

## Superoxide Dismutase Activity in the Oviductal and Uterine Fluid of the Bitch and the Effects of the Enzyme on Viability, Motility and Hyperactivation of Canine Sperm *In Vitro*

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**ABSTRACT.** Superoxide dismutase (SOD) activity in flushings from oviducts and uterine horns of 8 anestrous, 5 estrous and 7 diestrous bitches was measured. SOD activity in oviductal fluid in estrous bitches was significantly higher than that in anestrous and diestrous bitches ( $P < 0.01$ ). SOD activity in uterine fluid of diestrous bitches was, however, significantly higher than that in anestrous and estrous bitches ( $P < 0.01$ ). Additionally, sperm collected from normal dogs were incubated in MEM and in MEM containing SOD (SOD-MEM) for 24 hr. The percentages of sperm with viability, motility and hyperactivation in SOD-MEM were higher than those in MEM. SOD produced in oviduct and uterus may be able to maintain or improve sperm quality and fertility in the dog.

**KEY WORDS:** canine, oviduct, sperm, superoxide dismutase, uterus.

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Superoxide dismutase (SOD) in seminal plasma is the primary antioxidant enzyme that inhibits increases in reactive oxygen species (ROS) concentration in the semen and maintains sperm motility [1, 2, 5, 6]. Moreover, canine sperm motility is low when the level of antioxidant enzymes, such as SOD and catalase, in seminal plasma are decreased [11]. The estrous period in the bitch is longer than that in other mammals, lasting an average of 10 days, and oocytes in the oviduct are capable of being fertilized 5 days after the start of estrus [7, 21]. In the canine oviduct, sperm remains viable for at least 5 days [3]. Antioxidant enzymes are present in human [4], mouse [4] and bovine [14] oviductal fluid, and these enzymes are thought to protect sperm and oocytes against damage and oxidative stress caused by ROS in the female reproductive tract [14].

There are no reports about SOD activity of the reproductive tract in each phase of estrous cycle in the bitch. In this study, SOD activity in flushings from the oviducts and the uterine horns of anestrous, estrous and diestrous bitches was measured. In addition, SOD was added to canine sperm medium, and the effects on sperm viability, motility and hyperactivation of sperm were evaluated after 24 hr of incubation.

Oviducts and uterine horns from 8 anestrous, 5 estrous and 7 diestrous bitches (1–5 years old) were obtained by ovariohysterectomy (OVH) performed for contraceptive purposes at the Animal Medical Center of our university. Estrous

cycle stage was identified on the basis of vaginal bleeding and presence of large follicles in the removed ovaries. After OVH, right-side oviducts and uterine horns were flushed, and SOD activity in the flushings was measured. A 20- or 24-gauge retaining needle connected to a 1-ml injection syringe was inserted into the lumen of the uterus or oviduct. The lumen of the oviduct or uterus was flushed with 0.5 or 1.0 ml of HEPES solution, respectively. SOD activity in the flushings was measured with a SOD Assay Kit (Trevigen, Inc., Gaithersburg, MD, U.S.A.) as described previously [11]. Data were summarized as mean  $\pm$  standard error ( $\pm$  SE) U/ml. SOD activity in the oviduct or uterine horn flushings was compared between anestrous, estrous and diestrous bitches by one-way analysis of variance (ANOVA) to determine differences between the groups. When a significant difference was found by one-way ANOVA, Tukey-Kramer's post hoc test was used to perform intergroup comparisons. Statistical significance was set at  $P$  values less than 0.05.

Semen was collected by digital manipulation from 4 healthy dogs (2–6 years old) cared for in our university. Sperm was suspended to a concentration of  $1 \times 10^7$  sperm/ml in minimum essential medium (MEM) as a control at 38°C and in MEM supplemented with 30 units/ml SOD (Wako Pure Chemical Industries Co., Ltd., Osaka, Japan) (SOD-MEM). The concentrations of SOD added to the MEM were selected to reflect those measured in the oviduct flushings from the estrous and anestrous bitches. Sperm was incubated in loosely capped  $120 \times 15$  mm glass test tubes for 24 hr at 38°C in 5% CO<sub>2</sub> in air. The percentages of viable sperm, actively motile sperm [12] and sperm exhibiting a unique active, star-spin-like movement called hyperactivation [22] were determined 24 hr after the start of incubation. Data were summarized as mean percent ( $\pm$  SE) and compared using the paired- $t$  test. Statistical significance was set at  $P$  values less than 0.05.

