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Adipocyte Accumulation in Penile Corpus Cavernosum of the Orchiectomized Rabbit: A Potential Mechanism for Veno-occlusive Dysfunction in Androgen Deficiency

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ABSTRACT: Androgens are deemed to be critical for the development, growth, and maintenance of penile tissue as well as for erectile function. Androgens are also reported to inhibit differentiation of stroma progenitor cells into adipocytes and promote differentiation into smooth muscle. The objective of this study was to investigate whether androgen deprivation results in accumulation of adipocytes in the corpus cavernosum. Mature, New Zealand white male rabbits were subjected to sham surgery (control) or orchiectomy. Two weeks after surgery, erectile function was assessed by monitoring changes in intracavernosal blood pressure (ICP) in response to pelvic nerve stimulation. All ICP measurements were normalized to the mean systemic arterial blood pressure. In parallel studies, penile cross sections from control and orchiectomized rabbits were fixed and stained with either Masson's trichrome or hematoxylin and eosin to assess smooth muscle and connective tissue content. Alternatively, tissue sections were stained with Toluidine blue to assess accumulation of fat-containing cells. Orchiectomy resulted in loss of erectile function and penile atrophy, associated with reduced trabecular smooth muscle and increased connective tissue content. Most strikingly, tissue from orchiectomized animals exhibited accumulation of fat-containing cells (adipocytes) in the subtunical region of the corpus cavernosum. We hypothesize that androgen deprivation promotes differentiation of progenitor stroma cells into an adipogenic lineage producing fat-containing cells, thus altering erectile function.

Key words: Trabecular smooth muscle, veno-occlusion, fat-containing cells.

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Clinical and animal studies have suggested that veno-occlusion is modulated by the balance between the smooth muscle and connective tissue content of the corpus cavernosum (Nehra et al, 1996, 1998; Moreland, 1998). Rogers et al (2003) have suggested that regardless of the etiology of organic erectile dysfunction (neurogenic, traumatic, hormonal, vascular, etc), venous leakage is a common final condition resulting from smooth muscle atrophy. Furthermore, veno-occlusive dysfunction is the most common characteristic among nonresponders to medical management of erectile dysfunction. It has been hypothesized that androgen deprivation might produce tissue atrophy and trabecular smooth muscle cell death, causing an imbalance in the ratio between smooth muscle and the extracellular matrix, leading to veno-occlusive dysfunction (Traish et al, 1999, 2003; Rogers et al, 2003). However, the cellular and molecular mechanisms by which androgens modulate penile erectile function remain largely undefined.

It is generally accepted that androgens play an important role in the development, growth, and maintenance of function of male secondary sexual characteristics (Dorfman and Shipley, 1956). However, the role of androgens in erectile function remains controversial and poorly understood. Several studies in the rat model have reported that androgen deprivation results in reduction of nitric oxide synthase (NOS) expression and activity (Chamness et al, 1995; Garban et al, 1995; Lugg et al, 1995, 1996; Zvara et al, 1995; Penson et al, 1996; Schirar et al, 1997). However, these observations were not confirmed in the rabbit corpus cavernosum (Holmquist et al, 1994; Traish et al, 1999, 2003), suggesting species differences in NOS regulation by steroid hormones.

Mills et al (1998) suggested that androgen deprivation alters penile blood outflow in the rat, resulting in reduced erectile function (veno-occlusive dysfunction). Rogers et al (2003) showed that castrated rats exhibit depressed expression of NOS, reduced smooth muscle content, and altered dorsal nerve structure and endothelial morphology. These animals display venogenic erectile dysfunction as assessed by intracavernosal pressure (ICP) monitoring and blood flow studies. Testosterone replacement induced vascular smooth muscle growth and restored erectile

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function. We have demonstrated that androgen deprivation in the animal model by surgical or medical castration resulted in loss of trabecular smooth muscle and increase in deposition of extracellular matrix, producing diffuse fibrosis and erectile dysfunction (Traish et al, 1999, 2003). The androgen-dependent loss of erectile response is restored by androgen administration but not restored by administration of phosphodiesterase type-5 inhibitor (Traish et al, 2003). Because androgens are reported to inhibit differentiation of stroma progenitor cells into adipocytes and to promote differentiation into smooth muscle (Bhasin et al, 2003), we hypothesize that, in the corpus cavernosum, androgens regulate differentiation of progenitor cells into smooth muscle cells and inhibit differentiation into adipocytes. Thus, castration would favor accumulation of adipocytes in the corpus cavernosum and alter its function. The goal of this study was to investigate the hypothesis that androgen depletion favors adipogenic lineage and increases accumulation of fat-containing cells in the corpus cavernosum in vivo.

