

## Altered Growth Factor Expression in the Aging Penis: The Brown-Norway Rat Model

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**ABSTRACT:** The objective of the present study was to evaluate age-related changes in the protein and gene expression of modulators of erectile function (nitric oxide [NO] and endothelin-1 [ET-1]) and growth factors such as transforming growth factor (TGF- $\beta$ 1) and vascular endothelial growth factor (VEGF) in the penile tissue of Brown-Norway (BN) rats. Young and old BN male rats were euthanized, and the penile tissue was processed for immunohistochemical and molecular analyses. Total RNA was extracted, and an Access reverse transcription-polymerase chain reaction (RT-PCR) system was used for messenger RNA (mRNA) expression analysis. Immunohistochemical studies showed a decreased expression of endo-

thelial nitric oxide synthase (eNOS) protein and an increased staining for ET-1. Quantitative analysis of PCR products revealed decreased levels of VEGF mRNA expression in the old population of rats. The most significant decrease was detected between bands corresponding to splice forms 164 (21%) and 120 (18%). The observed alterations in the gene expression of growth factors such as VEGF may contribute to the abnormal age-related morphological and physiological alterations in the erectile tissue.

**Key words:** Aging, erectile dysfunction, nitric oxide, endothelins, vascular endothelial growth factor, transforming growth factor.

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Male erectile dysfunction (ED) is age related and highly prevalent, affecting 10%–52% of men (Feldman et al, 1994). Ayta et al (1999) estimated that over 152 million men worldwide had ED in 1995 and that 322 million men would have ED by the year 2025. As men reach late middle age and old age, they experience a reduced libido, a decline in the frequency of sexual activity, and a decline in erectile function (Martin, 1981; Davidson et al, 1983; Kinsey et al, 1948). Studies have shown that aging men have a diminished erectile response during erotic video stimulation (Solnick and Birren, 1977) and during nocturnal penile tumescence monitoring (Schiavi and Schreiner-Engel, 1988) compared with their younger counterparts. These clinical findings are supported by laboratory studies showing a significant decline in erectile response in aging rats (Garban et al, 1995). The changes documented in these human and rat studies indicate that the aging process may be accompanied by an impairment of the mechanisms involved in erectile function. Age-related cellular and molecular changes in the penis and their contribution to erectile pathophysiology remain to be elucidated.

Normal erectile function is characterized by a delicate in vivo balance between vasoconstricting and vasorelaxing mediators on corporal smooth muscle tone (Taub et al, 1993). Endothelium-derived nitric oxide (NO) and endothelin-1 (ET-1) have been recognized to modulate erectile function. NO is a key modulator of cavernosal smooth muscle relaxation, whereas ET-1 is believed to maintain penile flaccidity (Saenz de Tejada et al, 1991). Another endothelial-specific mitogen that has recently been the focus of ED research is vascular endothelial growth factor (VEGF) (Burchardt et al, 1999; Byrne et al, 2000; Liu et al, 2001). Expression of VEGF has been documented in rat erectile tissues, and ex vivo exposure to this growth factor induced a migratory and proliferative response in penile smooth muscle cells (Burchardt et al, 1999; Liu et al, 2001). Recent studies suggest significant interactions between these endothelium-derived substances in the modulation of penile erection. Mills et al (2001) demonstrated that the NO-mediated erectile response in rats may involve antagonism of ET-1–induced vasoconstrictive tone. VEGF has been shown to increase the ability of endothelial cells to produce NO (Hood et al, 1998). Aging is known to alter endothelial cell function. Age-related impairment in acetylcholine-mediated relaxation of rabbit penile corporal tissue suggests endothelial dysfunction in penile cavernosum (Haas et al, 1998). An age-associated decrease in NO synthase (NOS) activity and a reduction in NOS-containing nerve fibers in the penis in old rats indicate impairment of NO synthesis during nor-

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mal aging (Carrier et al, 1997). ET-1 is a vasoconstrictor peptide, and localization of ET-1 immunoreactivity has been demonstrated in the endothelium and in trabecular smooth muscle of the human penis (Saenz de Tejada et al, 1991). Increased ET-1 levels are associated with several age-related pathophysiological disorders (Kumazaki et al, 1994).

