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Expression of nitric oxide synthase in the testis and epididymis of horse

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Abstract

Expression of nitric oxide synthase in the testis and epididymis of horse

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This study examined the expression of three isoforms of nitric oxide synthase (NOS) in the testis and epididymis of Thoroughbred horse.

Western blot analysis showed that constitutive endothelial NOS (eNOS) and neuronal NOS (nNOS), and inducible NOS (iNOS)

were differentially expressed in the testis and epididymis.

Immunohistochemical studies demonstrated the presence of eNOS immunostaining in some germ cells in the seminiferous tubules and in vascular endothelial cells in the interstitial tissues. Interstitial cells, most likely Leydig cells, were also intensely immunopositive for eNOS. The pattern of immunostaining for nNOS was similar to that for eNOS in the testis. Weak expression of iNOS was detected in the seminiferous tubules of the testis, but intense expression was found in interstitial cells. iNOS was also strongly detected in the stereocilia, spermatocytes, epithelium and connective tissue of the epididymis of normal horses.

These findings suggest that three isoforms of NOS are expressed in the testis and epididymis of horse and that they play important roles in the biology of interstitial cells that produce testosterone, as well as in spermatogenesis in the seminiferous tubules.

Keywords: Nitric oxide synthase, Leydig cells, horse, spermatogenesis

CONTENTS

Ⅰ. Introduction

Nitric oxide (NO) is a short-lived free radical with biological functions in the nervous, cardiovascular, and immune systems (Moncada *et al*., 1991; Bredt and Snyder, 1992). NO is synthesized from L-arginine by activation of the enzyme nitric oxide synthase (NOS). NOS exists in two forms: (1) constitutive, Ca^{2+}-dependent forms that are rapidly activated by agonists that elevate intracellular free Ca^{2+}, including neuronal NOS (nNOS) and endothelial NOS (eNOS); and (2) a Ca^{2+}-independent inducible form (iNOS) (Moncada *et al*., 1991; Xie and Nathan, 1994). nNOS has been shown to be constitutively expressed in a variety of cell types including testicular interstitial cells (Wang *et al*., 2002) and neuronal cells. Like nNOS, eNOS is expressed in many cell types in addition to the vascular endothelium in which it was first identified. eNOS is also known to be associated with a variety of inflammatory processes (Cirino *et al.*, 2003). These phenomena imply that both constitutive nNOS and eNOS are normally expressed in cells other than the neuronal and vascular endothelial cells in which they were respectively first detected. In contrast to the low level of NO generated by constitutive NOS, iNOS produces high levels (nanomolar quantities) of NO in various cell types when expression is activated (Xie *et al*., 1992). NOS is known to play both beneficial and detrimental roles,

depending on the cell activation status. It is generally accepted that excess NO production, induced mainly by iNOS, causes tissue damage, whereas constitutive nNOS and eNOS function in normal physiological events, such as regulation of the microcirculation, synaptic plasticity, and neuroprotective processes (Iadecola, 1993; Dawson and Dawson, 1996).

Some reports indicate that NOS may be involved in the normal biosynthesis and secretion of the steroid hormones in the male reproductive system, demonstrating direct effects on the function of interstitial cells in the testis (Welch *et al.*, 1995; Sengoku *et al.*, 1998). On the other hand, excessive NOS may induce the production of large amounts of NO metabolites in response to a variety of stressors, possibly reducing the survival rate and motility of sperm cells (Rosselli *et al.*, 1995). High levels of NO may also reduce testosterone secretion, as indicated by the suppression of testosterone secretion in male rats treated with the NO donor, isosorbide dinitrate (ISDN) (Adams *et al.*, 1994). In addition, it has been reported that normal human spermatozoa exhibit eNOS immunostaining that correlates with sperm motility (O'Bryan *et al.*, 1998), and nNOS immunoreactivity has also been detected in normal rat epididymis (Dun *et al.*, 1996). The finding that iNOS was expressed in normal tissue as well as inflamed tissue in the rat testis suggests a unique role for NOS in the male reproductive organs (O'Bryan *et al.*, 2000).

Thus, although NOS is known to be involved in the physiology of the reproductive system, little is known about the expression patterns of the three isoforms of NOS found in the testis and epididymis of the horse. This study used Western blot analysis and immunohistochemistry to examine the expression of constitutive eNOS, nNOS, and iNOS in the testis and epididymis of the horse.

Ⅱ. Materials and Methods

1. Animals

Seven horses (2-year-old thoroughbreds) were kindly supplied by the stud farm of the Korea Racing Association (Jeju, Korea). The testis of each horse were surgically removed under local anesthesia.

