Arginase: Structure, Mechanism, and Physiological Role in Male and Female Sexual Arousal

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ABSTRACT

Mammalian arginases I and II require an intact binuclear manganese cluster for the hydrolysis of L-arginine to generate L-ornithine and urea. Although arginase isozymes differ in terms of their tissue distribution, cellular localization, and metabolic function, each employs a metal-activated hydroxide mechanism for catalysis. To date, the best arginase inhibitors are those bearing *N*-hydroxyguanidinium or boronic acid "warheads" that can bridge the binuclear manganese cluster. Strikingly, the trigonal planar boronic acids undergo nucleophilic attack by hydroxide ion to form tetrahedral boronate anions that mimic the tetrahedral intermediate and its flanking transition states in the arginase mechanism. Given their affinity and specificity for arginase, boronic acid inhibitors are especially useful for probing the role of arginase in living systems. Arginase can regulate L-arginine bioavailability to nitric oxide synthase by depleting the substrate pool for NO biosynthesis, so arginase inhibition can enhance the substrate pool for NO biosynthesis. Accordingly, arginase inhibition can enhance NO-dependent physiological processes, such as the smooth muscle relaxation required for sexual arousal: administration of arginase inhibitors in vitro and in vivo enhances erectile function and engorgement in the male and female genitalia. Therefore, arginase is a potential therapeutic target for the treatment of sexual arousal disorders in men and women.

Introduction

Arginase is a 105 kD homotrimeric enzyme that requires manganese for the hydrolysis of L-arginine to form L-ornithine and urea. Two genetically distinct isozymes have evolved with differing tissue distributions and subcellular locations in mammals.[1,2] Arginase I is found predominantly in the liver, where it catalyzes the final cytosolic step of the urea cycle and is responsible for the generation of ∼10 kg of urea per year by the average human adult.[3,4] Arginase II is a mitochondrial enzyme that does not appear to function in the urea cycle and is more widely distributed in numerous tissues, for example, kidney, brain, skeletal muscle, and liver.[5,6] Genetic "knockout" experiments suggest that arginase II functions in L-arginine homeostasis by regulating L-arginine concentrations for cellular biosynthetic reactions such as nitric oxide (NO) biosynthesis.[7] Since L-arginine is the substrate of both arginase and NO synthase, arginase activity can effectively inhibit NO-dependent processes by depleting the substrate pool available for NO biosynthesis (Figure 1), for example, as observed in the cellular immune response and the regulation of smooth muscle tone.[8−12] Conversely, arginase inhibition can effectively enhance NO biosynthesis and NO-dependent processes by enhancing L-arginine bioavailability to NO synthase.[13−17]

Given that NO-dependent smooth muscle relaxation in the male and female genitalia is required for erectile function and genital engorgements, and given that both NO synthase and arginase are localized in male and female genitalia,[14−16,18−22] it follows that arginase inhibition may enhance the NO-dependent physiological processes required for sexual arousal.[14−16] In this Account, I shall review the structural and chemical biology of arginase, the structure-based design of arginase inhibitors, and the use of these inhibitors to probe arginase function in the physiology of sexual arousal.

Structure and Chemical Mechanism

The X-ray crystal structure[23] of rat arginase I provided the first view of the spin-coupled binuclear manganese cluster detected in electron paramagnetic resonance (EPR) experiments (Figure 2).[24] The metal cluster resides at the bottom of a ∼15 Å-deep active site cavity in each monomer and the Mn_A^{2+}−Mn_B^{2+} internuclear separation is 3.3 Å, consistent with the zero-field splitting of the quintet state observed in the EPR spectrum.[25] An intact binuclear manganese cluster is required for the stabilization and orientation of the catalytic nucleophile, a metal-bridging hydroxide ion:[23] the dialysis of Mn_A^{2+} from native arginase I or the substitution of ligands to either Mn_A^{2+} or Mn_B^{2+} in arginase I variants results in up to 20 000-fold reductions in k_{cat} due to the disruption of the metal cluster.[26−28] Simultaneous coordination of a water molecule to both metal ions in the cluster facilitates ionization to form a metal-bridging hydroxide ion,[29] and the activity-linked pK_a of 7.9 in the pH−rate profile may correspond to this ionization.[30]

Although the variation of Mn_A^{2+} or Mn_B^{2+} stoichiometry by dialysis or mutagenesis severely compromises catalysis, the Michaelis constant, K_M, remains essentially invariant (1.0−2.5 mM).[26−28] Since K_M reflects enzyme−substrate affinity in the precatalytic enzyme−substrate complex and since K_M is relatively insensitive to perturbations in the binuclear manganese cluster, a strong inner-sphere coordination interaction between the L-arginine guanidinium group and the metal cluster seems unlikely in the precatalytic enzyme−substrate complex.[28] Consistent with this expectation, the recent structure determination of an enzyme−substrate−uncompetitive inhibitor complex confirms a nonmetal binding site for L-arginine (by definition, an uncompetitive inhibitor binds only to the preformed enzyme−substrate complex).[31] Based on analysis of the native enzyme structure, Kanyo and colleagues proposed that the substrate guanidinium group hydrogen bonds

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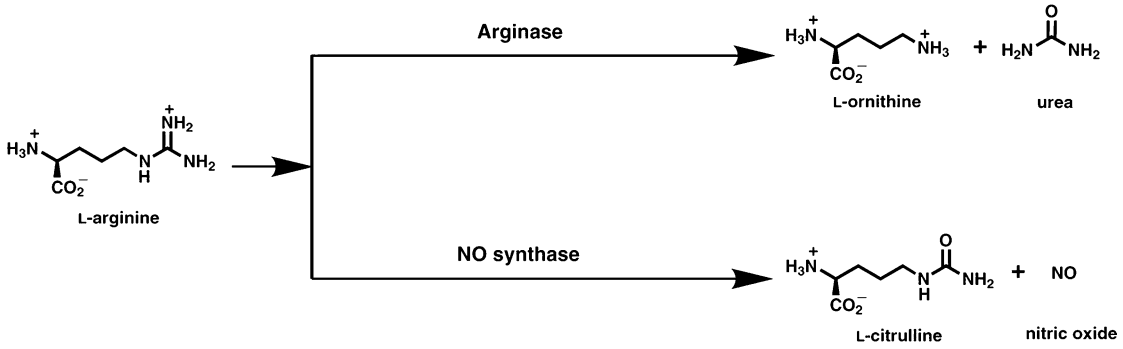


FIGURE 1. L-Arginine catabolism by arginase and NO synthase.