Although these animal studies suggest a link between ED, NO, ET-1, and penile end-organ changes in old animals (Garban et al, 1995; Carrier et al, 1997; Haas et al, 1998), most of these data have been gathered from studies employing old Fisher 344 (Garban et al, 1995) or Sprague-Dawley rats (Carrier et al, 1997), which have been documented to be unsuitable for the study of reproductive aging (Gruenewald et al, 1994). The aging male Fisher 344 rats are known to develop testicular Leydig cell tumors that secrete progesterone with increasing age (Gruenewald et al, 1994). The aging Brown-Norway (BN) rat offers the opportunity to study aging of the male reproductive system without the complexities consequential to such simultaneous pathological changes. These rats exhibit a combination of primary and secondary testicular failure that more closely resembles human reproductive aging than other rodent models (Gruenewald et al, 1994). A 23-month-old male BN rat can be considered analogous to a 65-year-old man, and tremendous alterations in the aging process occur between 23 and 28 months of life in this species (Gruenewald et al, 1994). The present study was designed to monitor the changes in the penile end-organ chemistry, especially the expression of certain key modulators (NO and ET-1) and growth factors (TGF- $\beta$ 1 and VEGF) during this critical period of aging in BN rats.

## Materials and Methods

### Experimental Animals

BN rats of different age groups ( $n = 6$ ; young, 6 months; old, 23–28 months) were obtained from the National Institute of Aging (Bethesda, Md) under a protocol approved by the Institutional Animal Care and Use Committee and were maintained on a 12-hour light-dark cycle with food and water available ad libitum. Rats were sacrificed by a lethal overdose of sodium pentobarbital (125 mg), and the penile tissue was rapidly harvested. The penis was detached at the crural bony attachments, and the distal penile shaft was employed for all the analyses. A small portion of the tissue was fixed in 10% buffered formalin for immunohistochemical studies, and the rest was frozen under liquid nitrogen and stored at  $-70^{\circ}\text{C}$  for gene expression studies.

### Trichrome Staining

A small portion of rat penis was fixed in 10% buffered formalin and processed for paraffin embedding. Paraffin sections ( $5\ \mu\text{m}$ ) were hydrated with distilled water and stained with Masson tri-

chrome stain, which determines the relative proportion of collagen to stromal smooth muscle (Carrier et al, 1997).

### Immunohistochemical Evaluations

Protein expression of NOS (eNOS) and ET-1 was evaluated in penile tissue of rats by immunohistochemical methods with commercially available (Calbiochem, San Diego, Calif) monoclonal antibodies that have been validated to react with rat tissues. Paraffin tissue sections ( $5\ \mu\text{m}$ ) were applied to charged slides, deparaffinized, and hydrated with phosphate-buffered saline containing 0.3% Triton X-100 for 10 minutes. After incubation for 30 minutes with normal goat serum for blocking nonspecific binding sites, slides were incubated overnight with specific antibodies (ET-1 and eNOS; 1:200 dilution) at  $4^{\circ}\text{C}$ . A conventional avidin-biotin complex (ABC) kit (Vector Laboratories, Burlingame, Calif) was used to immunostain the sections, and reaction products were visualized under a light microscope (Rajasekaran et al, 1998). The slides were examined by a blinded microscopist, and digital images were quantified by computer software (Matrox Imaging Library, Matrox, Dorval, Quebec, Canada).

### Evaluation of Gene Expression

Gene expression studies employed gene-specific primers (ET-1, eNOS, TGF- $\beta$ 1, and VEGF). Total RNA was extracted from the excised cavernosa and subjected to reverse transcription-polymerase chain reaction (RT-PCR) as follows.