2. Antibodies

The following monoclonal antibodies (mAb) were used in this study: mouse anti-eNOS, mouse anti-nNOS, and mouse anti-iNOS antibodies (all from Transduction Laboratories, Lexington, KY); and mouse anti-β-actin antibody (Sigma, St. Louis, MO).

3. Tissue sampling

The horses were castrated by surgical removal of the testis and epididymis. The tissues were dissected and samples were frozen

at −70℃ for protein analysis. Additional samples were processed for paraffin embedding after fixation in 10% buffered formalin for 48 hrs to prepare for histological examination.

4. Western blot analysis

The frozen tissue samples were thawed at room temperature, minced, lysed in a buffer consisting of 40 mM Tris-HCl, pH 7.4, 120 mM NaCl, and 0.1% Nonidet P-40 containing the protease inhibitors leupeptin (0.5 μg/ml), PMSF (1 mM), and aprotinin (5 μg/ml), and then homogenized. Samples were electrophoresed under denaturing conditions on 7.5% SDS-polyacrylamide gels, and the separated proteins were transferred to PROTRAN nitrocellulose transfer membrane (Schleicher and Schuell, Keene, NH). eNOS, nNOS, and iNOS were detected using mouse monoclonal anti-eNOS, mouse monoclonal anti-nNOS, and mouse monoclonal anti-iNOS antibodies diluted 1:5000 with TBS-T, respectively. The reaction was visualized by labeling with horseradish peroxidase-conjugated horse anti-mouse IgG secondary antibody (Vector, Burlingame, CA), followed by reaction with Amersham ECL reagents (Arlington Heights, IL). The immunoblot membranes were re-probed with a monoclonal antibody to β-actin using the methods described above. The immunoblot signals were quantitated using a densitometer (M

GS-700 imaging densitometer, Bio-Rad, CA).

5. Immunohistochemistry

Deparaffinized tissue sections were treated with 0.3% hydrogen peroxide in deionized water for 20 min to block endogenous peroxidase. After three washes with PBS, the sections were exposed to 10% normal horse serum, and then incubated with primary antibodies, either mouse monoclonal anti-eNOS, mouse monoclonal anti-nNOS, or mouse monoclonal anti-iNOS antibodies (diluted 1:200), for 1 hr at RT. After three washes, the appropriate biotinylated secondary antibody and the avidin-biotin-peroxidase complex (ABC) from the *Elite* kit (Vector, Burlingame, CA) were added sequentially. Immunostaining was developed with diaminobenzidine (DAB)-hydrogen peroxidase solution (0.001% 3,3′-diaminbenzidine and 0.01% hydrogen peroxidase in 0.05M Tris buffer). The sections were counterstained with hematoxylin before being mounted.