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Table 1. Superoxide dismutase (SOD) activities (mean  $\pm$  SE) in flushings of oviducts or uterine horns removed from 8 anestrus, 5 estrous and 7 diestrous bitches

	SOD activity (U/ml)	
	Oviduct	Uterine horn
Anestrus	18.4 $\pm$ 1.0 <sup>a)</sup>	10.8 $\pm$ 1.0 <sup>a)</sup>
Estrus	29.4 $\pm$ 1.6 <sup>b)</sup>	78.2 $\pm$ 6.2 <sup>b)</sup>
Diestrus	21.3 $\pm$ 1.2 <sup>a)</sup>	214.6 $\pm$ 18.7 <sup>c)</sup>

a)–c) Values in the same column with different superscript letters differ significantly ( $P < 0.01$ ).

The mean SOD activity in flushings from the oviducts or uterine horns of the anestrus, estrous and diestrous bitches is shown in Table 1. Mean SOD activity in the oviduct fluid was significantly higher in the estrous bitches than in the anestrus or diestrous bitches ( $P < 0.01$ ). Likewise, mean SOD activity in the uterine horn fluid was significantly higher in the diestrous bitches than in the anestrus or estrous bitches ( $P < 0.01$ ) and in the estrous bitches than in the anestrus bitches ( $P < 0.01$ ).

Changes in the mean percentages of viable sperm, actively motile sperm and hyperactivated sperm 24 hr after incubation are shown in Fig. 1. The percentage of viable sperm, actively motile sperm and hyperactivated sperm was significantly higher in SOD-MEM than in MEM ( $P < 0.05$ , 0.05 and 0.01, respectively).

In the present study, SOD activity was detected in canine oviductal fluid and was found to be higher in estrous bitches than in anestrus bitches. This is in contrast to other estrous cycles, such as the bovine estrous cycle, where SOD activity in oviductal fluid remains constant throughout [14]. These findings suggest that the kinetics of SOD activity in the estrous cycle is species specific. We also showed that sperm viability, motility and hyperactivation were improved by SOD supplementation and hyperactivated sperm movement is known to be necessary for sperm to reach the oocyte in the oviduct and penetrate its cumulus cell layer [15, 19]. The mitochondria of human sperm are known to produce ROS [13], and increases in semen ROS level can cause male infertility [18]. Our results here demonstrated that, at least in dogs, antioxidant enzymes have a beneficial effect on semen quality and therefore the ability for fertilization. We assume that sperm in the oviduct of the estrous bitch are able to maintain active movement for several days as a result of not only glycosaminoglycans [10] and oviductal epithelial cell membrane proteins [8] but also SOD. Transcripts encoding antioxidant enzymes, such as Cu-Zn-SOD, are expressed in human [4], bovine [9, 14] and mouse [4] oviductal epithelial cells (OEC). The present and previous results [4, 9, 14] demonstrated that this SOD production in OEC may be induced by estradiol-17 $\beta$  secreted from the ovaries, especially in the estrous phase in bitches.

Furthermore, SOD activity in the uterine horn was increased in estrous bitches and further increased in diestrous bitches compared with anestrus bitches. SOD activity in the human endometrial epithelium is known to increase from the