Total RNA was obtained by the Trizol method (GIBCO-BRL, Grand Island, NY), and an Access RT-PCR (Promega, Madison, Wis) system was used to amplify the products. RT-PCR of a single target RNA was performed in a single tube with Avian Myeloblastosis Virus (AMV) reverse transcriptase (AMV RT) for first-strand DNA synthesis; *Thermus flavus* (Tfl) DNA polymerase for second-strand complementary DNA (cDNA) synthesis; and 5 M oligodeoxythymidylate, 10 mM deoxynucleoside triphosphate, and 1 mM  $\text{Mg}^{2+}$  for DNA amplification in a volume of  $50\ \mu\text{L}$ . RT-PCR was performed in a DNA Thermal Cycler 480 (Perkin Elmer Cetus, Norwalk, Conn) with the following cycle parameters: 45 minutes of RT at  $48^{\circ}\text{C}$ , 2 minutes of AMV RT inactivation and RNA/cDNA/primer denaturation at  $94^{\circ}\text{C}$ , a 30-second denaturation at  $94^{\circ}\text{C}$ , a 60-second annealing step at  $60^{\circ}\text{C}$ , and a 120-second extension at  $68^{\circ}\text{C}$ . Forty cycles were used per amplification of each PCR product, and PCR reactions were confirmed to be within the exponential phase.

The PCR products were size fractionated by 1.2% agarose gel electrophoresis (agarose-1000, GIBCO-BRL) and stained with  $0.5\ \mu\text{g}/\text{mL}$  ethidium bromide (GIBCO-BRL), and the identity of the PCR products was confirmed using a 100-bp ladder (Promega) as the DNA standard. The primer sequences are shown in Table 1. Quantitation was performed by densitometry.  $\alpha$ -Actin was used as an internal control for RT-PCR reactions, and the products were analyzed on a 1.2% agarose minigel system. A computerized image analysis system was used to quantify the band intensity (Rajasekaran et al, 1998).

## Results

### Histologic Analysis

The light microscopy of penile midshaft tissue stained with Masson trichrome is shown in Figure 1. There was

Table 1. RT-PCR primer sequences and product size (location)\*

Primer	Primer Sequence	Size (bp)E	Reference
ET-1 (sense)	5'-CC AGC ACA TCC TGG AGA-3'		
ET-1 (antisense)	5'-CTC CAC CAG CTG CTG ATA-3'	378	(Hasselblatt et al, 1998)
eNOS (sense)	5'-CTG CTG CCC GAG ATA TCT TC-3'		
eNOS (antisense)	5'-AAG TAA GTG AGA GAG CCT GGC GCA-3'	432	(Schricker et al, 1996)
TGF-β1 (sense)	5'-CGG CAG CTG TAC ATT GAC TT-3'		
TGF-β1 (antisense)	5'-TCA GCT GCA CTT GCA GGA GC-3'	278	(el-Sakka et al, 1999)
VEGF-188 (sense)	5'-TGC ACC CAC GAC AGA AGG GGA-3'		
VEGF-188 (antisense)	5'-TCA CCG CCT TGG CTT CTC ACA T-3'	564	(Burchardt et al, 1999)
VEGF-164 (sense)	5'-TGC ACC CAC GAC AGA AGG GGA-3'		
VEGF-164 (antisense)	5'-TCA CCG CCT TGG CTT CTC ACA T-3'	492	(Burchardt et al, 1999)
VEGF-144 (sense)	5'-TGC ACC CAC GAC AGA AGG GGA-3'		
VEGF-144 (antisense)	5'-TCA CCG CCT TGG CTT CTC ACA T-3'	432	(Burchardt et al., 1999)
VEGF-120 (sense)	5'-TGC ACC CAC GAC AGA AGG GGA-3'		
VEGF-120 (antisense)	5'-TCA CCG CCT TGG CTT CTC ACA T-3'	360	(Burchardt et al, 1999)

\* eNOS indicates endothelial nitric oxide synthase; ET-1, endothelin-1; RT-PCR, reverse transcription-polymerase chain reaction; TGF, transforming growth factor; and VEGF, vascular endothelial growth factor.

no noticeable difference between the age groups in the smooth muscle content of the erectile tissue. Histologic examination revealed a marked reduction in the vascular endothelial integrity in the aged rat compared to its younger counterpart.