Materials and Methods

Surgical Castration of Animals

The Institutional Animal Care and Use Committee of the Boston University School of Medicine approved this study. New Zealand white male rabbits (4.5–5.0 kg) were divided into 2 groups. One group was subjected to sham surgery (control, n = 7), whereas the other group was bilaterally orchiectomized (n = 7), as described previously (Traish et al, 1999, 2003). For each group, 4 animals were used for hemodynamic studies and 3 animals were used for histologic studies.

Measurement of Plasma Testosterone

Blood samples were drawn before orchiectomy and then again on the day of assessment of erectile function. Plasma was processed from each sample, extracted with ether, and used in a commercially available enzyme-linked immunosorbent assay kit (Assay Designs Inc, Ann Arbor, Mich) to determine plasma testosterone levels.

Measurements of Systemic Arterial Pressure and ICP

Animals were anesthetized with intramuscular injection of ketamine (35 mg/kg) and xylazine (5 mg/kg). Anesthesia was maintained with 0.2-mL intravenous bolus injections of pentobarbital (25 mg/mL) as needed. A 20-gauge angiocatheter was placed into the carotid artery for measurement of systemic arterial blood pressure (SAP). A 21-gauge minicatheter was placed near the base of the penis for measurement of ICP. A midline abdominal incision was made to expose the perivesical space. The internal pudendal artery was identified, and the distal branch to the prostate, bladder neck, and cavernosal bodies was localized. The cavernosal nerve bears relation to the cavernosal artery on the posterolateral surface of the prostate. With the use of platinum wire electrodes, we electrically stimulated the cavernosal nerve at frequencies of 2.5 or 4 Hz with a train of square waves at 10 V and a pulse width of 8 milliseconds for a total duration of 30 seconds.

Determination of Penile Wet Weight

Animals were euthanized by intravenous administration of sodium pentobarbital (50 mg/kg). The penis was removed in its entirety by dissecting along the shaft to the crura and separating each crus from its point of attachment at the ischial tuberosity. The skin overlying the penile shaft, the surrounding skeletal muscle, and the corpus spongiosum were removed. The remaining corpus cavernosum, tunica albuginea, Bucks fascia, and glans of each penis was weighed on an analytical balance.

Histological Evaluation of Corpus Cavernosum Tissue

Cross sections (3–5 mm thick) from the medial region of the penile shaft were fixed in 10% formalin buffered with 75 mM phosphate for staining with hematoxylin and eosin or Masson's trichrome.

Masson's Trichrome Staining of Tissue Sections

Tissue staining by Masson's trichrome was carried out as described previously (Nehra et al, 1996; Traish et al, 1999). Briefly, fixed tissues were embedded in paraffin, sectioned (6 μm), and placed on Colorfrost Plus glass slides (Fisher Scientific, Pittsburgh, Pa). Tissue sections were deparaffinized with CitriSolv (Fisher Scientific) and rehydrated in graded ethanol solutions (100%–70%). Sections were then placed in Bouin's fixative for 1 hour at room temperature, transferred to 4% ferric ammonium sulfate for 5 minutes at 50°C, and rapidly rinsed with distilled water at 50°C. Sections were stained with 1% hematoxylin at 50°C for 30 to 60 seconds and destained in 2% ferric ammonium sulfate at room temperature until only nuclei retained stain. After washing in running water for 10 minutes, slides were immersed in 0.1% acid fuchsin for 1 minute and gently rinsed by repeatedly immersing in water 5 times. The slides were then placed in 1% phosphomolybdic acid for 10 minutes and then stained for 90 seconds in 0.25% aniline blue/0.5% phosphomolybdic acid. The slides were washed in water until the rinses became clear and then dehydrated in graded ethanol, cleared with CitriSolv, and coverslipped with Permount (Fisher Scientific).