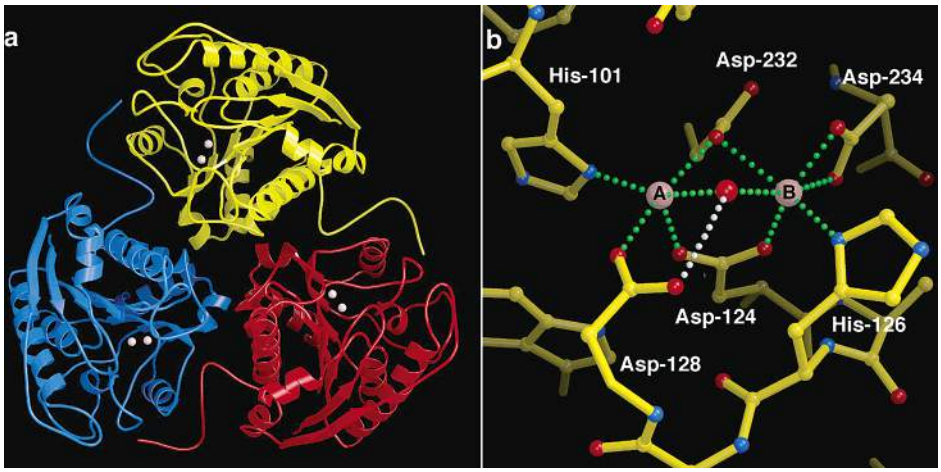


FIGURE 2. Panel a presents a ribbon plot of the arginase trimer. The binuclear manganese cluster is represented by a pair of spheres in each monomer. Panel b shows the binuclear manganese cluster of arginase. Metal coordination interactions are indicated by green dotted lines, and the hydrogen bond between the metal-bridging hydroxide ion (red sphere) and Asp128 is indicated by a white dotted line. Mn_A^{2+} is coordinated with square pyramidal geometry, leaving a vacant coordination site that permits octahedral coordination geometry as a means of transition state stabilization in catalysis. Mn_B^{2+} is coordinated with octahedral geometry. Reprinted with permission from ref 23. Copyright (1996) Nature Publishing Group (http://www.nature.com/nature/).

with Glu277.[23] Intermolecular interactions subsequently observed in the crystal structures of rat arginase I, human arginase I, and human arginase II complexed with reactive substrate analogues,[14−16,33] as well as the crystal structure of inactivated *Bacillus caldovelox* arginase complexed with L-arginine,[34] together indicate that the carboxylate side chain of Glu277 and the backbone carbonyl oxygen of His141 accept hydrogen bonds from the guanidinium η2-NH_2 group, and the hydroxyl side chain of Thr246 accepts a direct or water-mediated hydrogen bond from the guanidinium η1-NH_2 group.

This hydrogen bond array orients the substrate guanidinium group for nucleophilic attack by the metal-bridging hydroxide ion, which generates a metastable tetrahedral intermediate (Figure 3). Structural studies of arginase complexed with "reaction coordinate analogues" (reactive substrate analogues that undergo a chemical transformation that mimics a chemical step of catalysis[35]) reveal important clues regarding the formation of the tetrahedral intermediate. Consider the boronic acid analogues 2(*S*)-amino-6-boronohexanoic acid (ABH) and *S*-(2-boronoethyl)-L-cysteine (BEC), in which the trigonal planar boronic acid moiety replaces the trigonal planar guanidinium group of L-arginine (Table 1).[14,15,36−38] The electron-deficient boron atom in each of these L-arginine analogues is particularly susceptible to attack by a solvent nucleophile, such as the metal-bridging hydroxide ion, to yield the tetrahedral boronate anion—just as nucleophilic attack of hydroxide ion at the guanidinium group of L-arginine yields the tetrahedral intermediate in catalysis.

The crystal structure of rat arginase I complexed with ABH was the first to reveal the molecular strategy for transition state stabilization in catalysis.[14] The tetrahedral boronate anion of ABH (or, by analogy, the tetrahedral intermediate and its flanking transition states) makes multiple interactions with the binuclear manganese cluster and neighboring protein residues (Figure 4): the boronate hydroxyl group O1 symmetrically bridges Mn_A^{2+} and Mn_B^{2+} ($Mn_A^{2+}\cdots O$ and $Mn_B^{2+}\cdots O$ separations = 2.2 Å) and donates a hydrogen bond to Oδ2 of D128; the boronate hydroxyl group O2 makes a 2.4 Å coordination interaction with Mn_A^{2+} and donates hydrogen bonds to Glu277 and the backbone carbonyl of His141; the boronate hydroxyl group O3 makes a water-mediated hydrogen bond interaction with Thr246. The structures of rat and

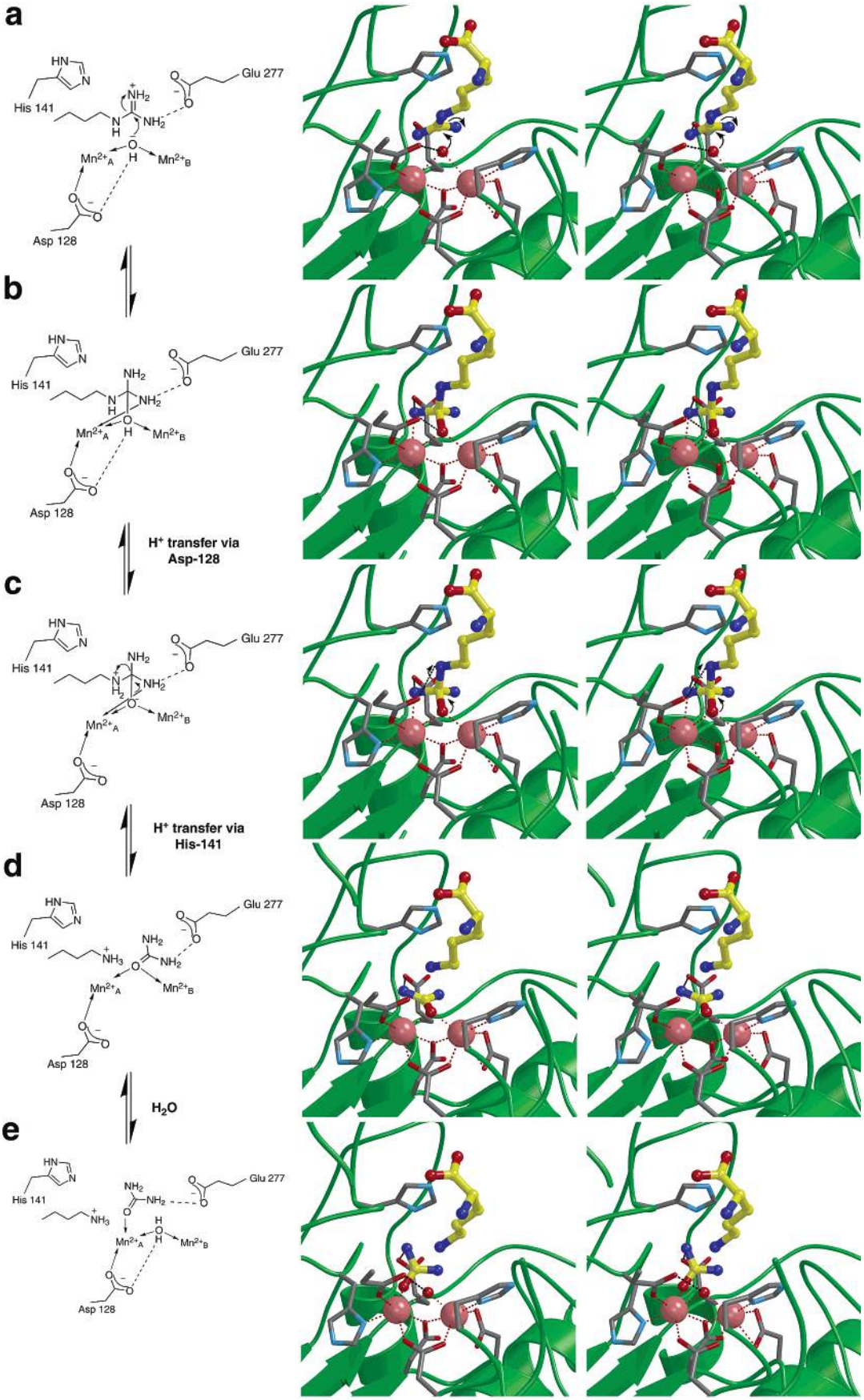
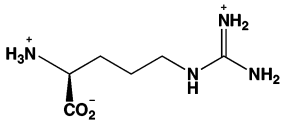
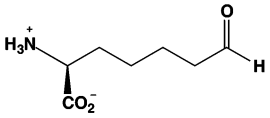
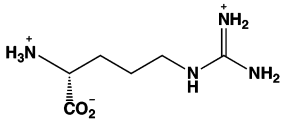
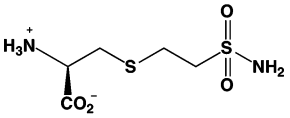
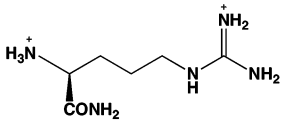
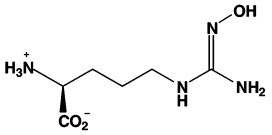
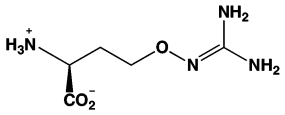
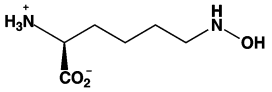
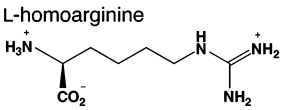
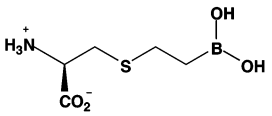
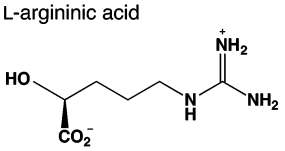
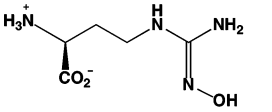
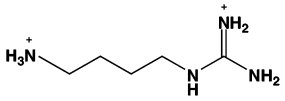
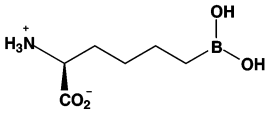