#### Immunocytochemistry

Penile tissue sections from both age groups of rats exhibited positive immunoreactivity to both NOS and ET-1 antibodies. Reaction products were predominantly associated with endothelial lining and, to a lesser extent, demonstrated in smooth muscle fibers. Distinct differences in the staining patterns were observed between young and old rats (Figure 2). For eNOS, a decreased immunostaining was observed in old rats compared to the young population. An intense immunostaining for ET-1 was noticed in old rats compared to young ones (Figure 2).

#### Gene Expression

Our results showed expression of eNOS, ET-1, TGF-β1, and VEGF in young as well as old animals. RT-PCR of VEGF messenger RNA (mRNA) produced 4 distinct products, corresponding to 4 different splice variants of VEGF mRNA (VEGF 120, 144, 164, and 188). No age-related differences in TGF-β1 mRNA levels were observed in the penile tissue. There was approximately a 13% decrease in eNOS and a 16% increase in ET-1 expression in old rats compared to younger ones (Figure 3A). For VEGF, the most noticeable age-related decrease in expression was detected between bands corresponding to splice forms 164 (21%) and 120 (18%) (Figure 3B; Table 2).

## Discussion

The aging BN rat has been documented as the most sensitive strain representing the effects of reproductive aging

(Gruenewald et al, 1994). We have analyzed penile end-organ changes in this rat model during a critical period of aging when tremendous morphological and physiological alterations are known to occur in this species. Our investigation using the BN rat strain has elucidated a novel role for VEGF in the aging penis that has not been identified in either Sprague-Dawley or Fisher 344 aging rats. Further, our results demonstrate age-related alterations in the expression of key modulators of erectile process, such as NO and ET-1, and growth factors, such as TGF-β1, which are in agreement with the previous studies in either Sprague-Dawley or Fisher 344 aging rat strains.

We observed age-related differential expression of both the endothelium-derived vasorelaxing and vasoconstrictive modulators of erectile function. The immunohistochemical data for these important modulators support their gene expression pattern. The decreased eNOS expression observed in old rats suggests impaired NO synthesis and endothelial smooth muscle relaxation of the penile cavernosum. The relaxation of the penile smooth muscle is controlled by nerves, neurotransmitters (cholinergic and noncholinergic), and endothelium-derived substances. Defects in the production or release of neurotransmitters or the presence of antagonists could cause inhibition of cavernosal smooth muscle relaxation, resulting in inhibition of erection. Aging is well known to alter endothelial cell function. Age-related impairment in acetylcholine-mediated relaxation of rabbit penile corporal tissue has been demonstrated, suggesting endothelial dysfunction in the penile cavernosum (Haas et al, 1998). A decreased NOS activity and a reduction in NOS-containing nerve fibers within the corpus cavernosum in the penis of aging male Sprague-Dawley rats have also been reported (Garban et al, 1995). Gene transfer of eNOS to the penis has been shown to augment the erectile response in the aged rat by enhanced eNOS protein synthesis

