of America, Inc, Verona, Wisconsin) during collection to remove gel. Sperm motility in collected semen was determined as previously described.[10]

Freezing of boar semen

Modena Extender (Swine Genetics International, Cambridge, Iowa) was added to collected semen at an amount 1.5 times the volume of the semen. The extended semen was poured into a Nalgene bottle (Fisher Scientific; Pittsburgh, Pennsylvania), placed in a Styrofoam box with gel packs to maintain a temperature of 18°C, and shipped overnight to Swine Genetics International. The day after collection, semen was frozen in 5-mL plastic macrotubules using commercial procedures (Swine Genetics International) and then stored at -196°C in liquid nitrogen until used for IVF procedures at Findlay University, Ohio. The proportion of live spermatozoa was determined prior to freezing and after thawing using 0.6% eosin red and 5.0% aniline blue dye.[11] At the commercial stud, semen with less than 75% live spermatozoa upon arrival is not frozen. On the basis of this criterion, one boar in the group fed selenomethionine was rejected, and data for this animal were not included in the statistical analyses.

Determination of sperm fertilizing capability

Previously described procedures[7,12] were used. Unless otherwise stated, chemicals were purchased from Sigma-Aldrich (St Louis, Missouri). The oocyte maturation medium was medium 199 with Earle's salts (Thermo Fisher Scientific, Inc, Pittsburgh, Pennsylvania), supplemented with 5 μg per mL follicle stimulating hormone (FSH), 1 μg per mL insulin, 50 ng per mL gentamicin sulfate, 10 ng per mL epidermal growth factor, and 10% (volume by volume) porcine follicular fluid. The IVF medium used was a modified Tris-buffered medium (mTBM),[13] and the in vitro culture (IVC) medium employed was North Carolina State University (NCSU) 23 medium containing 0.4% (weight by volume) bovine serum albumin.[14]

Porcine oocytes (Applied Reproductive Technologies; Madison, Wisconsin) surrounded by a compact cumulus cell mass and uniform ooplasm were washed three times in a 50-mm × 9-mm Falcon polystyrene dish (Thermo Fisher Scientific) using the oocyte maturation medium, and 60 oocytes were placed into each well of a Nunclon four-well multidish (Thermo Fisher Scientific) containing 500 μL of oocyte maturation medium overlaid with mineral oil. The oocytes were incubated at 39°C in an atmosphere of 5% carbon dioxide for 20 to 24 hours. Oocytes were then matured in oocyte maturation medium without FSH, insulin, or porcine follicular fluid for an additional 20 to 24 hours in the manner described.

After incubation, cumulus cells were removed from the oocytes by repeat pipetting with 0.1% hyaluronidase in NCSU 23 solution for 15 to 30 seconds. Oocytes were then washed three times in a Falcon polystyrene dish in 100-μL drops of mTBM and stored in 50 μL drops of mTBM under mineral oil. Semen samples were each thawed in a 15-mL polypropylene conical tube containing 10 mL of mTBM at 37°C, and centrifuged at 36*g* for 5 minutes. The supernatant was poured into a new tube and mTBM was added to bring the volume to 10 mL. Viable sperm cells were collected after centrifugation at 553*g* for 5 minutes. The supernatant was discarded and the pellet washed once more as described. Following the wash, the supernatant was discarded and 10 mL of mTBM was added to the pellet. Spermatozoa were counted using a Bright-Line hemacytometer (Thermo Fisher Scientific) and were then diluted with mTBM such that the final concentration was 1×10^6 cells per mL. Then, 50 μL of spermatozoa in mTBM was added to each well, mixed by gentle repeat pipetting, and the oocytes and spermatozoa were co-incubated for 6 to 8 hours at 39°C in an atmosphere of 5% carbon dioxide. For each boar, spermatozoa were added to each of three wells (triplicates), thus 180 oocytes per boar were used and a total of 2700 oocytes were employed for the study (720 total oocytes each for the control and selenomethionine-fed groups and 1260 total oocytes for the sodium selenite group). The IVF and embryo characteristics for each individual boar were determined by averaging the values for the three wells.