Staining of Fat-Containing Cells With Osmium Tetroxide

Penile tissues were fixed in 4.3% glutaraldehyde in 0.03 M Palade's veronal acetate buffer (pH 7.4) containing 0.07 M KCl and postfixed with 1% osmium tetroxide. Penile tissues were then embedded in epoxy, and semithin sections (1–2 μm) were stained with Toluidine blue by adding several drops of filtered aqueous 1% Toluidine blue/1% borax solution and heated at 70°C to 80°C for 45 seconds. After incubation, the slides were rinsed thoroughly with water.

Data Analysis

Plasma testosterone data for orchiectomized animals were analyzed by paired *t* test. Comparison of hemodynamic data between control and orchiectomized animals was analyzed by unpaired *t* test. Comparisons were considered to be significantly different at *P* less than or equal to .05.

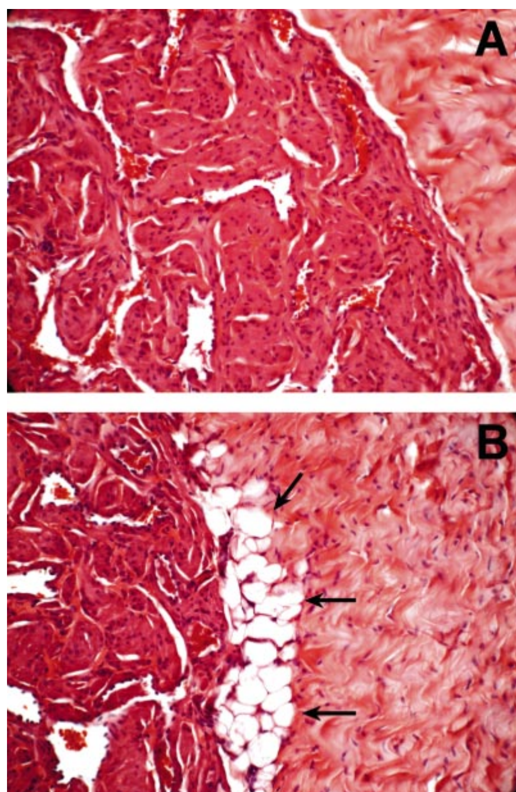

Figure 1. Hematoxylin and eosin staining of penile tissue. Male rabbits (4.5–5 kg) were subjected to sham surgery **(A)** or bilateral orchiectomy **(B)**. After 2 weeks, rabbits were euthanized and cross sections (3–5 mm) of penile tissue were removed from the medial region. Tissue was fixed in buffered 10% formalin, embedded in paraffin, sectioned (6 μm), and stained with hematoxylin and eosin. Note numerous empty cellular structures (probably adipocytes from which fat is washed out) under the tunica albuginea in the orchiectomized rabbit (arrows). Magnification, 125×.

Results

Effects of Orchiectomy on Plasma Testosterone Levels and Penile Weight

In the intact animals, testosterone plasma levels ranged between 2 and 2.5 ng/mL. Two weeks postorchiectomy, plasma testosterone levels decreased significantly to 0.3–0.35 ng/mL. Penile wet weight was significantly reduced from 2.56 ± 0.12 g in the control animals to 1.85 ± 0.18 g in the castrated animals.

Effects of Androgen Deprivation on Penile Corpus Cavernosum Morphology

Histologic examination of hematoxylin and eosin–stained penile tissue sections from intact (sham-operated control) animals showed normal tissue morphology with a characteristic network of cavernosal spaces (sinusoids) and trabecular smooth muscle bundles surrounded by the tunica albuginea (Figure 1A). In contrast, penile tissue sections from orchiectomized animals exhibited clusters of ‘‘empty’’ cellular structures in the subtunical region of the corpus cavernosum that were distinct from cavernous spaces (Figure 1B). These hollow cells resembled adipocytes. When penile tissue was subjected to Masson's trichrome staining, these hollow cells were consistently present in tissue from orchiectomized animals but absent in tissue from control animals (Figure 2).