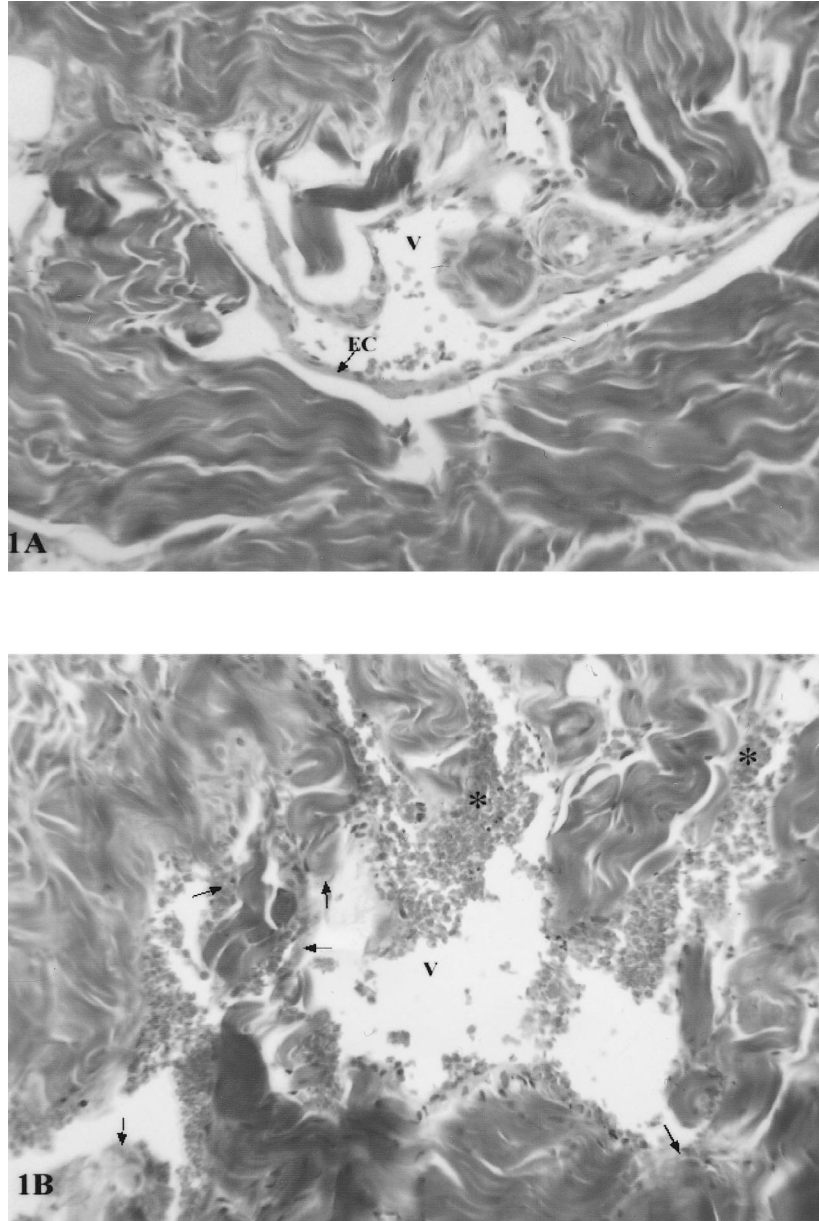


Figure 1. Photomicrographs of Masson trichrome stain in (A) young and (B) old Brown-Norway (BN) rat penile shaft tissue sections. The integrity of the vascular lumina (V) in young rats is maintained by the normal-appearing endothelial cell (EC; arrow) lining. The V of old rats lacked functional integrity, as evidenced by missing EC (arrow). Random migration of red blood cells (\*) into the interstitial spaces indicates sinusoidal pathology in old rats. Magnification 40 $\times$ .

(Champion et al, 1999; Bivalacqua et al, 2000). These observations corroborate our finding that basal release of NO is impaired during normal aging.

We have also demonstrated an increase in the gene and protein expression of ET-1 in the penis of aging BN rats. ET-1 is a vasoconstrictor peptide, and localization of ET-1 immunoreactivity has been demonstrated in the endothelium and in trabecular smooth muscle of the human penis (Saenz de Tejada et al, 1991). In vitro incubation of cavernosal tissue ET-1 exhibited a dose-dependent contractile activity. In rats, injection of ET-1 into the corpus

cavernosum induced a strong vasoconstrictive action on cavernosal vasculature as well as systemic circulation (Mills et al, 2001). Significantly elevated plasma levels of ET-1 were observed in diabetic impotence in comparison to a control population (Francavilla et al, 1997). These in vivo and in vitro studies suggest that the human penile cavernous tissue has the ability to synthesize its own endothelin, which may have an autoregulatory role in erectile function. Increased ET-1 levels are generally associated with age-related endothelial dysfunction (Dohi et al, 1995). A significant increase in ET-1 mRNA has

