


A Thesis

For The Degree of Master of Veterinary Medicine

Expression of Nitric Oxide Synthase  
Isoforms in the Ovaries of Landrace Pigs

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# Expression of Nitric Oxide Synthase Isoforms in the Ovaries of Landrace Pigs

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(Supervised by Professor Taekyun Shin)

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## Abstract

# Expression of Nitric Oxide Synthase Isoforms in the Ovaries of Landrace Pigs

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The expression of nitric oxide synthase (NOS) isoforms in the ovaries of pigs was examined to study the involvement of nitric oxide, a product of NOS activity, in the function of the ovary. Western blot analysis detected three types of NOS in the ovary, including constitutive neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS); eNOS

immunoreactivity was more intense compared with that of iNOS or nNOS.

Immunohistochemical studies demonstrated the presence of nNOS and eNOS in the surface epithelium, stroma, oocytes, and thecal cells. Positive immunoreactions for nNOS and iNOS were detected in the granulosa cells from multilaminar and antral follicles, but not in those of unilaminar follicles. Immunoreactivity for eNOS was also detected in the endothelial cells of blood vessels and in granulosa cells. iNOS was detected in the surface epithelium, oocytes, and theca of multilaminar and antral follicles.

Taking all of the findings into consideration, the observed differential expression of the three NOS isoforms in the ovary suggests a role for nitric oxide in modulating reproduction in pigs.

Key words: porcine, nitric oxide synthase, ovary

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# I. Introduction

Nitric oxide (NO) is a reactive free radical gas that is derived from L-arginine by the action of NO synthase (NOS) (Xie and Nathan, 1994). NO has diverse roles including intracellular signaling and vasoregulation (Bredt and Snyder, 1992), and exists in a variety of isoforms. A constitutive, calcium-dependent isoform (cNOS) is activated rapidly by agonists that elevate intracellular free calcium and is found in endothelial cells (eNOS) and the brain (nNOS) (Moncada et al., 1991). A calcium-independent inducible isoform (iNOS) can be induced after several hours of immunological stimulation and is detectable in macrophages, neutrophils, and endothelial cells (Knowles and Moncada, 1992).

Several studies have identified the presence of different isoforms of NOS in female reproductive tissues, including the ovary (Zackrisson et al., 1996), oviduct (Bryant et al., 1995), and uterus (Purcell et al., 1999). In addition, nitric oxide is known to be an important factors in the physiology and pathophysiology of reproduction (Rosselli et al., 1998).

In pigs, the expression of iNOS and eNOS has been

studied in ovaries (Takesue et al., 2003; Tao et al., 2004), in which iNOS was shown to be mainly localized in the oocytes, cumulus cells, and corpus luteum (Tao et al., 2004), whereas eNOS was detected by immunostaining in oocytes, granulosa cells, cumulus cells, corpus luteum, and corpus albicans (Hattori et al., 2004; Takesue et al., 2003; Tao et al., 2004). Recently, many studies have suggested that nNOS, one of the constitutive isoforms of NOS, is found in non-neuronal cells, including macrophages (Kim et al., 2000). This implies that nNOS, in addition to eNOS and iNOS, may contribute to the physiology of the ovary. However, little is known about the expression of nNOS in the ovary. The aim of this study, therefore, was to compare the expression patterns of eNOS, iNOS, and nNOS in the porcine ovary during follicular development in order to elucidate the phenotype of the cells in which each NOS isoform is expressed.

## II. Materials and Methods

### 1. Animals and tissue sampling

Ovary samples were collected from 6-month-old Landrace pigs at a local slaughterhouse, excluding pigs that were visually assessed as pregnant. Immediately after collection of each ovary, 0.5 cm pieces were dissected and placed at  $-70^{\circ}\text{C}$  until they were used for Western blotting analysis. Additional tissue pieces were processed for paraffin embedding after fixation in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4.

### 2. Histological analysis

Ovary tissues were sectioned ( $5\ \mu\text{m}$ ), deparaffinized in xylene, and rehydrated through a graded ethanol series to distilled water before staining with hematoxylin and eosin.