Ⅲ. Results

1. Histological findings

Histological examination confirmed that all testes from normal horses showed no pathological changes, and these were used for further study (Fig. 1). The testis includes the seminiferous tubules, consisting of basal cells, spermatocytes, spermatids, and spermatozoa (found in the lumen of the tubules). Interstitial tissue is present among seminiferous tubules, including Leydig cells, fibrocytes, free mononuclear cells, blood and lymph vessels. The mammalian epididymis is a dynamic accessory sex organ, dependent on testicular androgens for the maintenance of the differentiated state of its epithelium. Macroscopically, the epididymis is divided into a head (caput epididymis), body (corpus epididymis), and tail (cauda epididymis). It is surrounded by a thick tunica albuginea of dense irregular connective tissue covered by the visceral layer of tunica vaginalis.
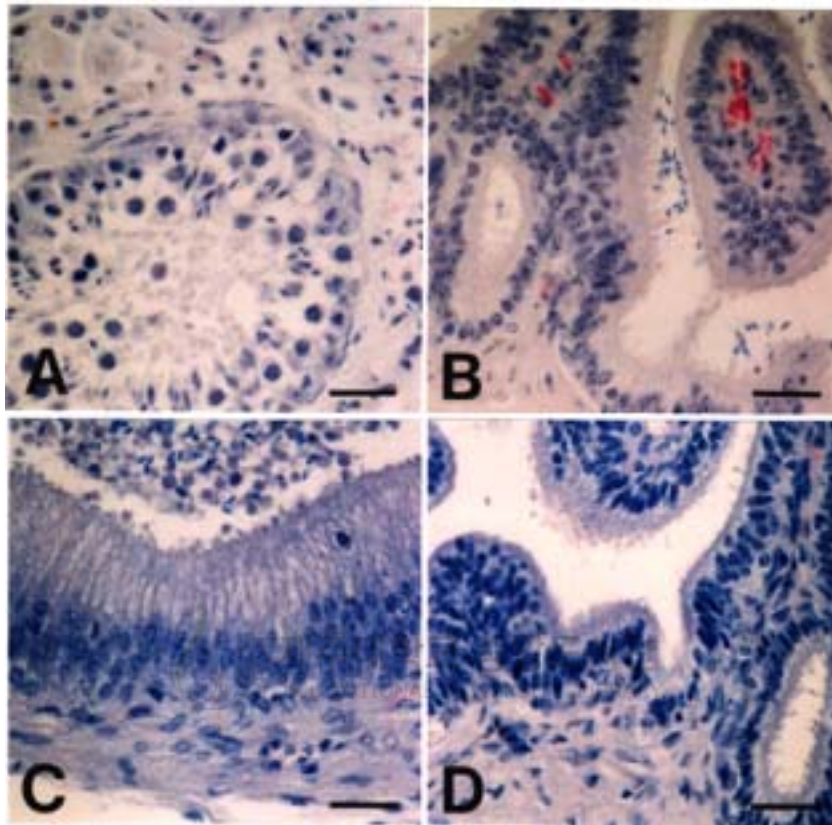
Figure 1. Histological findings in the male reproductive system of thoroughbred horses. There was no inflammation in the testis (A), caput epididymis (B), corpus epididymis (C), or cauda epididymis (D). Hematoxylin-eosin staining. Scale bar = 30 μm.

2. Western blot analysis of eNOS, nNOS, and iNOS in the reproductive system

Expression of eNOS, nNOS, and iNOS was confirmed in the testis and epididymis of the horse (Fig. 2A-2C). The expression of eNOS in the testis and corpus epididymis (Fig 2A, lanes 1 and 3) of normal horses was stronger than expression in the caput and cauda epididymis (Fig. 2A, lanes 2 and 4). The expression of nNOS was most strongly detected in the corpus epididymis (Fig. 2B, lane 3). Finally, the expression of iNOS was similar that of eNOS (Fig. 2C), with the highest levels of iNOS activity present in the testis and corpus epididymis (Fig. 2C, lanes 1 and 3).
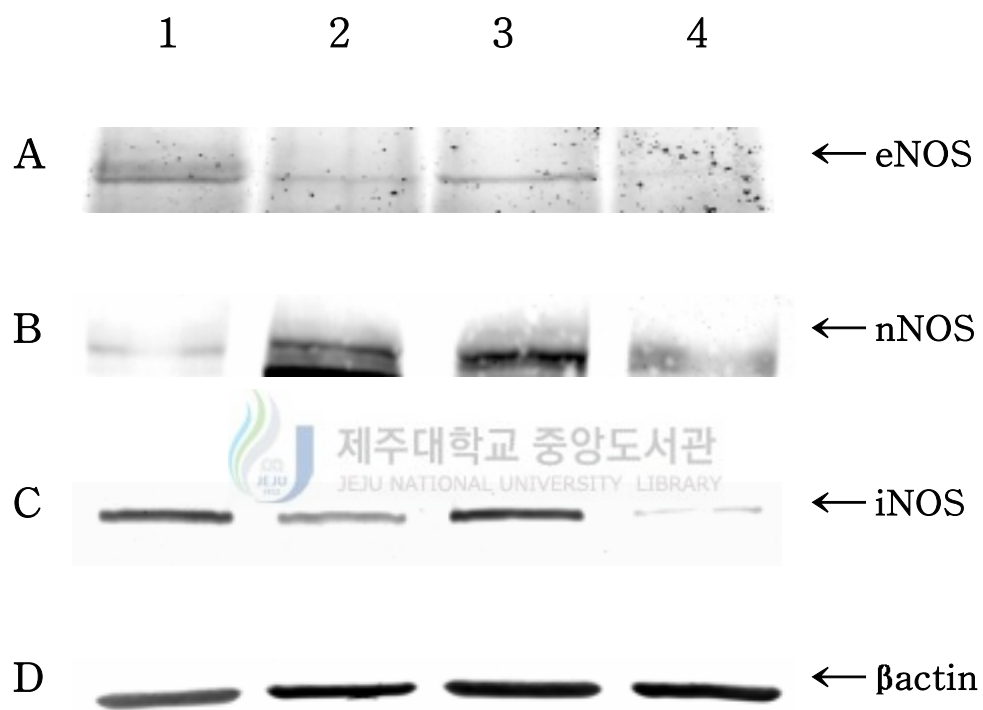
Figure 2. Representative Western blot analysis of NOS in the reproductive system of a normal thoroughbred horse. The expression of eNOS (140 kDa) in the testis (A, lane 1) and corpus epididymis (A, lane 3) was strongly detected as compared to in the caput and cauda epididymis (A, lanes 2 and 4). The expression of nNOS (155 kDa) was highest in the corpus epididymis (B, lane 3). The pattern of expression of iNOS (C) was similar that of eNOS. The highest levels of iNOS (130 kDa) activity were in the testis and corpus epididymis (C, lanes 1 and 3). β-actin was used for normalization of all tissues.