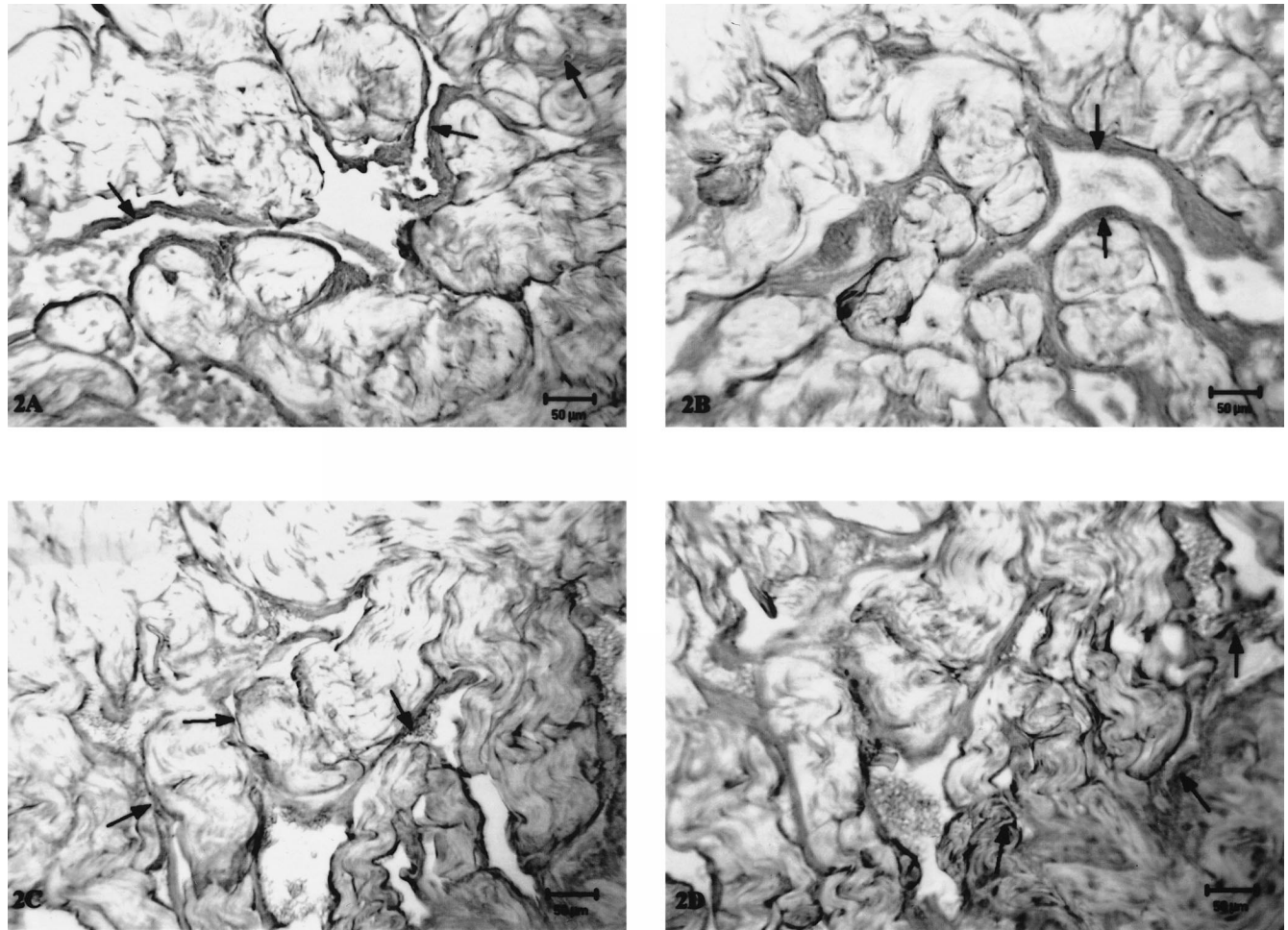


Figure 2. Photomicrographs of rat penile shaft sections immunohistochemically stained for endothelial nitric oxide synthase (eNOS) (A, C) and endothelin-1 (ET-1) (B, D) in young and old Brown-Norway (BN) rats. Reaction products were predominantly associated with the endothelial lining of penile cavernosal sinusoids (arrows) and, to a lesser extent, demonstrated in smooth muscle fibers. A marginal decrease in eNOS (C) and an increase in ET-1 (D) immunoreactivity were observed in cavernosal sinusoids of old rats when compared to tissues from young animals. Magnification 40 $\times$ .

also been reported in endothelial cells from aged donors, suggesting an up-regulation of ET-1 expression during the aging process (Kumazaki et al, 1994). Our findings that aging leads to an increased expression of ET-1 in the penis are consistent with these observations and suggest that elevated ET-1 may attenuate cavernosal smooth muscle relaxation and predispose to ED in the aging male.