### 3. Follicle classification

Ovarian follicles were divided into three classes as described previously (Van den Hurk and Van de Pavert, 2001): (1) unilaminar follicles (with one layer of granulosa cells), (2) multilaminar follicles (with multiple granulosa cell layers), and (3) antral follicles (with multiple granulosa cell layers enclosing an antrum). Nonatretic antral follicles had an intact membrana granulosa and no invagination of the theca layer into the granulosa layer. Atretic antral follicles had a thinner, fragmented granulosa cell layer.



### 4. Antibodies

The antibodies used in this study were as follows: mouse monoclonal anti-endothelial nitric oxide synthase (eNOS) antibody, rabbit anti-inducible nitric oxide synthase (iNOS) antisera, and rabbit anti-neuronal nitric oxide synthase (nNOS) antisera (all from Transduction Laboratories, Lexington, KY).

## 5. Western blot analysis

Samples of porcine ovary were dissected free of extraneous tissue, homogenized in lysis buffer (20 mM HEPES, pH 7.2, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 150 mM NaCl, 10  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride) and then centrifuged. The proteins were resolved by sodium dodecylsulfate polyacrylamide gel electrophoresis (8% SDS-PAGE) and blotted onto a nitrocellulose transfer membranes (Schleicher and Schuell, Keene, NH). The membranes were probed with anti-eNOS monoclonal antibody, rabbit anti-iNOS, or rabbit anti-nNOS antisera diluted 1:1000 in blocking solution. The antibodies used in the present study had been characterized in our previous study (Kim et al., 2000). The reaction was visualized by labeling with horseradish peroxidase-conjugated horse anti-mouse IgG or anti-rabbit IgG secondary antibody (Vector Labs, Burlingame, CA). The peroxidase reaction was developed with Amersham ECL reagents (Amersham, Arlington Heights, IL).

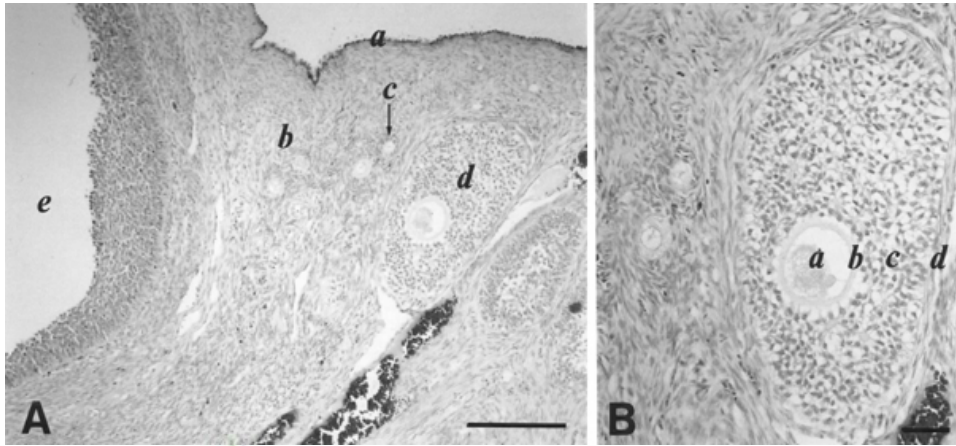
## 6. Immunohistochemistry

Immunostaining for eNOS, iNOS, and nNOS was performed as described previously (Kim et al., 2000). Briefly, paraffin-embedded sections (5  $\mu\text{m}$ ) of porcine ovary were deparaffinized and treated with citrate buffer (0.01 M, pH 6.0) in a microwave for 2 min. The sections were treated with 0.3% hydrogen peroxide in methyl alcohol for 20 min to block endogenous peroxidase activity. After three washes in phosphate-buffered saline (PBS), the sections were incubated with 10% normal horse or goat serum and thereafter incubated with mouse anti-eNOS antibody, rabbit anti-iNOS, or rabbit anti-nNOS antisera (1:200 dilution) for 1 h at room temperature. After three washes in PBS, the appropriate biotinylated secondary antibody and avidin-biotin peroxidase complex (Vector Elite; Vector Labs, Burlingame, CA) were added sequentially. The peroxidase reaction was developed with diaminobenzidine as a substrate. Before being mounted, the sections were counterstained with hematoxylin.