3. Immunohistochemical localization of eNOS, nNOS, and iNOS in the testis and epididymis of normal horses

Immunostaining for three isoforms of NOS was detected in the reproductive system of normal male thoroughbred horses. The histochemical reactions of the constitutive neuronal, endothelial, and inducible NOS in the testis and epididymis are summarized in Table 1.

In the testis, immunoreactivity for eNOS was intense in secondary spermatids, spermatocytes, and interstitial cells in the seminiferous tubules (Fig. 3A). In contrast, only weak immunostaining for eNOS was found in some Sertoli cells and primary spermatids (Fig. 3A). In the caput epididymis, eNOS was strongly detected in the stereocilia and basal cells, while epithelium and connective tissue showed weak staining and spermatocytes were negative (Fig. 3B). eNOS was also strongly stained in the stereocilia and spermatocytes of the corpus epididymis of normal horses. The epithelium and connective tissue of the corpus epididymis stained weakly for eNOS, but basal cells were negative (Fig. 3C). In the cauda epididymis, the expression of eNOS was strongly detected in the connective tissue and in the stereocilia, with weaker staining of the epithelium and spermatocytes, while basal cells were again negative (Fig. 3D).

Table 1. Histochemical staining pattern for three NOS isoforms in various cell types in the male reproductive system of normal two-year-old Thoroughbred horses. (−, negative; +, weak; ++, moderate; +++, intense)

		eNOS	nNOS	iNOS
Testis	Primary spermatid	+	+	−
	Secondary spermatid	++	++	−
	Spermatocyte	++	−	−
	Sertoli cell	+	−	−
	Interstitial cell	+++	+++	++
Caput epididymis	Stereocilia	++	++	+
	Epithelium	+	++	−
	Basal cell	+	+	−
	Spermatocyte	−	−	+
	Connective tissue	+	+	+
Corpus epididymis	Stereocilia	++	+	+
	Epithelium	+	++	+
	Basal cell	−	−	++
	Spermatocyte	++	++	++
	Connective tissue	+	−	+
Cauda epididymis	Stereocilia	+	+	+
	Epithelium	+	++	−
	Basal cell	−	−	−
	Spermatocyte	+	+	−
	Connective tissue	++	+	+

Intense immunoreactivity for nNOS was detected in the interstitial cells and secondary spermatids of the testis. nNOS was also weakly detected in the primary spermatids, but not in spermatocytes and Sertoli cells (Fig. 4A). In the caput epididymis, nNOS was strongly detected in the stereocilia and epithelium, and weakly detected in the basal cell and connective tissues, but spermatocytes in the caput epididymis were negative (Fig. 4B). In the corpus epididymis, nNOS was strongly detected in the epithelium and spermatocytes, but only weakly detected in the stereocilia, and not detected at all in the basal cells or connective tissues (Fig. 4C). In the cauda epididymis, nNOS was strongly detected in the epithelium, weakly detected in the stereocilia, spermatocytes, and connective tissue, and not detected at all in the basal cells (Fig. 4D).

Immunoreactivity of iNOS was intense only in the interstitial cells of the testis of normal horses (Fig. 5A). Spermatocytes and Sertoli cells were both negative for iNOS. In the caput epididymis, iNOS was detected in the stereocilia and connective tissue and weakly detected in spermatocytes, but not detected in epithelial cells or basal cells (Fig. 5B). Spermatocytes in the corpus epididymis showed strong iNOS immunostaining, with somewhat less intense staining present in the stereocilia, epithelium, and connective tissue (Fig. 5C). In the cauda epididymis, the expression of iNOS was detected in the connective tissue, spermatocytes, and stereocilia, but