FIGURE 3. Arginase mechanism. Reprinted with permission from ref 41. Copyright (2001) American Chemical Society.

Table 1. Selected Substrates and Inhibitors of Rat Arginase I

Substrate[a]	K_M (mM)	k_{cat}/K_M x 10^3 ($M^{-1}s^{-1}$)	Inhibitor	K_i or K_d (μM)
L-arginine 	1.0	2600	AOH[b] 	60
D-arginine 	—	—	SEC[c] 	52
L-argininamide 	12.4	150	NOHA[d] 	10
L-canavanine 	12.0	7.4	N-hydroxy-L-lysine[e] 	4
L-homoarginine 	7.2	2.8	BEC[f] 	2.2
L-argininic acid 	9.3	0.82	nor-NOHA[d] 	0.5
agmatine 	10.8	0.048	ABH[g] 	0.11

[a] Reference 42. [b] Reference 32. [c] Reference 40. [d] Reference 47. [e] Reference 46. [f] References 15 and 33; K_d = 0.27 μM against human arginase I. [g] References 33, 36−38; K_d = 5 nM against human arginase I and K_i = 8.5 nM against human arginase II.

human arginases I and human arginase II complexed with BEC reveal comparable intermolecular interactions.[15,16,33]

Given that ABH binding mimics transition state binding, it is clear that both Mn_A^{2+} and Mn_B^{2+} are critical for transition state stabilization: the metal-bridging hydroxyl group of the tetrahedral intermediate (corresponding to boronate hydroxyl group O1) remains close to its position as the former metal-bridging hydroxide ion of the native enzyme, and the vacant coordination site on Mn_A^{2+} in the native enzyme (Figure 2b) accommodates a sixth coordination interaction by the developing sp^3 lone electron pair on the former η2-NH_2 group of L-arginine (corresponding to boronate hydroxyl group O2) (Figure 3b).

In the crystal structure of the complex between inactivated, Mn_B^{2+}-depleted *B. caldovelox* arginase and L-arginine, the substrate η2-$NH_2\cdots Mn_A^{2+}$ separation is 2.5 Å.[34] Interpreting this as an inner-sphere interaction, Bewley and colleagues propose that the substrate coordinates to metal in the precatalytic enzyme−substrate complex.[34] However, such an interaction would require the η2-NH_2 group of L-arginine to undergo $sp^2 \rightarrow sp^3$ rehybridization prior to nucleophilic attack at the guanidinium carbon, which would break the "Y"-shaped guanidinium π system. Moreover, an inner-sphere metal coordination interaction in the precatalytic enzyme−substrate complex is difficult to reconcile with the insensitivity of K_M to perturbations in the metal cluster.[26−28] Given that the boronate hydroxyl O2$\cdots Mn_A^{2+}$ coordination interactions of 2.4 and 2.5 Å in the arginase−ABH and arginase−BEC complexes,[14,15] respectively, are somewhat

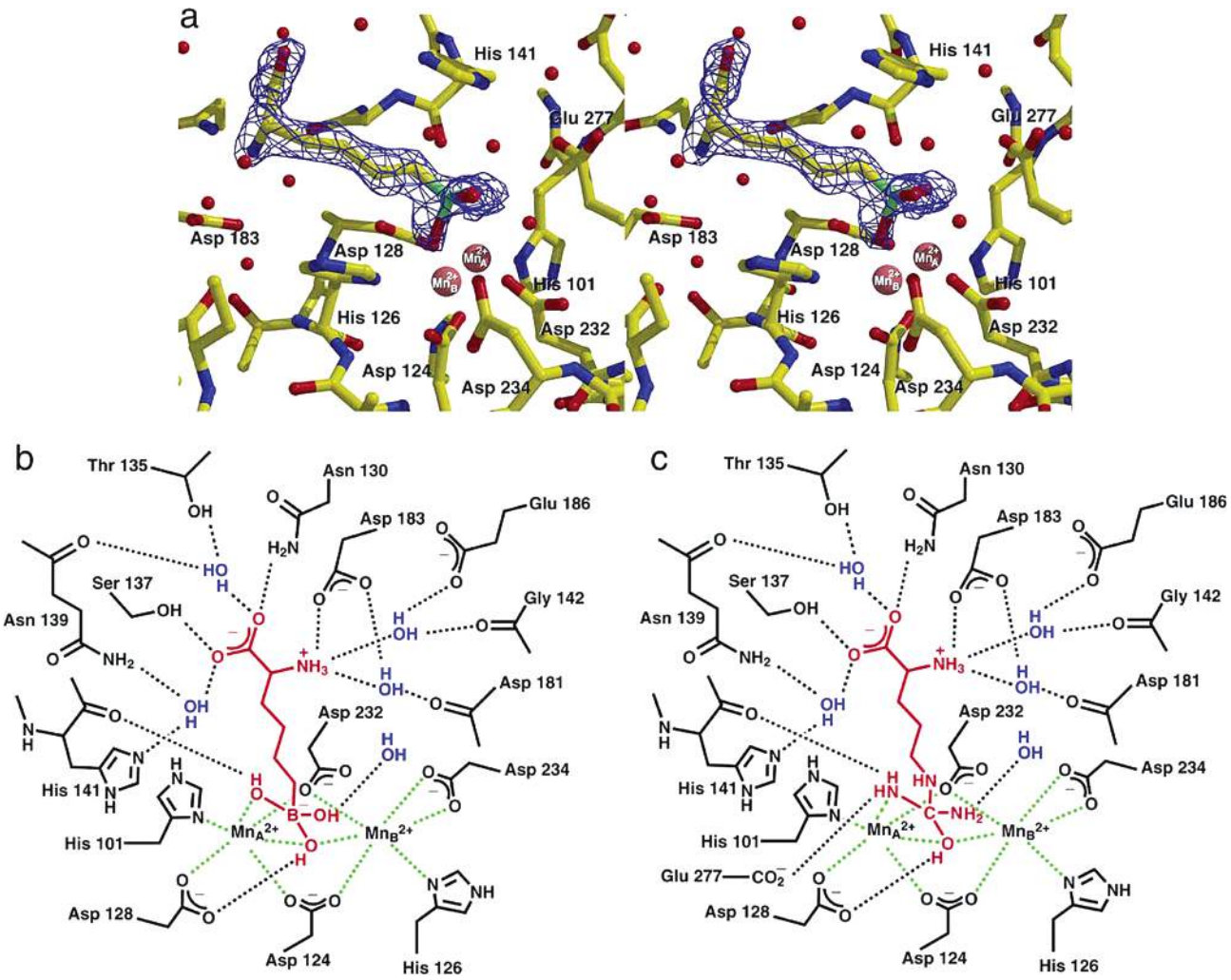
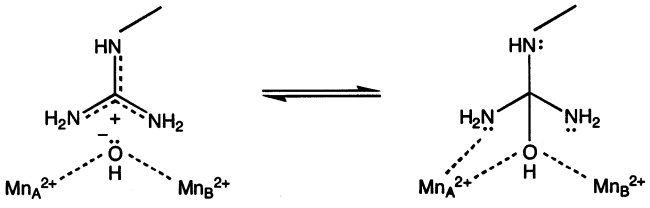