### III. Results

#### 1. Histologic structure of the ovary

The ovarian tissue was divided into an outer cortex and an inner medulla (Fig. 1). A simple squamous or cuboidal epithelium (Fig. 1A-a) covered the cortex of the ovary. The cortical stroma (Fig. 1A-b) contained the ovarian follicles. Unilaminar follicles (Fig. 1A-c), multilaminar follicles (Fig. 1A-d, B), and antral follicles (Fig. 1Ae) were seen in the cortex. From the interior to exterior, the multilaminar follicle was comprised of the oocyte (Fig. 1B-a), zona pellucida (Fig. 1B-b), granulosa cells (Fig. 1B-c), and theca (Fig. 1B-d).

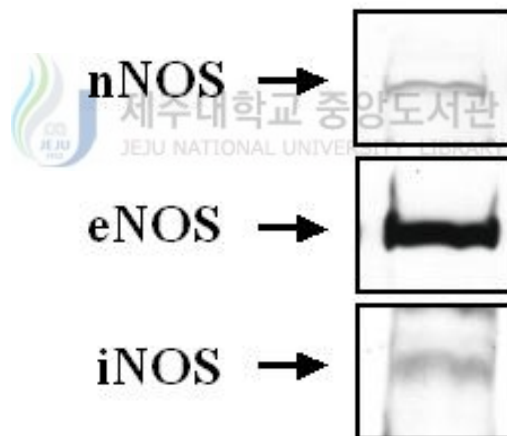



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**Figure 1.** Histological findings in porcine ovaries. A, Surface epithelium (a); stroma (b); unilaminar follicle (c); multilaminar follicle (d); antral follicle (e). B, Multilaminar follicle, oocyte (a); zona pellucida (b); granulosa cells (c); theca (d). A and B were stained with hematoxylin and eosin. Scale bars: in A, 200  $\mu\text{m}$ ; in B, 50  $\mu\text{m}$ .

## 2. Western blot analysis of three isoforms of NOS in the ovary

The expression levels of nNOS, eNOS, and iNOS were assessed semiquantitatively by densitometry after Western blotting. Immunoreactivity for all three isoforms of NOS was detected in the porcine ovary; in particular, eNOS immunoreactivity was more intense relative to that of iNOS or nNOS (Fig. 2).



**Figure2.** Western blot analysis for expression of nNOS, eNOS and iNOS in porcine ovaries. Arrows indicate the position of nNOS (155 kDa), eNOS (140 kDa), and iNOS (130 kDa) respectively.

### 3. Immunohistochemical localization of nNOS, eNOS, and iNOS in the ovary

Expression of nNOS was detected in the surface epithelial cells and stromal cells (Fig. 3A). In the unilaminar, multilaminar, and antral follicles, nNOS immunoreactivity was localized to the oocytes. Immunostaining for nNOS was present in the granulosa cells of multilaminar follicles, but was absent in those of unilaminar follicles (Fig. 3D). Moreover, a positive immunoreaction for nNOS was observed in the theca of multilaminar follicles (Fig. 3D). The expression of nNOS in the theca and granulosa cells of antral follicles (Fig. 4A) was strong compared with that in multilaminar follicles (Fig. 3A). In atretic follicles, nNOS immunoreactivity was localized to the fibrous theca layer.