Because normal processing of paraffin-embedded tissue with organic solvents results in removal of fat droplets, we fixed penile tissue in glutaraldehyde and postfixed with osmium tetroxide, which binds to unsaturated lipids and results in a brown or gray-black stain. Tissues were then embedded in epoxy resin to verify that the hollow cells were indeed adipocytes. Epoxy-embedded tissue sections were then stained with Toluidine blue to visualize the remaining cellular structures. Although not all cells retained their lipid content, this fixation and staining procedure confirmed the presence of fat globules in a majority of the hollow cells that were observed in paraffin-embedded tissue sections (Figure 3). Interestingly, a few fat cells were always present in the penile tissue of control animals. However, the quantity and distribution of fat-containing cells was greatly increased in tissue sections from orchiectomized animals (Figure 3B).

Effect of Androgen Deprivation on Erectile Function

In intact animals, the mean baseline ICP/SAP ratio was 0.16 ± 0.10. Pelvic nerve stimulation at 2.5 and 4 Hz caused a marked increase in the ICP/SAP ratio in intact animals, with values increasing to 0.48 ± 0.14 at 2.5 Hz and 0.69 ± 0.11 at 4 Hz (Figure 4). In orchiectomized animals, the mean baseline ICP/SAP value was 0.19 ± 0.02 and was not significantly different from that of intact animals. However, as shown in Figure 4, a marked decrease in erectile function was observed in orchiectomized animals. The ICP/SAP ratios were 0.18 ± 0.02 at 2.5 Hz and 0.32 ± 0.11 at 4 Hz for this group. Androgen deprivation via surgical castration did not result in significant changes in mean systemic blood pressure (73.8 ± 1.4 mm Hg in the orchiectomized group vs 72.8 ± 2.4 mm Hg in the control group).

Discussion

Normal erectile function is dependent on trapping incoming blood within the cavernosal bodies to develop increasing pressure and volume. This physiologic process, termed the veno-occlusive mechanism, depends on the integrity of neural, vascular, and endocrine components, as well as the fibroelastic properties of the cavernosal tissue. The penile corpus cavernosum is a vascular bed, and any alterations to its structure can produce vascular insufficiency, a common etiology of erectile dysfunction (Krane et al, 1989). It has been noted that cavernosal

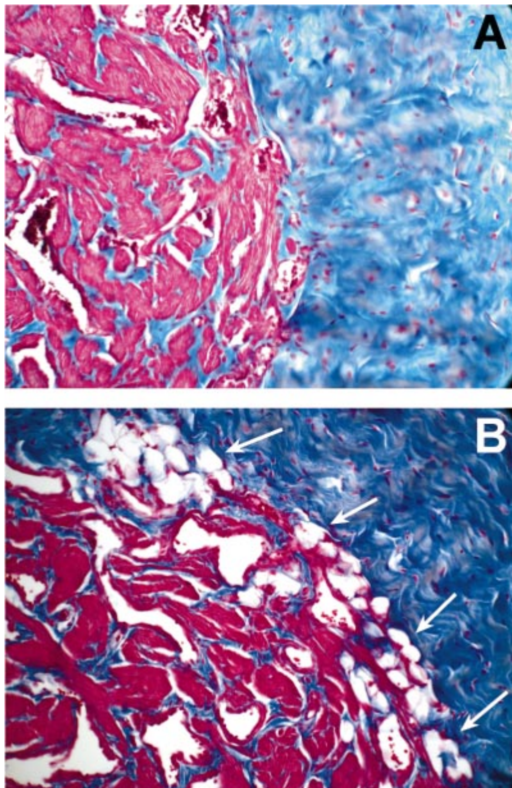

Figure 2. Masson's trichrome staining of penile tissue. Penile tissue from sham-operated **(A)** or orchiectomized **(B)** rabbits (see Figure 1) was fixed in buffered 10% formalin, embedded in paraffin, sectioned (6 μm), and subjected to Masson's trichrome staining. Smooth muscle is stained red and connective tissue is stained purple-blue. Arrows in panel B indicate empty cellular structures (see Figure 1). Magnification, 125×.