FIGURE 4. In panel a, the electron density map of the rat arginase I—ABH complex conclusively reveals the binding of ABH as the tetrahedral boronate anion. Panel b shows the intermolecular interactions in the rat arginase I—ABH complex. Metal coordination and hydrogen bond interactions are designated by green and black dashed lines, respectively. Panel c shows postulated intermolecular interactions of the tetrahedral intermediate in arginase catalysis presumed by analogy with panel b. Reprinted with permission from ref 14. Copyright (1999) Nature Publishing Group (http://www.nature.com/nsmb).

long for inner-sphere interactions and given that the L-arginine η2-NH_2···Mn_A^{2+} interaction of 2.5 Å is comparable,[34] it could be argued that the enzyme—substrate complex involves a weak outer-sphere interaction with Mn_A^{2+} *if* the η2-NH_2 group of L-arginine undergoes some degree of $sp^2 \rightarrow sp^3$ rehybridization in the active enzyme—substrate complex. Regardless, the sp^3 lone electron pair on the η2-NH_2 group is nearly fully developed in the transition state and is fully developed in the tetrahedral intermediate:



That Mn_A^{2+} serves a role unique in transition state stabilization illustrates Pauling's notion of transition state stabilization in enzyme catalysis[39] and clarifies the requirement for two manganese ions in arginase catalysis: both metals activate the bridging hydroxide ion nucleophile (Figure 3a), Mn_A^{2+} stabilizes the developing sp^3 lone electron pair on the η2-NH_2 group as the tetrahedral intermediate is approached (Figure 3b), and both metals stabilize the hydroxyl group of the tetrahedral intermediate and facilitate its ionization prior to the collapse of the tetrahedral intermediate (Figure 3b,c).

Following a proton transfer to the leaving amino group mediated by Asp128, which would be consistent with the binding conformations of boronic acid,[14−16] aldehyde,[32] and sulfonamide[40] analogues, the collapse of the tetrahedral intermediate yields products L-ornithine and urea (Figure 3d). The subsequent addition of a water molecule to the binuclear manganese cluster facilitates urea departure and the complex with L-ornithine, urea, and a metal-bridging water molecule (Figure 3e) has been stabilized for crystallographic analysis.[41] Urea departure may trigger the ionization of the metal-bridging water molecule to regenerate the nucleophilic metal-bridging hydroxide ion. The side chain of His141, which is 4.2 Å

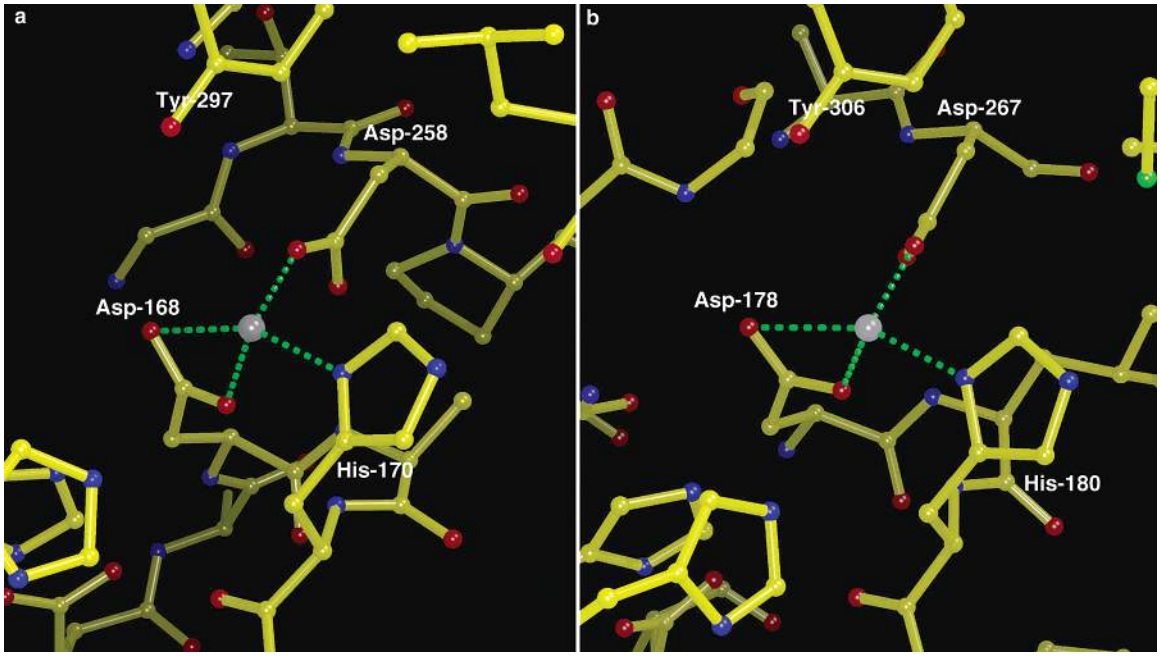


FIGURE 5. The zinc binding sites of *A. aeolicus* histone deacetylase-like protein (a) and human histone deacetylase-8 (b) correspond to the Mn_B^{2+} site of arginase (Figure 2b).

away from the metal-bridging hydroxide ion in the native enzyme, may help shuttle the ionized proton to bulk solvent.[23]

Substrate and Inhibitor Specificity

Arginase exhibits exquisite specificity for the hydrolysis of its sole biological substrate, L-arginine. Modifications to substrate structure or stereochemistry significantly attenuate catalysis: D-arginine is not a substrate, and derivatization or deletion of the α-substituents of L-arginine yields alternative substrates with severely compromised kinetic properties (Table 1).[42] The crystal structures of the rat arginase I−ABH complex[14] and the inactivated *B. caldovelox* arginase−L-arginine complex[34] reveal the structural basis for substrate and inhibitor specificity: an array of direct and water-mediated hydrogen bonds saturate all four acceptor positions on the α-carboxylate group and all three donor positions on the α-amino group. These interactions, as illustrated for the rat arginase I−ABH complex in Figure 4b, are generally conserved in the binding of numerous amino acid inhibitors, thereby rationalizing the binding and weak inhibition observed for inert amino acids.[31,43] Interestingly, the majority of hydrogen bond interactions with the α-substituents are water-mediated, as is the case for the binding of BEC to human arginases I and II.[16,33]