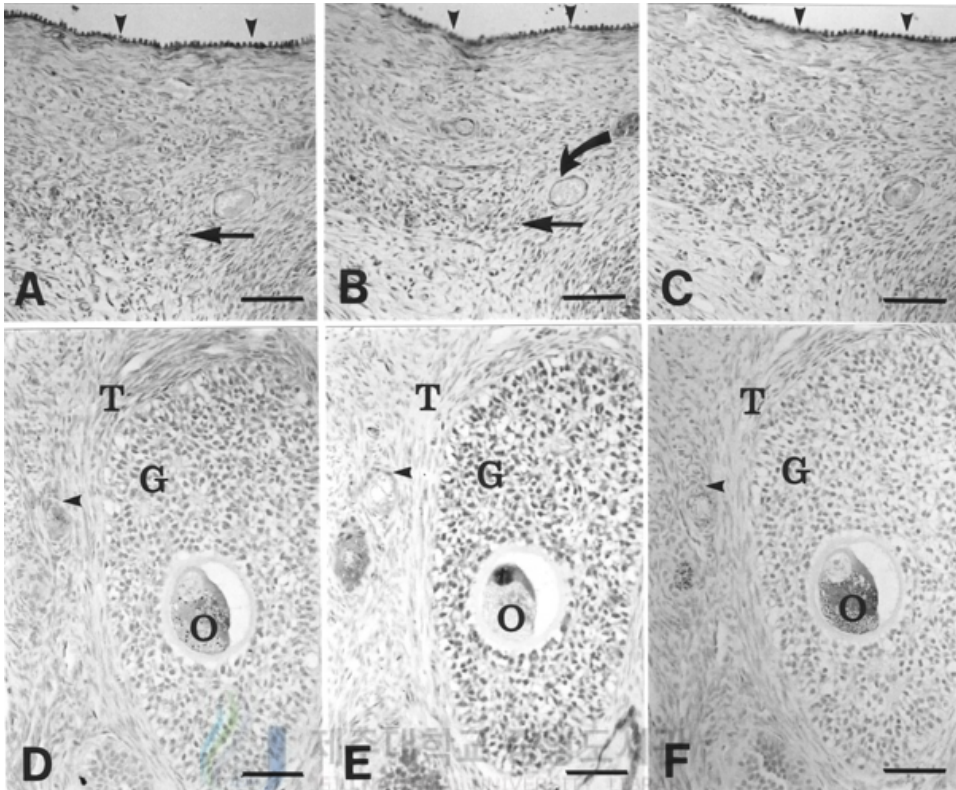
The immunostaining pattern of eNOS was largely the same as that of nNOS; however, eNOS was additionally detected in the granulosa cells of unilaminar follicles and in the endothelial cells of blood vessels (Fig. 3B, E; Fig. 4B).

Expression of iNOS was detected in surface epithelial cells (Fig. 3C). In the unilaminar, multilaminar, and antral follicles, the iNOS immunoreactivity was localized to the oocytes. Immunostaining for iNOS was weakly detected in the granulosa cells of multilaminar follicles, but was not detected

in those of unilaminarfollicles (Fig. 3F). A positive immunoreaction for iNOS was present in the theca of atretic follicles (Fig. 4C), but was absent in those of multilaminar follicles (Fig. 3C). In atretic follicles, iNOS immunoreactivity was localized to the fibrous theca layer (Table. 1).