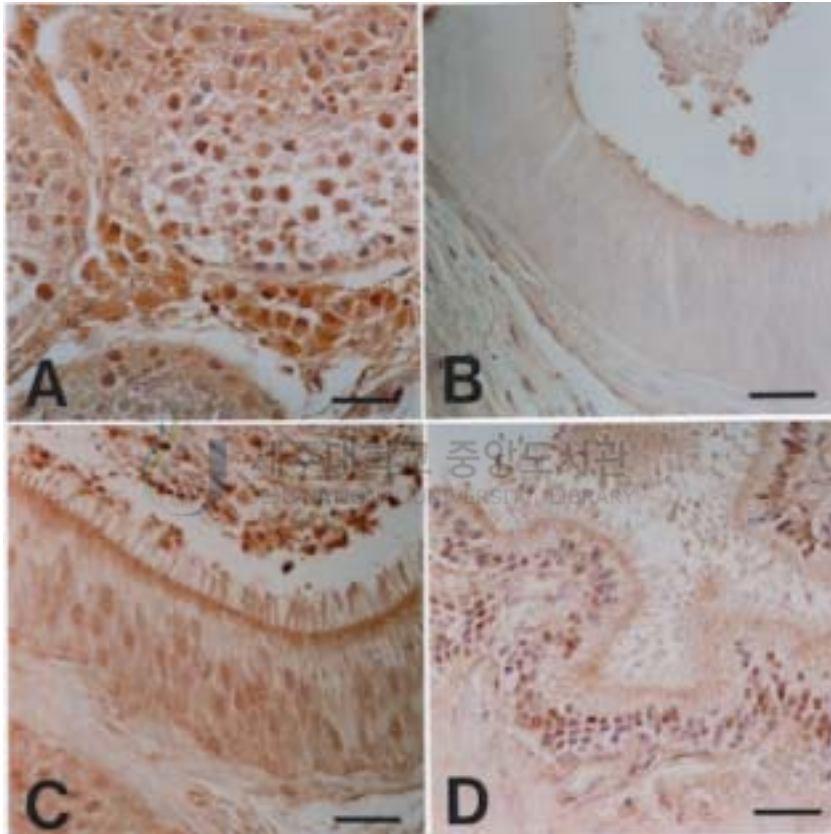not in epithelium or basal cells (Fig. 5D).