To date, the best arginase inhibitors are those bearing *N*-hydroxyguanidinium or boronic acid "warheads" that bridge the binuclear manganese cluster (Table 1). Intriguingly, an intermediate of NO biosynthesis, N^{ω}-hydroxy-L-arginine (NOHA; Table 1), is a modest inhibitor of arginase,[44,45] and the crystal structure of its complex with rat arginase I reveals that the N^{ω}-hydroxyl group displaces the metal-bridging hydroxide ion and bridges the binuclear manganese cluster.[41] Mansuy and colleagues noted that the distance between the α-carbon and the N^{ω}-OH group in a series of N^{ω}-hydroxy amino acids is critical for inhibitory activity,[46] which led to the design of N^{ω}-hydroxy-nor-L-arginine (nor-NOHA; Table 1).[47] The crystal structure of the complex between rat arginase I and nor-NOHA reveals that the N^{ω}-hydroxyl group of the inhibitor displaces the metal-bridging hydroxide ion.[41]

The simplest arginase inhibitor is the fluoride ion,[48,49] which is an uncompetitive inhibitor with $K_i = 1.3$ mM.[48] By definition, an uncompetitive inhibitor binds to the enzyme−substrate complex, and the crystal structure of the rat arginase I−L-arginine−$(F^-)_2$ complex has been determined.[31] This unusual mode of inhibition involves the displacement of the metal-bridging hydroxide ion by a fluoride ion, and the addition of a fluoride ion to the formerly vacant coordination site on Mn_A^{2+}. Metal-bound fluoride ions are further stabilized by short hydrogen bond interactions with the substrate guanidinium group, so substrate binding is clearly required to stabilize inhibitor binding: this is the hallmark of an uncompetitive inhibitor.

Divergence of Arginase and Histone Deacetylase from a Primordial Mononuclear Metalloenzyme

Arginase bears a surprising evolutionary resemblance to the mammalian histone deacetylases (HDACs) and their bacterial homologues, but the HDACs exhibit strikingly different metal ion specificity and stoichiometry for catalysis. The HDACs contain a single Zn^{2+} ion that is required for the hydrolysis of acetylated lysine residues located near the N-termini of nucleosomal histones, a process that is generally accompanied by decreased

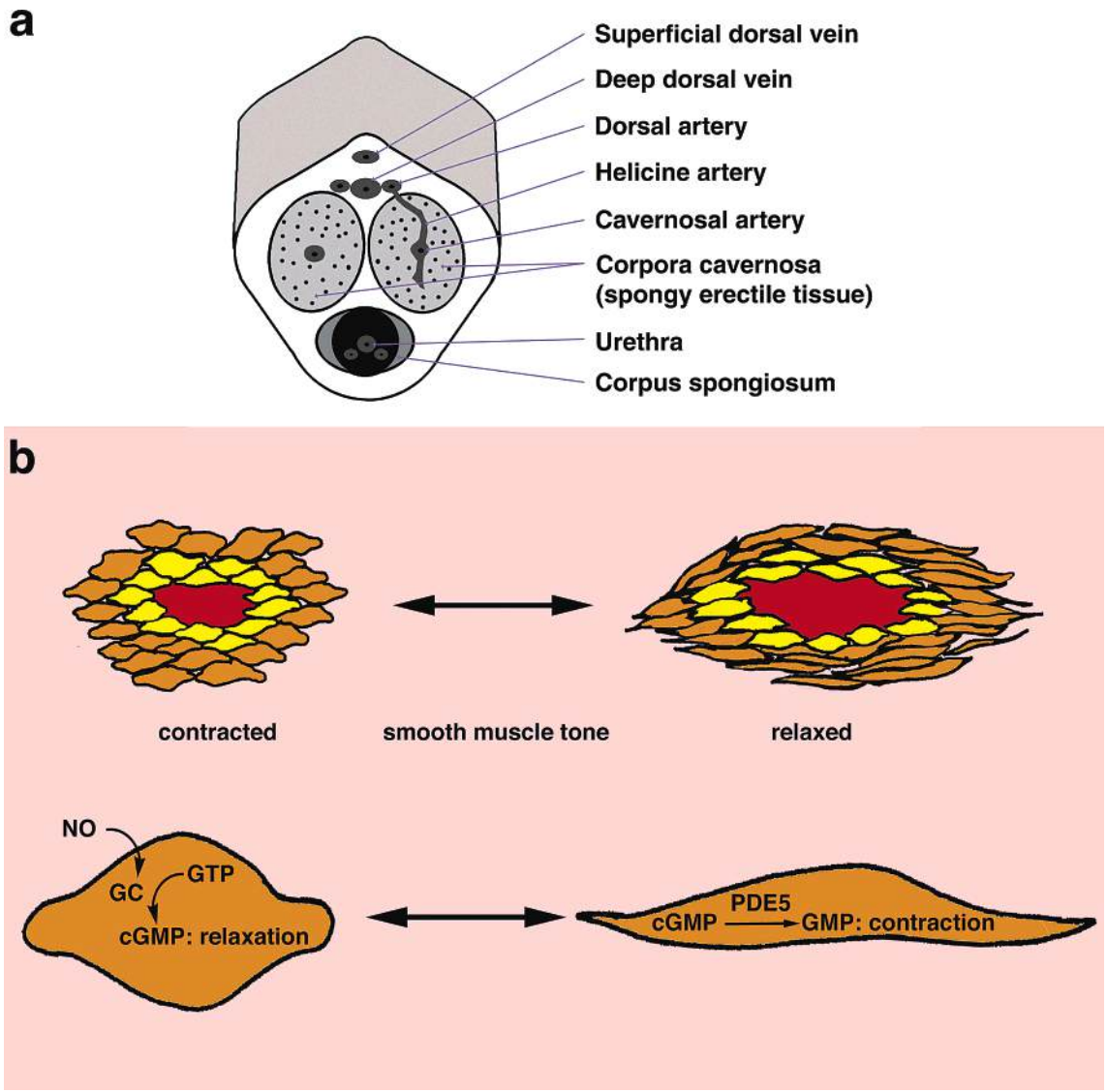


FIGURE 6. In panel a, a cross-section of the human penis illustrates the anatomy of the corpus cavernosum, which becomes engorged with blood during erection. In panel b, smooth muscle relaxation in genital tissues such as the male or female corpus cavernosum results in engorgement and erection. NO activates guanylate cyclase (GC), which generates cyclic guanosine monophosphate (cGMP), which mediates intracellular processes that lead to relaxation. Phosphodiesterase V (PDE5) degrades cGMP and leads to smooth muscle contraction. Inhibitors of PDE5 sustain cGMP concentrations and thereby enhance smooth muscle relaxation (e.g., this is the therapeutic strategy of Viagra). Insufficient NO flux in this signaling pathway could result in insufficient cGMP concentrations, in which case a phosphodiesterase V inhibitor will be unable to enhance smooth muscle relaxation in the absence of enhanced NO flux.