After 12 hours from the end of IVF, a portion of the potential embryos were washed three times in 100-μL drops of IVC medium and then placed in 10 μL of phosphate buffered saline containing 1 μg per mL bisbenzimide Hoechst 33342 stain. After 15 minutes of staining, the oocytes were de-stained in IVC medium for 5 minutes and examined under a fluorescent microscope (346 nm excitation wavelength; 460 nm emission wavelength). Oocytes were characterized for penetration by spermatozoa (swollen spermatozoon head), polyspermic (more than one swollen spermatozoon head) penetration, or undergoing male pronucleus (MPN) formation (visual identification of an MPN).

The remaining zygotes were washed three times in 100 μL of IVC medium, placed in 100-μL drops of IVC medium in a 100-mm × 9-mm Falcon polystyrene dish, and incubated at 39°C in an atmosphere of 5% carbon dioxide under mineral oil. After 48 hours post IVF, embryos were placed in fresh IVC medium in the manner described. Cleavage and blastocyst formation were evaluated using a stereomicroscope after 48 and 144 hours post IVF, respectively.

Statistical analyses

Data were analyzed by analysis of variance using the GLM procedure of SAS (SAS Institute Inc, Cary, North Carolina). Boar was the experimental unit for the analyses and the model included treatment as the main effect. Replicate and time were not included in the model. With three treatments, only two orthogonal comparisons were allowed, so single degree of freedom contrasts were used to compare selenium-supplemented boars (selenomethionine and sodium selenite supplementation) versus control boars, and selenomethionine boars versus sodium selenite boars. Data were checked and satisfied the assumptions of analysis of variance.

Results

Results of the investigation are shown in Table 1. There were no effects ($P > .05$) of dietary treatment on the percentage of motile spermatozoa immediately after semen collection, on the proportion of live cells post thaw, or on the percentages of oocytes penetrated, polyspermic penetration, or MPN formation, when frozen-thawed boar spermatozoa were used for IVF. There was a tendency ($P = .09$) for selenium-supplemented boars to have a greater MPN than controls. The percentage of embryos cleaved by 48 hours post IVF was affected by treatment ($P = .01$), with selenium-supplemented boars tending ($P = .07$) to have greater values than

Table 1: Effects of dietary supplementation with selenomethionine or sodium selenite sources of selenium* or no selenium supplementation on in vitro fertilization and early embryonic development after boar spermatozoa were frozen and stored at -196°C

					P†		
	Selenomethionine	Sodium selenite	Control	SE	Overall	Selenomethionine and sodium selenite versus control	Selenomethionine versus sodium selenite
No. of boars	4	7	4	NA	NA	NA	NA
Motile spermatozoa (%)‡	98.0	97.6	97.8	.7	.29	.91	.12
Live spermatozoa (%)§	43.3	42.7	41.3	2.9	.84	.56	.87
Penetrated oocytes (%)¶	90.0	84.8	89.4	3.9	.49	.67	.31
Polyspermic penetration (%)	16.3	15.2	18.7	5.4	.90	.65	.88
Male pronucleus formation (%)	81.4	78.7	66.1	6.4	.22	.09	.74
Embryonic development (%)							
Cleaved by 48 hours post IVF	32.6	23.1	22.1	2.4	.01	.07	< .01
Blastocyst by 144 hours post IVF	21.3	18.3	10.0	2.0	< .01	< .01	.27