Our results also showed alterations in the expression of certain growth factors such as TGF- $\beta$ 1 and VEGF in the penis of aging BN rats. Recently, Dahiya et al (1999) demonstrated differential gene expression of growth factors such as TGF- $\beta$ 1 that were implicated in the onset of ED in aging rats. TGF- $\beta$ 1 is known to be a profibrogenic agent in the penis, and elevated expression of this factor has been reported to play a role in the pathophysiology of the penis (Nehra et al, 1999). Although we were able to demonstrate the expression of both of these growth factors, only VEGF expression exhibited noticeable alterations between the age groups. These findings are in

agreement with recent reports that the secretion of this growth factor in the penis decreases with age in rats (Liu et al, 2001). VEGF is one of the most potent angiogenic, vascular permeability factors, and the presence of this growth factor has been documented in rat and human penile tissue as well as in cultured cavernosal cells (Burchardt et al, 1999). In our study, RT-PCR evaluation showed 4 splice variants of VEGF mRNA, a finding that is consistent with a previous report (Burchardt et al, 1999). Among these isoforms of VEGF, noticeable changes were observed in the expression of VEGF 164, which is considered the most active isoform (Petrova et al, 1999). Recent reports indicate that this isoform of VEGF may be a potential candidate for gene therapy for ED (Byrne et al, 2000). The exact mechanism by which VEGF regulates erectile function is not clear. Conceivably, it might involve regulation of neurovascular or smooth muscle changes within the penis. Besides its angiogenic role, VEGF has been shown to regulate eNOS