Figure 3. Immunostaining for eNOS in the testis and caput, corpus, and cauda epididymis of a normal thoroughbred horse. eNOS was detected in all cells including spermatocyte, Sertoli cells, and interstitial cells in the testis (A). In the caput epididymis, eNOS was detected in the stereocilia, basal cells and connective tissue, but not in epithelial cells or spermatocytes (B). eNOS immunoreactivity was also seen in stereocilia, spermatocytes, and connective tissue of the corpus epididymis (C). In the cauda, eNOS was expressed in all cells (D). Counterstained with hematoxylin. Scale bar = 30 µm.
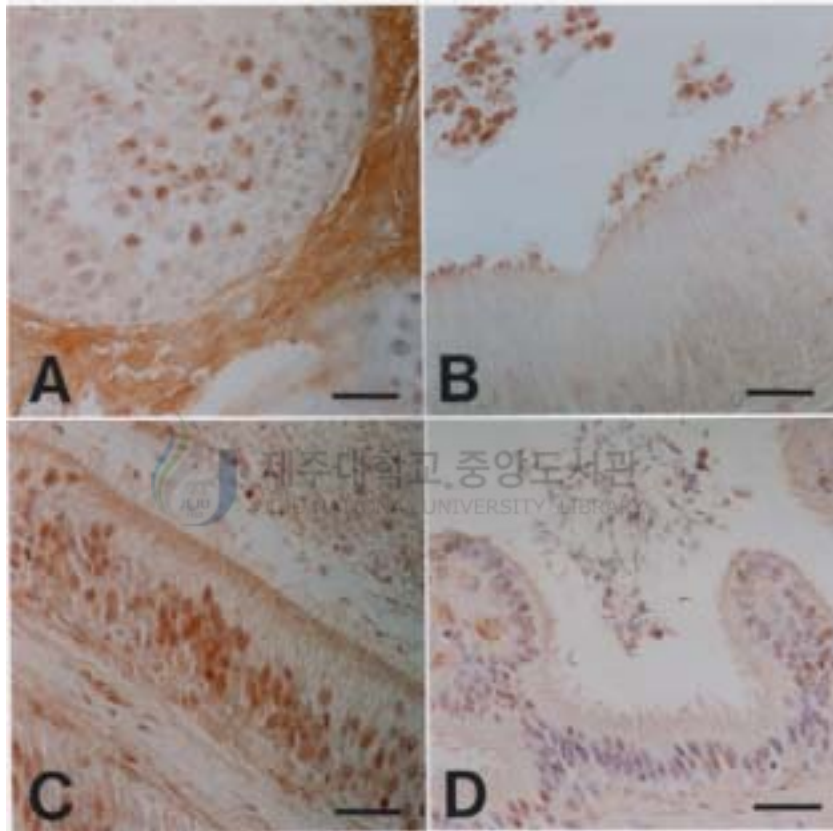
Figure 4. Immunostaining for nNOS in the testis and caput, corpus, cauda epididymis of a normal thoroughbred horse. nNOS was detected in spermatocytes and interstitial cells of the testis (A). Immunoreactivity for nNOS was strongly detected in the stereocilia and spermatocytes of the corpus epitidymis (C). nNOS was also detected in the stereocilia, spermatocytes, and epithelial cells of the caput and cauda epididymis (B, D). Counterstained with hematoxylin. Scale bar = 30 μm.
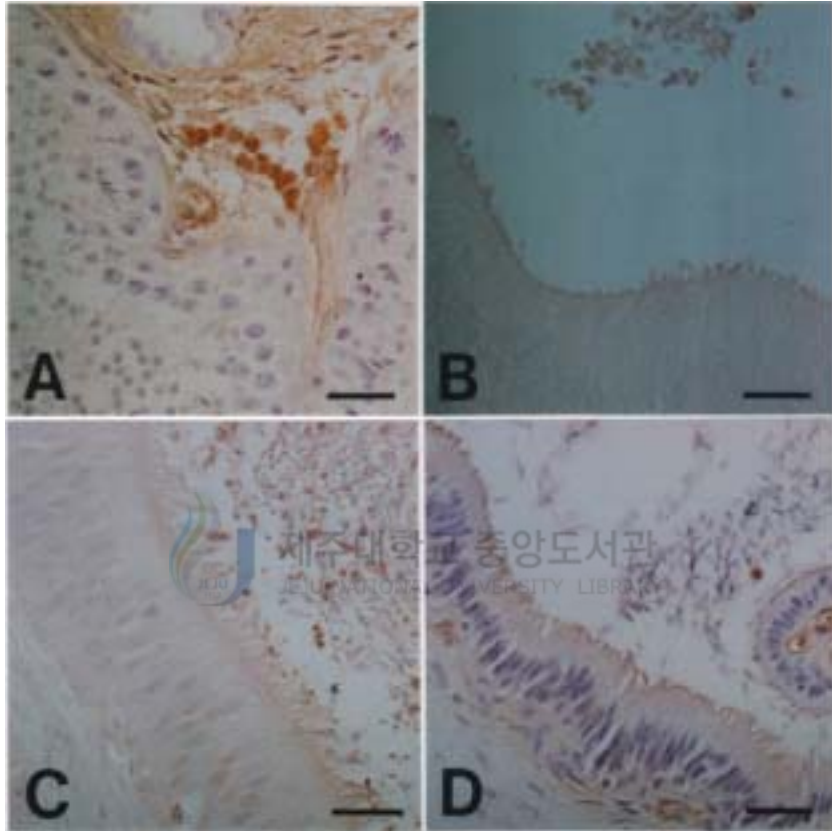
Figure 5. Immunostaining of iNOS in the testis and caput, corpus, and cauda epididymis of a normal thoroughbred horse. Strong immunoreactivity for iNOS was detected in the interstitial cells of the testis (A). In the epididymis, iNOS was strongly expressed in basal cells and spermatocytes of the corpus epididymis (C). In the caput and cauda epididymis, iNOS was detected in the stereocilia and connective tissue (B, D). Counterstained with hematoxylin. Scale bar = 30 μm.

Ⅳ. Discussion

This is the first report to show that constitutive eNOS, nNOS, and inducible NOS are differentially expressed in the seminiferous tubules of normal thoroughbred horses. In the testis, constitutive NOS was strongly detected in the interstitial cells, while in the epididymis both eNOS and nNOS expression were detected mainly in spermatocytes and epithelium. Although it has been reported that iNOS is overexpressed in ischemia-reperfusion injuries of the testis (Ozokutan *et al.*, 2000), this is the first report of iNOS expression in the interstitial cells of the testis, as well as in basal cells and spermatocytes in the epididymis of the normal horse. This is also the first study to show that constitutive and inducible NOS are expressed in the testes of horses with induced orchitis. In orchitis, constitutive NOS and iNOS were detected predominantly in the infiltrating round cells.