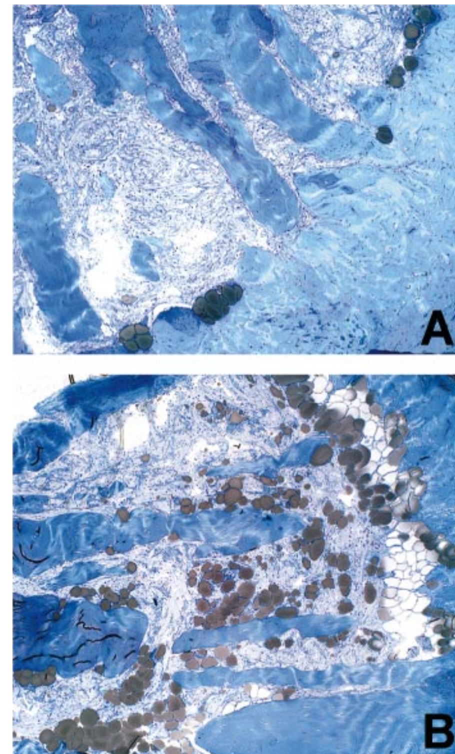

Figure 3. Toluidine blue staining of penile tissue. Penile tissue from sham-operated **(A)** or orchiectomized **(B)** rabbits (see Figure 1) was fixed in glutaraldehyde, postfixed in 1% osmium tetroxide, and embedded in epoxy. Tissue sections (1–2 μm) were then subjected to Toluidine blue staining. Note the presence of fat globules within cells that are readily visible by the gray staining of the osmium tetroxide. Magnification, 50×.

tissue from men with erectile dysfunction of various etiologies, whether hormonal, neurological, or vascular, exhibited reduced lacunar spaces, reduced smooth muscle content, and a concomitant increase in connective tissue deposition (Mersdorf et al, 1991; Karadeniz et al, 1996). It is proposed that such changes in penile tissue structural integrity contribute to veno-occlusive dysfunction.

Several hypotheses have been put forth to explain the decline in erectile function after orchiectomy. These include reduced NO synthesis from decreased expression of NOS isoforms (Chamness et al, 1995; Garban et al, 1995; Lugg et al, 1995, 1996; Zvara et al, 1995; Penson et al, 1996; Schirar et al, 1997) and changes in responsiveness of the vascular smooth muscle to neurotransmitters. Alternatively, we previously proposed that orchiectomy results in an increased extracellular matrix with a concomitant decrease in smooth muscle content (Traish et al, 1999, 2003). These changes contribute to a reduced compliance of the cavernosal bodies that prevents engagement of the veno-occlusive mechanism.

Along with the previously noted histopathologic changes in smooth muscle and connective tissue content, the most striking observation made in this study is the ac-

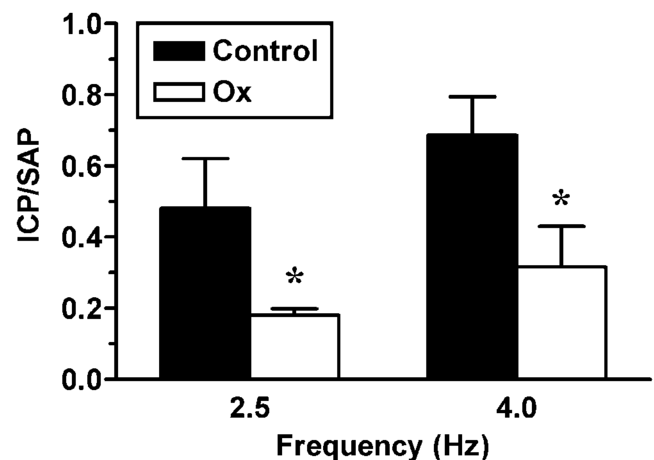

Figure 4. Effect of orchiectomy on erectile function. Systemic arterial and intracavernosal blood pressure (ICP) was continuously recorded in anesthetized male rabbits that were intact (Control, n = 4) or orchiectomized (Ox, n = 4). The maximum rise in ICP was determined at the indicated frequencies of pelvic nerve stimulation and normalized to systemic arterial blood pressure (SAP). Data are mean ± SEM (*$P < .05$ compared with control).

cumulation of fat-containing cells in the corpus cavernosum. These alterations in cavernosal tissue composition and structure are accompanied by a reduced erectile response to pelvic nerve stimulation. It is interesting to note that because veno-occlusion depends on the compression of the subtunical venules to impede blood outflow during sexual stimulation, it is possible that the presence of fat cells in the subtunical region of the corpus cavernosum might contribute to venous leakage in the orchiectomized animal.