transcriptional activity.[50,51] The catalytic core of class I and class II HDACs is related to the histone deacetylase-like protein (HDLP) from *Aquifex aeolicus* (35% identity), the prokaryotic acetoin utilization proteins (28% identity), and prokaryotic acetylpolyamine amidohydrolase (15% identity).[52] The X-ray crystal structure of HDLP unexpectedly revealed the "arginase fold" despite only ~15% amino acid sequence identity, and the recently determined structures of human HDAC-8 reveal the same topological similarity.[53−55]

The crystal structures of HDLP and HDAC-8 show that the catalytic Zn^{2+} ion binds to a site corresponding to the higher-affinity Mn_B^{2+} site of arginase: Zn^{2+} ligands Asp168/Asp178, His170/His180, and Asp258/Asp267 of HDLP/HDAC-8 correspond to Mn_B^{2+} ligands Asp124, His126, and Asp234 of arginase (compare Figure 5 with Figure 2b). In HDLP/HDAC-8, the residues corresponding to Mn_A^{2+} ligands Asp128 and Asp232 of arginase are not conserved, so a "Mn_A^{2+} site" has not evolved for metal binding in the histone deacetylases. It is notable that the weaker Mn_A^{2+} site of arginase (K_d = 8 μM)[26,27] corresponds to the more "mutated" metal site in the histone deacetylases, suggesting that the evolution of the Mn_A^{2+} site and the divergence of Zn^{2+}/Mn^{2+} specificity occurred after the evolutionary divergence of arginase and histone deacetylase from a common primordial ancestor.

Arginase and the Physiology of Sexual Arousal

A recent survey indicates that 31% of men and 43% of women aged 18 to 59 years report varying degrees of sexual dysfunction.[56] Rooted in physiological or psycho-

logical causes or both, the various manifestations of this malady present an insidious threat to satisfactory reproductive health. In men, sexual dysfunction is succinctly characterized as erectile dysfunction (impotence), whereas in women, sexual dysfunction is more broadly classified in four main categories: hypoactive sexual desire, sexual arousal disorder, orgasmic disorder, and sexual pain disorder.[57] Female sexual arousal disorder, defined as an inability to achieve or maintain sufficient sexual excitement, including clitoral erection and genital engorgement, is physiologically analogous to male erectile dysfunction in that a deficiency in genital blood circulation compromises the hemodynamics of erection/engorgement.

Erectile dysfunction can result from physiological defects in the complex cascade of enzyme-catalyzed reactions governing blood flow into and out of the corpus cavernosum, a muscularized chamber of expandable tissue that becomes engorged with blood in the erect penis or clitoris (Figure 6a). Nitric oxide is the principal mediator of erectile function[18] and governs nonadrenergic, noncholinergic neurotransmission in penile corpus cavernosum smooth muscle.[19,58−61] NO causes rapid relaxation of smooth muscle tissue and thereby facilitates the engorgement of the corpus cavernosum (Figure 6b). Thus, NO synthase is clearly a critical enzyme in the physiology of sexual arousal.

A growing body of evidence suggests that arginase is also a critical enzyme in the physiology of sexual arousal, since it is coexpressed with NO synthase in smooth muscle tissue.[13−15,62] Given that arginase and NO synthase compete for the same substrate, L-arginine, arginase appears to attenuate NO synthase activity and NO-dependent smooth muscle relaxation by depleting the substrate pool of L-arginine that would otherwise be available to NO synthase (Figure 1).[13−16] Indeed, the overexpression of arginase II in the corpus cavernosum of diabetic men likely contributes to the erectile dysfunction that often accompanies this disease.[63] Conversely, arginase inhibition by the boronic acid inhibitors ABH or BEC (Table 1) sustains cellular L-arginine concentrations,[13] which in turn enhances NO synthase activity and NO-dependent smooth muscle relaxation in tissue bath experiments with rabbit and human penile corpus cavernosum (Figure 7)[14,15] and penile erection in live rabbits (Figure 8).[16] Thus, arginase is strongly implicated as a regulator of erectile function, and human penile arginase is accordingly a potential target for the treatment of male sexual dysfunction.

While the etiology of female sexual dysfunction is more complex, female sexual arousal disorder resulting from compromised circulation in the genitalia might similarly respond to arginase inhibition. Defined as genital arousal disorder, this malady can result from autonomic nerve damage or multiple sclerosis (notably, arginase I is significantly overexpressed in a murine model of multiple sclerosis[17]). Given the localization of NO synthase in clitoral corpus cavernosum[20] and vagina,[21] the subsequent identification of arginase activity in vaginal tissue extracts[16] suggests that arginase may similarly serve to regulate NO-dependent smooth muscle relaxation and engorgement

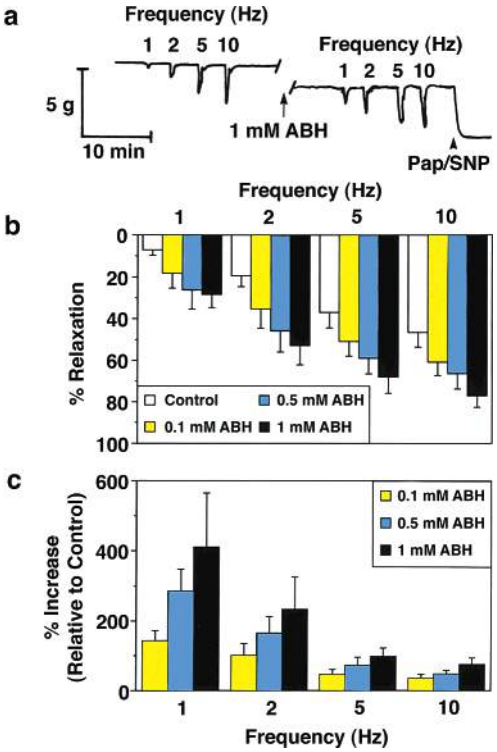


FIGURE 7. Effect of ABH on smooth muscle relaxation triggered by electric field stimulation (EFS) in organ bath experiments with penile corpus cavernosum. Panel a shows a representative polygraph tracing of responses to EFS in the absence and presence of 1 mM ABH; tissue tone is represented as grams of tension on the ordinate. In the absence of EFS, ABH causes moderate relaxation due to basal NO synthase activity. Panel b provides a summary of data acquired from four organ bath experiments. Panel c shows total increases in cavernosal smooth muscle relaxation caused by ABH. Reprinted with permission from ref 14. Copyright (1999) Nature Publishing Group (http://www.nature.com/nsmb).

in the female genitalia. Administration of the arginase inhibitor ABH to live female rabbits enhances genital engorgement following pelvic nerve stimulation, giving rise to 2-fold increases in genital tissue oxyhemoglobin concentrations as measured by near-infrared spectroscopy[16] and 34% enhancement of vaginal blood flow as measured by laser Doppler flowmetry.[64] Thus, arginase is strongly implicated as a regulator of genital hemodynamics and engorgement in the female, and arginase is therefore a potential target for the treatment of female sexual arousal disorder. Notably, ABH administration has no discernible effect on systemic arterial blood pressure in either male or female rabbits, so its effect on other NO-mediated vascular phenomena appears to be limited.[16]

With L-arginine bioavailability to NO synthase being implicated in male and female sexual arousal, it is perhaps not surprising that dietary supplements ("nutraceuticals") have been explored as remedies for sexual dysfunction. Dietary L-arginine supplements enhance intracavernosal pressure in the rat,[65] and 6 out of 10 men taking 2800 mg of L-arginine per day subjectively report improved erectile function in a pilot study.[66] In another study, 73.5% of women taking a nutritional supplement containing 2500 mg of L-arginine subjectively report "improved satisfaction