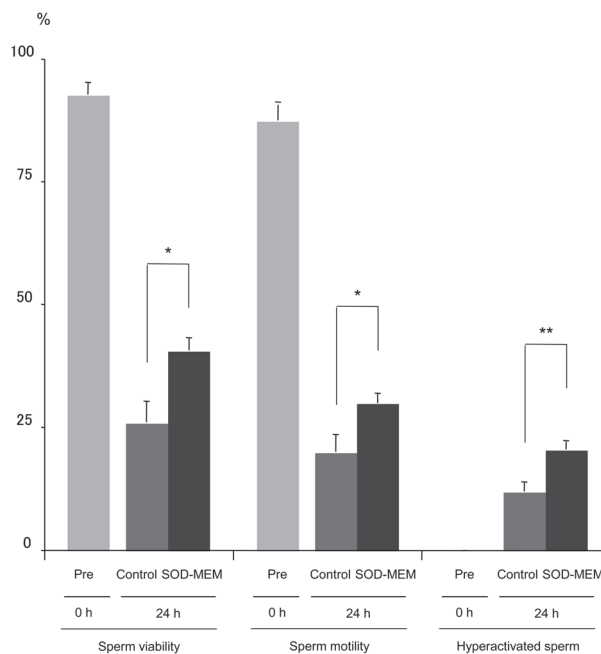


Fig. 1. Percentages of viable, motile and hyperactivated spermatozoa (mean  $\pm$  SE) before (Pre) and 24 hr after incubation in minimum essential medium (MEM; control) or MEM supplemented with 30 U/ml superoxide dismutase (MEM-SOD). \* $P < 0.05$  and \*\* $P < 0.01$  compared with control values.

early to mid-late proliferative phases of the menstrual cycle and increase further in the mid-secretory phase [16, 20]. Cu-Zn-SOD and Mn-SOD are expressed in the human endometrial epithelium throughout the menstrual cycle as well as in early pregnancy [20]. In addition, antioxidants including SOD have been reported to promote the *in-vitro* development of mouse embryos by protecting them from oxidative stress [17]. Therefore, SOD may also protect embryos from oxidative stress in dogs and allow for successful implantation or growth *in utero*.

In conclusion, we demonstrated that SOD activity in dogs differs on the basis of location or estrous phase, as evidenced by high SOD activity in the oviduct of estrous bitches and in the uterus of estrous and diestrous bitches. These findings indicate that SOD production increases in response to signaling by sex hormones from OEC or in the endometrial epithelium. SOD produced in the oviduct and uterus may be able to maintain or improve sperm quality or male fertility via protection from oxidative stress.

## REFERENCES

1. Alkan, I., Simsek, F., Haklar, G., Kervancioglu, E., Ozveri, H., Yalcin, S. and Akdas, A. 1997. Reactive oxygen species production by the spermatozoa of patients with idiopathic infertility: relationship to seminal plasma antioxidants. *J. Urol.* **157**: 140–143. [Medline] [CrossRef]
2. Chen, H., Chow, P. H., Cheng, S. K., Cheung, A. L., Cheng, L.