Previous studies in the intact rabbit have noted that administration of the endocrine disrupters bisphenol A and tetrachlorodibenzodioxin (TCDD) resulted in an abnormal deposition of fat-containing cells in the subtunical region of the corpus cavernosum (Moon et al, 2001, 2004). However, these reports noted an increase in the trabecular smooth muscle content and thickening of the tunica albuginea. The increase in smooth muscle content is in contrast to that observed with castrated animals (Traish et al, 1999, 2003). Organ bath studies that use cavernosal tissue from bisphenol A and TCDD-treated animals showed a reduced relaxation response to nitroprusside and acetylcholine. However, no hemodynamic studies were presented to demonstrate the effects of these agents on the erectile response. Interestingly, neonatal rats exposed to the estrogen receptor agonist diethylstilbestrol also showed accumulation of fat-containing cells in the penile corpus cavernosum, whereas animals treated with vehicle exhibited no fat-containing cells (Goyal et al, 2004a,b). The authors suggested that estrogen treatment coupled with low plasma androgen levels contributed to alterations in penile morphology, infertility, and potentially erectile dysfunction (Goyal et al, 2004a,b). However, no hemodynamic or organ bath studies were presented to document that these changes in erectile tissue morphology are related to the erectile response in the estrogen-treated rats.

Masson's trichrome staining of penile tissue confirmed our previous observations that the area occupied by the smooth muscle cells (stained red) was decreased and that of connective tissue (stained blue-purple) increased as a result of orchiectomy (Traish et al, 1999, 2003). Furthermore, the data presented in this study suggest that additional histopathologic changes in the subtunical region of the corpus cavernosum in which fat-containing cells accumulated might interfere with the veno-occlusive mechanism and contribute to erectile dysfunction. It is possible that androgens might modulate the differentiation of the progenitor stroma cells into the myogenic lineage and that androgen deficiency (after orchiectomy) shifts this differentiation into the adipogenic lineage, contributing to accumulation of fat cells in the penis.

Bhasin et al (2003) proposed that androgens promote the commitment of pluripotent stem cells into a muscle lineage, whereas androgen deprivation promotes differentiation of pluripotent stem cells into an adipocyte lineage. In a recent study, Singh et al (2003) showed that differentiation of pluripotent cells C3H10T1/2 is androgen dependent. Both testosterone and dihydrotestosterone decreased the number of adipocytes and down-regulated the expression of the adipogenic markers PPAR-γ-2 and C/EBPα. However, these mechanisms have yet to be investigated in tissue or cells from the corpus cavernosum. It is possible that pluripotent stem cells are present in the corpus cavernosum and that these cells respond to androgen deprivation by differentiation to an adipogenic lineage.

Another possibility is the dedifferentiation of the corpus cavernosum trabecular smooth muscle cells into other phenotypes. In several experimental systems, vascular smooth muscle was shown to undergo dedifferentiation into other phenotypes (Johnson et al, 2001; Ruker-Martin et al, 2002). Interestingly, Corradi et al (2004) showed that inhibition of 5α-reductase activity induces stromal remodeling and smooth muscle dedifferentiation in the prostate. Because 5α-reductase converts testosterone to 5α-dihydrotestosterone (5α-DHT), these data suggest that 5α-DHT deficiency promotes smooth muscle dedifferentiation. However, there is no data in the literature on the dedifferentiation of the trabecular smooth muscle in the corpus cavernosum. Future studies that use expression of muscle biochemical markers, as well as changes in ultrastructure as determined by electron microscopy, will be needed to test this possibility in the corpus cavernosum under androgen deprivation and supplementation.