* From weaning until approximately 1.5 years of age, boars consumed control diets (no supplementary selenium; n = 4) or diets supplemented with 0.3 ppm selenium from selenomethionine (Sel-Plex; Alltech, Inc, Nicholasville, Kentucky; n = 5) or sodium selenite (Premium Selenium 270; North American Nutrition Co, Inc, Lewisburg, Ohio; n = 7). Collected semen from one boar fed selenomethionine had less than 75% live spermatozoa and was not frozen. Data from this boar were not included in the statistical analysis.
† Data were analyzed by analysis of variance. A *P* value of < .05 was considered statistically significant; *P* < .10 was considered a trend.
‡ Determined immediately post collection as previously described.[7]
§ Live spermatozoa as determined post thawing using 0.6% eosin red and 5.0% aniline blue.
¶ Spermatozoa from each boar were added to each of three wells (triplicates), each containing 60 oocytes; values for an individual boar represent the average of the triplicate values.
IVF = in vitro fertilization; NA = not applicable.

controls, and selenomethionine-fed boars having greater (*P* < .01) values than selenite-supplemented boars. The percentage of embryos that progressed to the blastocyst stage of development by 144 hours post IVF was also affected by treatment (*P* < .01), and values for selenium-supplemented boars were greater (*P* < .01) than controls.

Discussion

The dietary requirement for selenium in breeder boars is 0.3 ppm.[8] Thus, in the current experiment, control boars consumed a diet considered nutritionally deficient in selenium. Our results suggest that dietary supplementation of boar diets with selenium may have a beneficial effect on the ability of sperm cells to maintain fertility after the freeze-thaw process. Moreover, it appears that selenium supplied in an organic form (ie, selenomethionine), rather than an inorganic form (ie, sodium selenite) is superior for maintaining fertility in stored semen. This concept is supported by previous work.[7] Indeed, in that study, extended semen from boars supplemented with selenomethionine at a concentration of 0.3 ppm had numerically greater, but not statistically different (*P* = .11) IVF rates than did boars supplemented with 0.3 ppm selenium from sodium selenite or unsupplemented control boars, and the greater sperm motility and enhanced IVF rates displayed by selenomethionine-fed boars were maintained during liquid storage for 8 days at 18°C.[7]

Determining the mechanism responsible for the positive effects of dietary selenium on fertility reported herein will require further experimentation. However, oxidative stress occurs when the antioxidant capacity of the boar is exceeded by the production of physiologically harmful reactive oxygen species. Cryopreservation decreases the antioxidant capacity of boar semen and decreases sperm fertility, negative effects of which are ameliorated by exogenous supplementation of semen with antioxidants.[15] Selenium is an integral component of glutathioneperoxidase (GPx), and gene expression for GPx4, the primary GPx in the mammalian testis, was upregulated in boars fed selenomethionine, compared with boars fed sodium selenite.[16] Thus, enhanced in vitro fertility demonstrated in this study might be a result of increased antioxidant capacity and protection against reactive oxygen species in selenium-supplemented boars.

The vast majority of US sows are bred using artificial insemination (AI)[17] with fresh, liquid semen that is stored at 16°C to 18°C for use within 5 days. Use of frozen semen in swine production has numerous theoretical advantages over use of fresh, liquid semen. For example, frozen semen can be stored indefinitely, and the genetics of outstanding sires can be available for many years after death of the boar. Although good fertility of frozen-thawed porcine semen has been reported in some research trials,[18,19] in general, lower farrowing rates and litter sizes are obtained after insemination with frozen-thawed semen than with fresh, liquid semen.[20] Thus, the current use of frozen semen for AI in the US swine industry is very limited. The results of the current experiment suggest that dietary inclusion of selenium may be a practical approach for improving reproductive performance in AI breeding programs employing frozen boar semen.

Implications

- Under the conditions of this study, in vitro fertility is better in boars supplemented with selenium.

- Inclusion of selenium in the diet of boars may be an effective approach for enhancing reproductive performance on swine farms using cryopreserved semen.

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Conflict of interest

None reported.

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References

1. Marin-Guzman J, Mahan DC, Chung YK, Pate JL, Pope WF. Effects of dietary selenium and vitamin E on boar performance and tissue responses, semen quality, and subsequent fertilization rates in mature gilts. *J Anim Sci.* 1997;75:2294–3003.

2. Marin-Guzman J, Mahan DC, Pate JL. Effect of dietary selenium and vitamin E on spermatogenic development in boars. *J Anim Sci.* 2000;78:1537–1543.