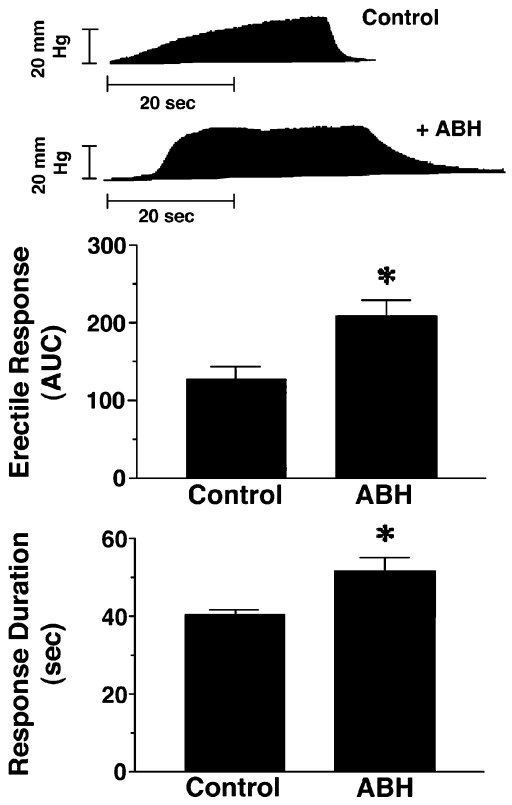


FIGURE 8. The arginase inhibitor ABH enhances the erectile response (area-under-the-curve of intracavernosal pressure as a function of time) and the duration of the erectile response following pelvic nerve stimulation in the male rabbit. Reprinted with permission from ref 16. Copyright (2003) American Chemical Society.

with their overall sex life".[67] However, efficacy has generally been limited in well-controlled clinical trials,[68] perhaps due to the "arginase problem"—L-arginine nutraceuticals supply substrate to both arginase and NO synthase, so arginase activity still attenuates NO biosynthesis and NO-dependent physiological effects.

Concluding Remarks

Although it seems that the advent of Viagra (sildenafil citrate) first popularized frank discussions of sex and chemistry, the molecular mechanisms of sexual arousal have been probed for many years prior. Indeed, Brindley used himself as a test subject for the evaluation of injectable penile erection drugs nearly two decades ago.[69] Since Brindley's pioneering contributions, pharmacological approaches using injectable and topically applied vasodilators such as nitroglycerin or prostaglandin E1 have shown efficacy in the treatment of male and female sexual arousal disorders.[70,71] However, such approaches bypass the neural circuitry between behavior and biochemistry that turns on the NO-dependent physiological processes required for sexual arousal.

Inhibitors of phosphodiesterase V (Figure 6b) are notable in that they do not bypass the neural circuitry of cavernosal smooth muscle relaxation and sexual arousal; instead, phosphodiesterase V inhibitors prevent the degradation of the second messenger, cGMP. Inhibitors of arginase are likewise notable in that they do not bypass the neural circuitry of sexual arousal; instead, they prevent L-arginine degradation, which then enhances L-arginine concentrations for biosynthesis of the first messenger, NO. Targeting arginase with inhibitors to enhance NO biosynthesis may be a useful strategy for the treatment of other diseases linked to L-arginine homeostasis, such as multiple sclerosis or other diseases of the immune response. For example, in a murine model for human multiple sclerosis characterized by significant arginase I overexpression, ABH-treated mice developed milder disease symptoms with delayed onset, reduced disease score, and expedited recovery.[17] Future research will undoubtedly illuminate additional disease pathologies in which this ubiquitous manganese metalloenzyme is implicated.

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Human Arginase II: Crystal Structure and Physiological Role in Male and Female Sexual Arousal[†,‡]

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ABSTRACT: Arginase is a binuclear manganese metalloenzyme that catalyzes the hydrolysis of L-arginine to form L-ornithine and urea. The X-ray crystal structure of a fully active, truncated form of human arginase II complexed with a boronic acid transition state analogue inhibitor has been determined at 2.7 Å resolution. This structure is consistent with the hydrolysis of L-arginine through a metal-activated hydroxide mechanism. Given that human arginase II appears to play a role in regulating L-arginine bioavailability to NO synthase in human penile corpus cavernosum smooth muscle, the inhibition of human arginase II is a potential new strategy for the treatment of erectile dysfunction [Kim, N. N., Cox, J. D., Baggio, R. F., Emig, F. A., Mistry, S., Harper, S. L., Speicher, D. W., Morris, S. M., Ash, D. E., Traish, A. M., and Christianson, D. W. (2001) *Biochemistry 40,* 2678−2688]. Since NO synthase is found in human clitoral corpus cavernosum and vagina, we hypothesized that human arginase II is similarly present in these tissues and functions to regulate L-arginine bioavailability to NO synthase. Accordingly, hemodynamic studies conducted with a boronic acid arginase inhibitor in vivo are summarized, suggesting that the extrahepatic arginase plays a role in both male and female sexual arousal. Therefore, arginase II is a potential target for the treatment of male and female sexual arousal disorders.

Arginase is a binuclear manganese metalloenzyme that catalyzes the hydrolysis of L-arginine to form L-ornithine and urea through a metal-activated hydroxide mechanism (Figure 1a) (*1*, *2*). In mammals, two isozymes are identified: arginase I is found predominantly in hepatocytes where it catalyzes the final cytosolic step of the urea cycle (*3*), and arginase II is extrahepatic (e.g., kidneys, small intestine, lactating mammary gland, penile corpus cavernosum) (*3*−*6*) and localizes subcellularly in the mitochondrial matrix of kidney cells (*7*). Arginase isozymes differ from each other in terms of their catalytic, molecular, and immunological properties. Unlike arginase I, the primary function of arginase II appears to be in L-arginine homeostasis (*8*−*10*), regulating L-arginine or L-ornithine pools for subsequent biosynthetic transformations (*11*).

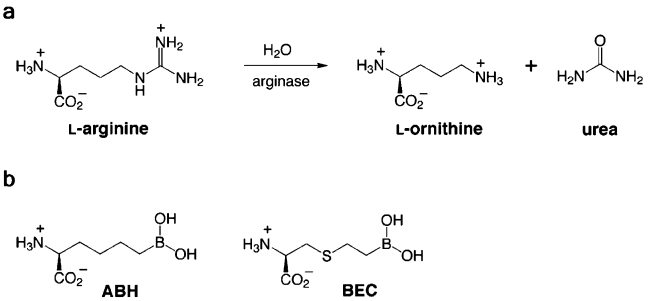

FIGURE 1: (a) Arginase reaction. (b) Arginase inhibitors 2(*S*)-amino-6-boronohexanoic acid (ABH) and *S*-(2-boronoethyl)-L-cysteine (BEC).

Being the predominant extrahepatic isozyme, arginase II can play a key role in the regulation of L-arginine bioavailability to NO synthase, given that both enzymes compete for the same substrate, L-arginine. In principle, arginase activity should attenuate NO biosynthesis and NO-dependent processes by attenuating L-arginine bioavailability to NO synthase, and arginase inhibition should enhance NO biosynthesis and NO-dependent processes by enhancing L-arginine bioavailability to NO synthase. Accordingly, recent studies demonstrate that potent boronic acid inhibitors of arginase enhance NO-dependent smooth muscle relaxation in rabbit and human penile corpus cavernosum (*6*, *12*). Additionally, the gene expression, protein level, and catalytic activity of arginase II are elevated in diabetic corpus

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[‡] The atomic coordinates for human arginase II complexed with *S*-(2-boronoethyl)-L-cysteine have been deposited in the Protein Data Bank, www.rcsb.org, with accession code 1PQ3.