The role of androgens in erectile function is highly controversial. Although the prevalence of erectile dysfunction increases with age (Feldman et al, 1994), it is not clear whether an association exists between the progressive decrease in the circulating levels of androgens in aging males and erectile dysfunction. Rhoden et al (2002a,b) suggested that although erectile dysfunction showed a clear association with aging, there was no consistent correlation with total plasma testosterone. In men, bioavailable and free testosterone levels decline by about 1.0% and 1.2% per year, respectively, after the age of 40. Clinical symptoms associated with aging and androgen insufficiency are characterized by decreased muscle mass and strength, increased fat mass, loss of libido, erectile dysfunction, impaired cognitive function, and depression. Testosterone supplementation has been shown to be effective in improving androgen deficiency in the hypogonadal older male (Aversa et al, 2000, 2003).

Clinical studies have suggested that surgical or medical castration results in loss of libido and erectile function (Ellis and Grayhack, 1963; Peters and Walsh, 1987; Rousseau et al, 1988; Eri et al, 1994a,b; Greenstein et al, 1995; Hirshkowitz et al, 1997; Marumo et al, 1999). A meta-

analysis of 16 studies on the usefulness of androgen replacement therapy for erectile dysfunction by Jain et al (2000) found a statistically significant difference in favor of testosterone over placebo, implying a role for testosterone supplementation in select groups. Recently, Aversa et al (2000) studied 52 men with erectile dysfunction without vascular risk factors in a double-blind correlation analysis. They noted a direct correlation between cavernosal artery resistive index values and free testosterone, a relationship that was maintained after adjusting for age, sex hormone–binding globulin, and estradiol. They concluded that men with erectile dysfunction and low free testosterone might have impaired relaxation of penile smooth muscle, thus providing clinical evidence for the importance of androgen in regulating erectile function. Subsequently, Aversa et al (2003) performed a perspective, randomized, placebo-controlled study in 20 men with erectile dysfunction who failed sildenafil treatment (100 mg dose) on 6 consecutive attempts and had free testosterone in the lower quartile of the lower range. One month after treatment with transdermal testosterone and sildenafil on demand, they found significantly increased scores in the erectile function domain of the International Index of Erectile Function. These data suggest a critical role for testosterone in erectile physiology. Several clinical studies have indicated that the majority of patients undergoing luteinizing hormone–releasing hormone agonist treatment suffer complete or partial loss of erectile function (Peters and Walsh, 1987; Rousseau et al, 1988; Eri et al, 1994a,b; Marumo et al, 1999).

Several lines of evidence for a role of androgens in erectile function are also suggested from animal studies. Baba et al (2000a,b) demonstrated that the ICP decreased significantly in castrated animals (vs control) after both pelvic nerve stimulation or intracavernosal papaverine injection. More importantly, testosterone replacement restored penile hemodynamics. Rogers et al (2003) demonstrated that castrated animals developed venous leakage and veno-occlusive dysfunction and that testosterone treatment restored erectile function. The authors noted a decrease in smooth muscle content and degeneration of trabecular smooth muscle morphology. In the rat model, Mills et al (1992, 1994, 1996, 1998, 1999) and Reilly et al (1997a,b,c) proposed that androgens are critical for maintaining erectile function and might act specifically to support the responsiveness of the vascular smooth muscle to vasoactive agents, maintaining both the inflow and the outflow of blood from the cavernous spaces during erection. Other studies in the rat model have suggested that androgens modulate erectile function by up-regulating NOS activity in the corpus cavernosum (Muller et al, 1988; Lugg et al, 1995; Zvara et al, 1995; Penson et al, 1996). We have reported that androgen insufficiency in the rabbit animal model produced significant loss of trabecular smooth muscle and increased connective tissue deposition but had little effect on neural NOS expression or activity (Traish et al, 1999, 2003).

The effects of androgens on erectile physiology are complex, and androgens modulate various cellular components. We suggest that in the corpus cavernosum, androgens affect 1) smooth muscle cell content and smooth muscle reactivity to vasodilators, 2) connective tissue metabolism, and 3) the differentiation of progenitor stroma cells into myogenic and lipogenic lineages. Thus, androgen deprivation results in erectile dysfunction by altering penile corpus cavernosum structural and functional integrity at multiple cellular components.

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