Recent *in vitro* studies have indicated that the inflammatory vasodilator NO is capable of inhibiting steroidogenesis by Leydig cells (Adams *et al.*, 1996). It has also been reported that aluminum chloride decreases production of testicular testosterone in mice (Guo *et al.*, 2001). Elevated aluminum levels in spermatozoa and seminal plasma were previously shown to be correlated with decreased sperm motility (Dawson *et al.*, 1998); however in a later study, NO levels were also shown to be increased in the related tissue of

mice (Guo et al., 2001).

Interstitial macrophages in the testis are an important source of nitric oxide produced by iNOS. It is well known that macrophages express iNOS upon activation and release NO in cultured cell systems. In a previous study, a majority of the macrophages that expressed ED1 and all Leydig cells were immunopositive for iNOS in both control and LPS-treated rat testes (Gerdprasert *et al.*, 2002). It is thus likely that the iNOS-immunoreactive cell populations detected in the interstitial tissue of horse testes in this study were comprised of Leydig cells and macrophages. When the testis is inflamed, as during adjuvant-induced orchitis in the present study, it is postulated that iNOS from macrophages in the testicular interstitium may exacerbate tissue damage. However, possible involvement of constitutive eNOS and nNOS in the macrophages is not excluded, because these two isoforms are also involved in tissue injury in other model systems (Shin, 2001).

There are many reports that eNOS is associated with cell death in certain cell types. It is postulated that over-activation of eNOS mediates the cell death program through the generation of NO, and may play a role in the cell-selection process that occurs in the seminiferous tubules. Spermatogenesis in the tubules is essentially a result of ongoing cell proliferation, cell selection, and cell death (de la Monte *et al.*, 2003; Qui *et al.*, 2003).

The functional role of NOS in interstitial cells and Leydig cells remains to be elucidated. In the present study, three isoforms of NOS were intensely immunostained in the interstitial cells of the testis, implying that NOS is involved in Leydig cell biology. If this is the case, it is possible to postulate that protein kinase C (PKC), most likely PKC theta (PKCθ) (Shin *et al.*, 1998), is intimately associated with NOS regulation in interstitial cells. There is much evidence that PKC and NOS, among other signaling molecules, are expressed in the interstitial tissue, including in Leydig cells (Jin and Shin, 1998; Kim and Shin, 1999; Shin *et al.*, 1998). In one study, differential expression of PKC and PKC was identified in the horse testis (Jin and Shin, 1998). Differential expression of PKC in interstitial cells (probably Leydig cells) and PKC in spermatids suggests that PKC delta and PKC theta play distinct roles in the regulation of testosterone synthesis and spermatid development, respectively. Additional evidence indicating that PKC delta subtypes are associated with NOS activation derives from in vitro systems. It has been shown that lipopolysaccharide (LPS) stimulation increases intracellular calcium and activates PKC in cultured cells, thus inducing iNOS gene expression, which leads to production of high levels of NO, demonstrating that a significant signaling role for NO is septic shock (Chung *et al.*, 2000; Nakajima *et al.*, 2003).

The findings of the present study suggest that three isoforms

of NOS are expressed in interstitial cells of the testis of the horse, and play important roles in the biology of testosterone-producing interstitial cells, as well as in spermatogenesis in the seminiferous tubules.

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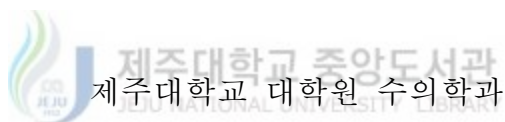
초   록

더러브렛 말의 고환과 부고환에서 nitric oxide synthase(NOS)의 발현

(지도교수 : 김 희 석)

하 태 영

제주대학교 대학원 수의학과

고환은 남성호르몬 생산 및 정자 생산의 기관으로 동물에서는 우수한 품종개발을 위해 많은 연구가 진행되고 있다. 고환에서의 정자생산은 곡정세관에서 주로 이루어지며 주변의 간질세포에서 생산된 여러 생리 활성 물질 중 상당수는 곡정세관에 영향을 미치기도 한다. 이때 관여하는 물질에는 신호전달 효소계를 들 수 있는데 nitric oxide synthase도 그 중 하나이다.