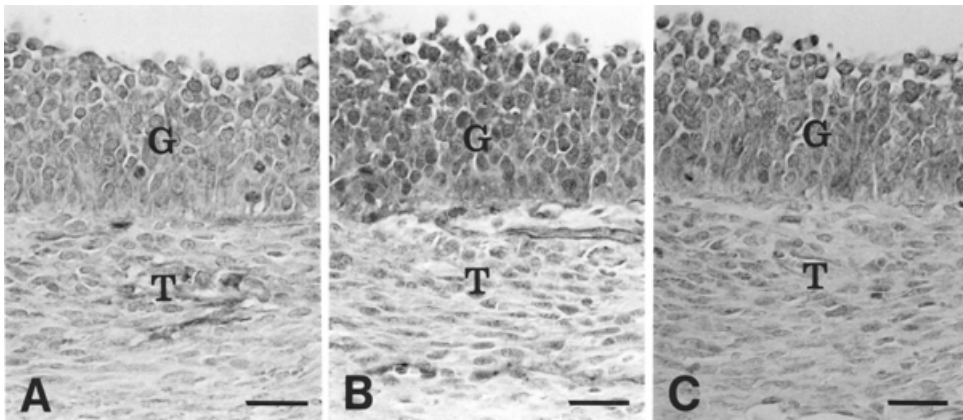




**Figure 3.** Immunohistochemical localization of nNOS (A, D), eNOS (B, E), and iNOS (C, F) in porcine ovaries. G, granulosa; O, oocyte; T, theca. nNOS (A) and eNOS (B) were expressed in the surface epithelial cells (arrowheads) and stroma cells (straight arrow). Only eNOS was expressed in the vascular endothelial cells (curved arrow). iNOS (C) was also expressed in the surface epithelial cells (arrowheads). In unilaminarfollicles, nNOS (D), eNOS (E), and iNOS (F) were expressed in the oocyte, and eNOS was expressed in the granulosa cells (E, arrowhead), while nNOS (D, arrowhead)

and iNOS (F, arrowhead) showed no immunoreactivity in granulosa cells. nNOS (D) and eNOS (E) were expressed in the granulosa cells, oocytes, and theca interna of multilaminar follicles. iNOS (F) was expressed in the granulosa cells and oocytes of multilaminar follicles. A-F: Counterstained with hematoxylin. Scale bars = 60  $\mu$ m.





**Figure 4.** Immunohistochemical localization of nNOS (A), eNOS (B), and iNOS (C) in the antral follicle. G, granulosa; T, theca. nNOS (A), eNOS (B), and iNOS (C) were expressed in the granulosa cells and theca of antral follicles. A-C: Counterstained with hematoxylin. Scale bars = 30  $\mu$ m.

**Table 1.** Immunohistochemical localization of neuronal (nNOS), endothelial (eNOS), and inducible (iNOS) isoforms of nitric oxide synthase (NOS) in the ovaries of pigs. The intensity of staining is indicated by (-), where staining was absent, up to (+++), for maximal staining.

Tissue / Cell type		nNOS	eNOS	iNOS
Surface epithelium		+	+	+
Intersitium	Stroma	+++	++	-
	Blood vessels			
	Endothelia	-	+	-
	Tunica media	-	-	-
Unilaminar follicles	Oocyte	+	+	+
	Granulosa cell	-	+	-
Multilaminar follicles	Oocyte	+	+	+
	Granulosa cell	++	+++	+
	Theca	+	+	-
Antral follicles	Oocyte	+	+	+
	Granulosa cell	+++	+++	++
	Theca	+	++	+
Atretic follicles	Fibrosed theca	+	+	+

## IV. Discussion

This study is the first to demonstrate that three isoforms of NOS, including nNOS, eNOS, and iNOS, are expressed in porcine ovaries during follicular development. There is a general consensus that each NOS isoform is expressed in the ovarian follicles of pigs (Takesue et al., 2003; Tao et al., 1996). It has been shown that within large-sized follicles (7-10 mm in diameter) of porcine ovaries, eNOS was expressed in the oocytes, vascular endothelial cells, granulosa cells, theca cells, and cumulus cells; but no eNOS immunoreactivity was observed in the cumulus cells of medium follicles (3-6 mm in diameter) (Tao et al., 2004). This suggests that eNOS expression is associated with stages of ovarian follicular development in pigs. In the present study, the observed patterns of eNOS immunostaining in the ovary were largely consistent with those of previous reports (Takesue et al., 2003; Tao et al., 2004).

Although the expression of iNOS in porcine ovaries is well known, our findings contrast in part with the previous report (Tao et al., 2004). In the present study, iNOS was mainly localized to the oocytes of unilaminar and multilaminar follicles, and to granulosa and theca cells. However, it was

previously reported that iNOS, particularly in large follicles, was localized to the oocytes and cumulus cells (Tao et al., 2004). This discrepancy might be a result of the different antisera used in the present study or a difference in the immunodetection systems used.