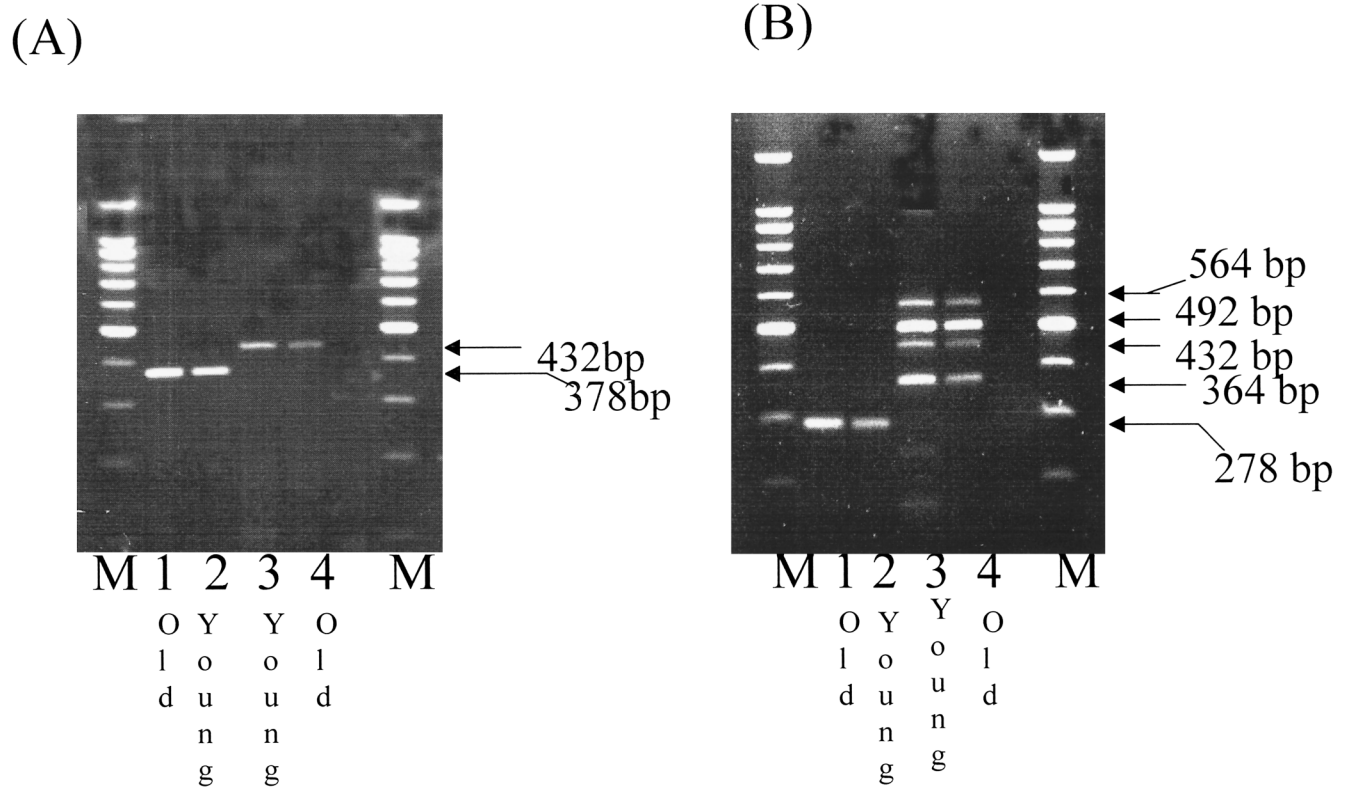


Figure 3. **(A)** A representative gel picture showing the gene expression of endothelin-1 (ET-1) and endothelial nitric oxide synthase (eNOS) in Brown-Norway (BN) rat penile cavernosal tissues. A decrease in eNOS (lane 2) and an increase in ET-1 (lane 3) expressions were observed in old rats when compared to young animals (lanes 1 and 4). **(B)** A representative gel picture showing the gene expression of transforming growth factor (TGF- $\beta$ 1) and vascular endothelial growth factor (VEGF) in BN rat penile cavernosal tissues. An increase in TGF- $\beta$ 1 (lane 2) and a decrease in VEGF (lane 3) expression were observed in old rats when compared to young animals (lanes 1 and 4). M indicates molecular weight markers.

Table 2. *Semiquantitative densitometry of RT-PCR products\**

RT-PCR Product	Size (bp)	IDV	% (based on IDV)	% Difference Between Young and Old Rats
ET-1 (Y)†	378	68 924	24.4	
ET-1 (O)‡	378	58 360	20.7	15.16
eNOS (Y)	432	46 515	16.5	
eNOS (O)	432	40 705	14.4	12.73
TGF- $\beta$ 1 (Y)	278	26 327	8.9	
TGF- $\beta$ 1 (O)	278	24 241	8.2	7.87
VEGF-188 (Y)	564	24 764	8.3	
VEGF-188 (O)	564	22 440	7.6	8.43
VEGF-164 (Y)	492	39 353	13.3	
VEGF-164 (O)	492	31 049	10.5	21.05
VEGF-144 (Y)	432	24 380	8.2	
VEGF-144 (O)	432	22 147	7.5	8.54
VEGF-120 (Y)	360	27 647	9.3	
VEGF-120 (O)	360	23 357	7.9	15.05

\* eNOS indicates endothelial nitric oxide synthase; ET-1, endothelin-1; IDV, integrated density value; RT-PCR, reverse transcription-polymerase chain reaction; TGF, transforming growth factor; and VEGF, vascular endothelial growth factor.

† Y = young rat.

‡ O = old rat.

expression in endothelial cells (Petrova et al, 1999). The observed decrease in VEGF expression in the aging penis suggests that derangement in the synthesis of this growth factor may contribute to age-induced morphological and physiological alterations in the erectile tissue.

In conclusion, aging leads to alterations in the expression of key modulators and growth factors involved in the regulation of erectile function. Regulation of angiogenesis and eNOS may play a crucial role in the aging penis.

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