Nitric Oxide synthase(NOS)는 nitric oxide(NO)를 생산하는데 결정적인 역할을 하는 효소로 알려져 있으며 정상상태에서 이들 효소들은 다양한 생리학적 기능을 담당하고 있다. NOS는 조직내

세포에서 상존하는 형(Constitutive form)과 자극이 있을 경우
생성되는 유도형(Inducible form)으로 크게 나눌 수 있고, 상존형은
혈관내피세포 유래의 endothelial NOS(eNOS), 신경세포 유래의
neuronal NOS(nNOS)를 들 수 있다. 이러한 NOS는 여러 세포에서
발현되는 것이 확인되고 있으나 아직까지 고환, 특히 말의 고환에서
NOS가 어떤 형태로 발현되는 지는 알려진 바 많지 않다.

따라서, 이번 연구는 더러브렛 말의 정상 고환과 부고환에서
상존형 eNOS와 nNOS 그리고 유도형인 inducible NOS(iNOS)가 어떤
형태로 발현되는지를 확인하고자 하였다.

더러브렛 숫 말(2년생 7두)은 한국마사회 제주육성목장에서
공급받아 외과적 처치를 통해 고환과 부고환으로 분리하였으며
조직검사를 위해 10% 중성포르말린에 고정한 후 파라핀 표본을
만들었으며 면역조직화학적 방법과 Western blot기법을 이용하여
이러한 효소가 어떻게 발현되는 지를 조사하였다.

Western blot 결과 상존형 NOS인 eNOS, nNOS 와 유도형
NOS인 iNOS는 각각 고환과 부고환에서 강하게 발현하는 것을 알 수
있었다.

Immunohistochemistry결과 eNOS는 정자와 간질세포,
상피세포에서 강하게 발현하였고, nNOS는 eNOS의 발현 양상과
유사하였다. iNOS는 고환에서는 오직 간질세포에서만 발현하였으며
부고환에서는 정자, 상피세포, 간질세포에서 발현하였다.

이상의 결과를 종합하여 볼 때 3종류의 NOS는 정상적인
숫말의 고환에서 정자 발생에 관여할 뿐만 아니라 정자의 활동에도

관여할 것으로 생각되었다. 또한 간질세포는 상존형 및 유도형 NOS를 모두 강하게 발현함으로써 남성호르몬의 생산에 NOS는 중요한 역할을 할 것으로 추정되며, 이들 효소의 조절은 말의 번식연구에 중요한 인자가 될 수 있음을 확인하였다.

감사의 글

한편의 논문이 나오기까지 정신적인 지주로서 많은 도움을 주시고 관심으로 이끌어 주신 김희석 지도교수님께 우선 감사의 말씀을 드리며, 부족한 내용이나마 자세하게 검토해 주시고 애정어린 충고를 잊지 않으시던 신태균 교수님, 이두식 교수님과 격려와 조언으로 자상하게 논문심사를 해 주신 경북대학교 변명대 교수님, 충북대학교 김일화 교수님께도 진심의 감사를 드리는 바입니다.

그리고 박사과정을 줄곧 지켜 보시며 항상 아낌없는 성원을 보내주신 제주대학교 수의학과 교수님들에게도 심심한 감사의 마음을 전합니다.

또한 연구하는 동안 내내 열과 성을 다해 수고해 준 제주대학교 수의학과 안미정씨를 비롯하여 바쁜 와중에서 귀중한 시간을 할애하여 정성껏 도와 준 같은 실험실 후배들에게도 이자리를 빌어 고마운 뜻을 보냅니다.

아울러 본 실험을 위해 물심양면으로 도움을 주신 한국마사회 회장님과 선후배 ,동료 여러분들에게도 감사를 드리며 특히 제주 육성목장에서 근무하는 양재혁 수의사를 포함한 직원들께도 특별한 고마움을 표합니다. 본 논문을 완성하는 과정에서 여러분들이 주신 따뜻한 고마움과 배움으로부터의 지혜를 통해 너무나 소중하고 뜻깊은 결실을 보게되어 이 또한 제게는 무한한 기쁨이 아닌가 생각합니다.

아무쪼록 본 논문이 국내 마필생산에 견인차 역할을 할 뿐만

아니라 추가적인 연구들에 자극이 되어 우수한 경주마 생산의
밑거름이 될 수 있었으면 하는 바램입니다.

끝으로 여러가지 어려움 속에서도 늘 용기와 힘이 되어 준
아내와 우리 가족들은 물론 언제나 자식의 건강을 염려하시며
기도하여 주신 어머님, 장모님과 이미 고인이 되신 아버님, 장인어른
영전에 모든 영광을 바칩니다.