NOS has diverse functional roles in the ovary. The expression of NOS in the ovarian follicles implies that nitric oxide, generated from iNOS, is involved in the ovulatory process in rats (Shukovski and Tsafirri, 1994). This interpretation is further supported by the observation that inhibition of iNOS reduced ovulation by a maximum of 54% (Shukovski et al., 1994). In addition, it is also suggested that eNOS (Rosselli et al., 1998; Shukovski and Tsafirri, 1994) and nNOS, from the present findings, also participate in the process of ovulation.

The eNOS and iNOS isoforms (but not nNOS) have previously been immunolocalized to mammalian ovaries (Jablonka-Shariff and Olson, 1997; Tao et al., 2004; Van Voorhis et al., 1995). In this study, nNOS immunoreactivity was observed in the stroma, oocytes, theca cells, and granulosa cells of multilaminar and antral follicles. Recently, it has been accepted that nNOS is expressed in non-neuronal cells, including macrophages. However, the exact role that

nNOS plays in the ovary remains to be determined.

The findings, together with previous research, indicate that the expression of NOS is in part dependent on the stage of ovarian follicle development. At the early stage of follicular development, little NOS immunostaining was detected in granulosa or theca cells. In the later stages, including Graafian follicles, immunostaining for three isoforms of NOS was detected in the granulosa and theca cells. This finding suggests that, in the porcine ovary, granulosa and theca cells may serve as sources of nitric oxide.

In conclusion, this study revealed that three isoforms of NOS were expressed in the porcine ovary, suggesting that nitric oxide might be involved in the process of follicular development and/or the ovulatory process.

## V. References

Bredt, D.S. and Snyder, S.H. 1992. Nitric oxide, a novel neuronal messenger. *Neuron* 8, 3-11.

Bryant, C.E., Tomlinson, A., Mitchell, J. A., Thiemermann, C. and Willoughby, D.A. 1995. Nitric oxide synthase in the rat fallopian tube is regulated during the oestrous cycle. *J. Endocrinol.* 146, 149-157.

Hattori, M.A., Arai, M., Saruwatari, K. and Kato, Y. 2004. Estrogen regulation of nitric oxide synthesis in the porcine oocyte. *Mol. Cell. Biochem.* 260, 13-19.

Jablonka-Shariff, A. and Olson, L.M. 1997. Hormonal regulation of nitric oxide synthases and their cell-specific expression during follicular development in the rat ovary. *Endocrinology* 138, 460-468.

Kim, S., Moon, C., Wie, M.B., Kim, H., Tanuma, N., Matsumoto, Y. and Shin, T. 2000. Enhanced expression of constitutive and inducible forms of nitric oxide synthase in autoimmune encephalomyelitis. *J. Vet. Sci.* 1, 11-17.

Knowles, R.G. and Moncada, S. 1994. Nitric oxide synthases in mammals. *Biochem. J.* 298, 249-258.

Moncada, S., Palmer, R.M.J. and Higgs, E.A. 1991. Nitric oxide: Physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.* 43, 109-134.

Purcell, T.L., Given, R., Chwalisz, K. and Garfield, R.E. 1999.



Nitric oxide synthase distribution during implantation in the mouse. *Mol. Hum. Reprod.* 5, 467-475.

Rosselli, M., Keller, P.J. and Dubey, R.K. 1998. Role of nitric oxide in the biology, physiology and pathophysiology of reproduction. *Hum. Reprod. Update* 4, 3-24.

Shukovski, L. and Tsafiriri, A. 1994. The involvement of nitric oxide in the ovulatory process in the rat. *Endocrinology* 135, 2287-2290.

Takesue, K., Tabata, S., Sato, F. and Hattori, M.A. 2003. Expression of nitric oxide synthase-3 in porcine oocytes obtained at different follicular development. *J. Reprod. Dev.* 49, 135-140.