3. Marin-Guzman J, Mahan DC, Whitmoyer R. Effect of dietary selenium and vitamin E on the ultrastructure and ATP concentration of boar spermatozoa, and the efficacy of added sodium selenite in extended semen on sperm motility. *J Anim Sci.* 2000;78:1544–1550.

4. Jacyno E, Kawecka M, Kamyczek M, Kolodziej A, Owsianny J, Delikator B. Influence of inorganic SE + vitamin E and organic SE + vitamin E on reproductive performance of young boars. *Agric Food Sci Finland.* 2002;11:175–184.

5. Kolodziej A, Jacyno E. Effect of selenium and vitamin E supplementation on reproductive performance of young boars. *Archiv fur Tierzucht.* 2005;48:68–75.

6. Mahan DC, Kim YY. Effect of inorganic or organic selenium at two dietary levels on reproductive performance and tissue selenium concentrations in first-parity gilts and their progeny. *J Anim Sci.* 1996;74:2711–2718.

7. Speight SM, Estienne MJ, Harper AF, Crawford RJ, Knight JW, Whitaker BD. Effects of dietary supplementation with an organic source of selenium on characteristics of semen quality and in vitro fertility in boars. *J Anim Sci.* 2012;90:761–770.

8. National Research Council. *Nutrient Requirements of Swine*. 10th ed. Washington, DC: National Academy Press; 1998:237–238.

9. US Food and Drug Administration. Food additives permitted in feed and drinking water of animals: Selenium. *Fed Reg*. 1987;52:10887.

10. Estienne MJ, Harper AF, Day JL. Characteristics of sperm motility in boar semen diluted in different extenders and stored for seven days at 18°C. *Reprod Biol.* 2007;7:221–231.

11. González LO, Fischman ML, Boquet M, Acerbo MC, Miguez MS, Cisale HO, Ferrari MR. Boar semen: complementary techniques for its evaluation. *InVet*. 2013;15:37–46.

12. Whitaker BD, Knight JW. Exogenous γ-glutamyl cycle compounds supplemented to *in vitro* maturation medium influence *in vitro* fertilization, culture, and viability of porcine oocytes and embryos. *Theriogenology*. 2004;62:311–322.

13. Abeydeera LA, Wang WH, Cantley TC, Prather RS, Day BN. Glutathione content and embryo development after in vitro fertilization of pig oocytes matured in the presence of a thiol compound and various concentrations of cysteine. *Zygote*. 1999;7:203–210.

14. Petters RM, Wells CD. Culture of pig embryos. *J Reprod Fertil.* 1993;Suppl 48:61–73.

15. Pena FJ, Johannisson A, Wallgren M, Rodriguez-Martinez H. Antioxidant supplementation in vitro improves boar sperm motility and mitochondrial membrane potential after cryopreservation of different fractions of the ejaculate. *Anim Reprod Sci.* 2003;78:85–98.

16. Speight SM, Estienne MJ, Harper AF, Barb CR, Pringle TD. Effects of organic selenium supplementation on growth performance, carcass measurements, tissue selenium concentrations, characteristics of reproductive organs, and testis gene expression profiles in boars. *J Anim Sci.* 2012;90:533–542.

17. Knox R, Levis D, Safranski T, Singleton W. An update on North American boar stud practices. *Theriogenology*. 2008;70:1202–1208.

18. Eriksson BM, Petersson H, Rodriguez-Martinez H. Field fertility with exported boar semen frozen in the new flat-pack container. *Theriogenology.* 2002;58:1065–1079.

19. Roca J, Carvajal G, Lucas X, Vazquez JM, Martinez EA. Fertility of weaned sows after deep intrauterine insemination with a reduced number of frozen-thawed spermatozoa. *Theriogenology*. 2003;60:77–87.

20. Johnson LA, Weitze KF, Fiser P, Maxwell WMC. Storage of boar semen. *Anim Reprod Sci.* 2000;62:143–172.