- Y. and O, W. S. 2003. Male genital tract antioxidant enzymes: their source, function in the female, and ability to preserve sperm DNA integrity in the golden hamster. *J. Androl.* **24**: 704–711. [Medline]
3. Concannon, P., Whaley, S., Lein, D. and Wissler, R. 1983. Canine gestation length: variation related to time of mating and fertile life of sperm. *Am. J. Vet. Res.* **44**: 1819–1821. [Medline]
  4. El Mouatassim, S., Guerin, P. and Menezo, Y. 2000. Mammalian oviduct and protection against free oxygen radicals: expression of genes encoding antioxidant enzymes in human and mouse. *Eur. J. Obstet. Gynecol. Reprod. Biol.* **89**: 1–6. [Medline] [CrossRef]
  5. Foote, R. H., Brockett, C. C. and Kaproth, M. T. 2002. Motility and fertility of bull sperm in whole milk extender containing antioxidants. *Anim. Reprod. Sci.* **71**: 13–23. [Medline] [CrossRef]
  6. Gavella, M., Lipovac, V., Vucic, M. and Rocic, B. 1996. Relationship of sperm superoxide dismutase-like activity with other sperm-specific enzymes and experimentally induced lipid peroxidation in infertile men. *Andrologia* **28**: 223–229. [Medline] [CrossRef]
  7. Holst, P. A. and Phemister, R. D. 1971. The prenatal development of the dog: preimplantation events. *Biol. Reprod.* **5**: 194–206. [Medline]
  8. Huang, V. W., Zhao, W., Lee, C. L., Lee, C. Y., Lam, K. K., Ko, J. K., Yeung, W. S., Ho, P. C. and Chiu, P. C. 2013. Cell membrane proteins from oviductal epithelial cell line protect human spermatozoa from oxidative damage. *Fertil. Steril.* **99**: 1444–1452.
  9. Jang, H. Y., Ji, S. J., Kim, Y. H., Lee, H. Y., Shin, J. S., Cheong, H. T., Kim, J. T., Park, I. C., Kong, H. S., Park, C. K., Yang, B. K. 2010. Antioxidative effects of astaxanthin against nitric oxide-induced oxidative stress on cell viability and gene expression in bovine oviduct epithelial cell and the developmental competence of bovine IVM/IVF embryos. *Reprod. Domest. Anim.* **45**: 967–974. [Medline] [CrossRef]
  10. Kawakami, E., Arai, T., Oishi, I., Hori, T. and Tsutsui, T. 2000. Induction of dog sperm capacitation by glycosaminoglycans and glycosaminoglycan amounts of oviductal and uterine fluids in bitches. *J. Vet. Med. Sci.* **62**: 65–68. [Medline] [CrossRef]
  11. Kawakami, E., Takemura, A., Sakuma, M., Takano, M., Hirano, T., Hori, T. and Tsutsui, T. 2007. Superoxide dismutase and catalase activities in the seminal plasma of normozoospermic and asthenozoospermic Beagles. *J. Vet. Med. Sci.* **69**: 133–136. [Medline] [CrossRef]
  12. Kawakami, E., Tsutsui, T., Yamada, Y. and Yamauchi, M. 1984. Cryptorchidism in the dog: occurrence of cryptorchidism and semen quality in the cryptorchid dog. *Jpn. J. Vet. Sci.* **46**: 303–308. [Medline] [CrossRef]
  13. Koppers, A. J., De Iuliis, G. N., Finnie, J. M., McLaughlin, E. A. and Aitken, R. J. 2008. Significance of mitochondrial reactive oxygen species in the generation of oxidative stress in spermatozoa. *J. Clin. Endocrinol. Metab.* **93**: 3199–3207. [Medline] [CrossRef]
  14. Lapointe, J. and Bilodeau, J. F. 2003. Antioxidant defenses are modulated in the cow oviduct during the estrous cycle. *Biol. Reprod.* **68**: 1157–1164. [Medline] [CrossRef]
  15. Meyers, S. A., Yudin, A. I., Cherr, G. N., VandeVoort, C. A., Myles, D. G., Primakoff, P. and Overstreet, J. W. 1997. Hyaluronidase activity of macaque sperm assessed by an *in vitro* cumulus penetration assay. *Mol. Reprod. Dev.* **46**: 392–400. [Medline] [CrossRef]
  16. Narimoto, K., Noda, Y., Shiotani, M., Tokura, T., Goto, Y., Takakura, K., Mori, T., Fujimoto, K. and Ogawa, K. 1990. Immunohistochemical assessment of superoxide dismutase expression in the human endometrium throughout the menstrual cycle. *Acta Histochem. Cytochem.* **23**: 487–498. [CrossRef]
  17. Nonogaki, T., Noda, Y., Narimoto, K., Umaoka, Y. and Mori, T. 1991. Protection from oxidative stress by thioredoxin and superoxide dismutase of mouse embryos fertilized *in vitro*. *Hum. Reprod.* **6**: 1305–1310. [Medline]
  18. Padron, O. F., Brackett, N. L., Sharma, R. K., Lynne, C. M., Thomas, A. J. Jr. and Agarwal, A. 1997. Seminal reactive oxygen species and sperm motility and morphology in men with spinal cord injury. *Fertil. Steril.* **67**: 1115–1120. [Medline] [CrossRef]
  19. Suarez, S. S., Katz, D. F. and Overstreet, J. W. 1983. Movement characteristics and acrosomal status of rabbit spermatozoa recovered at the site and time of fertilization. *Biol. Reprod.* **29**: 1277–1287. [Medline] [CrossRef]
  20. Sugino, N., Shimamura, K., Takiguchi, S., Tamura, H., Ono, M., Nakata, M., Nakamura, Y., Ogino, K., Uda, T. and Kato, H. 1996. Changes in activity of superoxide dismutase in the human endometrium throughout the menstrual cycle and in early pregnancy. *Hum. Reprod.* **11**: 1073–1078. [Medline] [CrossRef]
  21. Tsutsui, T. and Shimizu, T. 1975. Studies on the reproduction of the dog. IV. On the fertile period of ovum after ovulation. *Jpn. J. Anim. Reprod.* **21**: 65–69. [CrossRef]
  22. Yanagimachi, R. 1970. The movement of golden hamster spermatozoa before and after capacitation. *J. Reprod. Fertil.* **23**: 193–196. [Medline] [CrossRef]