Tao, Y., Fu, Z., Zhang, M., Xia, G., Yang, J. and Xie, H. 2004. Immunohistochemical localization of inducible and endothelial nitric oxide synthase in porcine ovaries and effects of NO on antrum formation and oocyte meiotic maturation. *Mol. Cell. Endocrinol.* 222, 93-103.

Van den Hurk R. and Van de Pavert S.A. 2001. Localization of an activin/activin receptor system in the porcine ovary. *Mol. Reprod. Dev.* 60, 463-471.

Van Voorhis, B.J., Moore, K., Strijbos, P.J., Nelson, S., Baylis, S.A., Grzybicki, D. and Weiner, C.P. 1995. Expression and localization of inducible and endothelial nitric oxide synthase in the rat ovary. Effects of gonadotropin stimulation in vivo. *J. Clin. Invest.* 96, 2719-2726.

Xie, Q.W. and Nathan, C. 1994. The high-output nitric oxide

pathway: role and regulation. *J. Leukoc. Biol.* 56, 576-582.

Zackrisson, U., Mikuni, M., Wallin, A., Delbro, D., Hedin, L. and Brannstrom, M. 1996. Cell-specific localization of nitric oxide synthases (NOS) in the rat ovary during follicular development, ovulation and luteal formation. *Hum. Reprod.* 11, 2667-2673.



초 록

# 돼지 난소내 Nitric oxide synthase isoforms의 발현

(지도교수 : 신 태 균)

김 희 철

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돼지 난소에서 nitric oxide synthase (NOS)의 최종 생산물인 NO가 관여하는지 연구하기 위하여, 돼지 난소에서 NOS isoforms의 발현을 조사하였다.

Western blot 결과 상존형인 neuronal NOS (nNOS)와 endothelial NOS (eNOS) 그리고 유도형인 inducible NOS (iNOS)가 돼지 난소에서 관찰되었고, eNOS의 면역 반응이 iNOS와 nNOS보다 강하게 발현되었다.

면역조직화학결과 nNOS와 eNOS는 모든 발육 단계의 난포에서 난소표면상피세포, 기질세포, 난모세포, 난포막세포에서 발현하였다. nNOS와 iNOS는 다층난포와 강난포의 과립막세포에서 발현하였지만, 단층난포의 과립막세포에서는 발현하지 않았

으며, 반면에 eNOS는 혈관내피세포와 단층난포에서 강난포까지의 과립막세포에서 발현하였다. 또한 iNOS는 난소표면상피와 난모세포, 그리고 다층난포와 강난포의 난포막세포에서 발현하였다.

이상의 결과 난소에서 NOS isoforms의 다른 발현을 통해, 생식조절에서 nitric oxide가 중요한 역할을 할 것으로 생각된다.

주요어 : 돼지, nitric oxide synthase, 난소



## 감사의 글

어느덧 한 해를 마무리하는 시기가 왔습니다.

수의학과를 졸업하고 석사과정에 입문하여, 이제 작은 결실을 이루었습니다. 2001년도 대학졸업하고, 바로 대학원에 입학하여 철없이 지내던 중 군대를 갔다 오고, 2003년 8월에 제대하여 지금까지 공부를 하면서, 인격적으로 미숙한 저를 인성교육을 해주시고 학문적으로 많은 가르침을 주신 신태균 교수님께 감사드립니다. 그리고, 저의 논문에 조언을 아끼지 않으신 지영훈 교수님과 끝까지 정성껏 지도해주신 이용덕 교수님께 감사드립니다.

제가 수의학이란 학문을 접하기 시작하여 지금까지 저에게 많은 가르침을 주신 김희석 교수님, 박진홍 교수님, 배종희 교수님, 이경갑 교수님, 임윤규 교수님, 이영재 교수님, 우호춘 교수님, 손원근 교수님, 이주명 교수님, 강태영 교수님, 윤영민 교수님, 주홍구 교수님, 이국경 교수님, 김재훈 교수님, 박현정 교수님, 그리고 지금 미국에 계신 이두식 교수님, 정종태 교수님께도 감사의 마음을 전합니다.



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