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cavernosum, and NO biosynthesis is enhanced by treatment with a boronic acid inhibitor of arginase (*13*).

The cDNA of human arginase II has been isolated and characterized and encodes a 354-residue protein, including a putative, 22-residue N-terminal mitochondrial targeting sequence (*14*, *15*). The deduced amino acid sequence of human arginase II is 62% identical to that of rat arginase I and 60% identical to that of human arginase I; all active site residues implicated in catalysis, including the metal ligands, are strictly conserved. Mature human arginase II exists predominantly as a 129 kDa trimer (*16*), which is larger than the 105 kDa trimer of rat arginase I (*17*). Mature human arginase II contains one inserted residue, 85A, in the loop between helices B1 and B2 and 12 additional residues at the C-terminus. The K_M of L-arginine for human arginase II is approximately 4.8 mM at physiological pH (*16*), which is not significantly different from the K_M of 1.4 mM measured for rat arginase I (*18*). Amino acid inhibitors with modest isozyme selectivity have been identified (Figure 1b): 2(*S*)-amino-6-boronohexanoic acid (ABH) and *S*-(2-boronoethyl)-L-cysteine (BEC) bind tightly to rat arginase I at pH 8.5 with K_d values of 0.11 and 2.2 μM, respectively, measured by isothermal titration calorimetry (*6*, *20*) and are potent inhibitors of human arginase II at pH 9.5 with K_i values of 8.5 and 30 nM, respectively, measured by kinetic assay (*19*).

Here, we report the X-ray crystal structure of a fully active, truncated form of human arginase II complexed with the inhibitor BEC at 2.7 Å resolution. The truncation variant is ΔM1-V23/ΔH331-I354 (114 kDa trimer), which was constructed to circumvent aggregation problems observed with the wild-type enzyme (*16*). The arginase II−BEC structure provides important insights regarding structure−affinity relationships for inhibitors binding to human arginase II and rat arginase I (*21*). Importantly, arginase II−BEC complexation causes 20−50% enhanced NO-dependent relaxation of penile corpus cavernosum smooth muscle in electrophysiological organ bath experiments, suggesting that arginase inhibition may enhance male erectile function (*6*). Given that NO synthase is identified in human clitoral corpus cavernosum (*22*) and vagina (*23*), arginase II may also colocalize in these tissues; if so, an arginase inhibitor may similarly enhance smooth muscle relaxation and female sexual arousal. Accordingly, we report the results of hemodynamic experiments conducted in vivo that suggest a role for arginase II in regulating both male and female sexual arousal.

MATERIALS AND METHODS

Arginase Inhibitors. The arginase inhibitor ABH was synthesized with some modifications to published procedures (*24*). Briefly, (*S*)-1-*tert*-butyl-2-[bis-(*tert*-butoxycarbonyl)-amino]-5-hexenoate was prepared (*25*) from the commercially available L-glutamic acid derivative for hydroboration, followed by treatment with methanol to quench unreacted borane and protected with (1*S*, 2*S*, 3*R*, 5*S*)-(+)-pinanediol (*26*, *27*). Complete deprotection with BCl_3 yielded ABH (*24*, *28*). The arginase inhibitor BEC was purchased from QVentas (Newark, DE).

Expression, Purification, and Assay of Truncated Human Arginase II. The ΔM1-V23/ΔH331-I354 variant of human arginase II was constructed by site-directed mutagenesis and transformation using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) using mature human arginase II cDNA (*16*). The following primers were used to generate the variant: sense mutagenic primer, CAGACAAGAGAAG-GAGGGCATTAGGTCTATGACCAACTTCCTACTCCC and antisense mutagenic primer, GGGAGTAGGAAGTTG-GTCATAGACCTAATGCC CTCCTTCTCTTGTCTG (mutated codons are underlined). The variant was expressed in *Escherichia coli* BL21(DE3) as described for the wild-type enzyme (*16*). The purification protocol for the variant was the same as that reported for the wild-type enzyme (*16*), with a minor modification: following DEAE chromatography and concentration using Millipore centrifugal filters, the variant was precipitated with ammonium sulfate from a 50 mM HEPES-KOH (pH 7.5) solution and centrifuged. The resultant pellet was resolubilized in 50 mM HEPES-KOH (pH 7.5). Arginase activity was assayed as described (*16*).

Crystallography. Crystallization was performed at 4 °C by the hanging drop vapor diffusion method. Drops containing 3 μL of 7 mg/mL human arginase II, 5 mM BEC, 50 mM bicine (pH 8.5), and 100 μL of $MnCl_2$ were mixed with 3 μL of precipitant buffer (0.10 M Tris-HCl (pH 8.3−8.5), 3.0 M $(NH_4)_2SO_4$) and equilibrated over a 1 mL reservoir of 0.10 M Tris-HCl (pH 8.3−8.5), 3.0 M $(NH_4)_2SO_4$, 20% (v/v) glycerol. X-ray diffraction data to 2.7 Å resolution were collected from a single flash-cooled crystal of the arginase II−BEC complex at the Cornell High Energy Synchrotron Source (beamline F-1), and intensity data integration and reduction were performed using DENZO and SCALEPACK, respectively (*29*). Crystals belong to space group $P3_2$ with unit cell dimensions $a = 142.99$ Å, $b = 142.99$ Å, and $c = 127.32$ Å, with two trimers in the asymmetric unit.

Initial phases were determined by molecular replacement with AMoRe (*30*, *31*) using the structure of the native rat liver arginase trimer (*21*) as a search probe. Using intensity data in the 20−3 Å shell, a cross rotation search yielded two peaks with correlation coefficients of 14.1. Subsequent translation searches using these two highest peaks produced two solutions with correlation coefficients of 16.1. Rigid body refinement with these solutions yielded $R = 0.494$. Iterative rounds of model building and refinement were performed using O (*32*) and CNS (*33*), respectively. The final structure contains 407 water molecules and 15 sulfate anions. In the final stages of refinement, the inhibitor BEC was built into clear and unbiased electron density, and the complex was refined to convergence with $R = 0.227$ ($R_{free} = 0.247$). Data collection and refinement statistics are reported in Table 1.

Tissue Arginase Activity Measurements. Rabbit vaginal tissue was divided into proximal and distal segments, frozen in liquid nitrogen, and pulverized. The tissue powder was homogenized 1:4 (wt/vol) at 4 °C in 20 mM HEPES (pH 7.4), 0.25 M sucrose containing mammalian protease inhibitor cocktail (Sigma-Aldrich Co., St. Louis, MO). The homogenate was centrifuged at 3000*g* for 20 min at 4 °C, and the resulting supernatant was used to determine arginase activity, as previously described (*6*).

Hemodynamic Studies of Male and Female Genitalia in Vivo. All protocols were approved by the Institutional Animal Care and Use Committee at the Boston University School of Medicine. Male and female New Zealand White rabbits (4.0−4.5 kg) were anesthetized with ketamine (35 mg/kg) and xylazine (5 mg/kg). Systemic blood pressure was

Table 1: Data Collection and Refinement Statistics

resolution (Å)	2.7	R_{cryst}[d]	0.227
total reflections (*N*)	404391	R_{free}[d]	0.247
unique reflections (*N*)	79653	rms deviations	
completeness (%) (last shell)	99.3 (98.9)[a]	bonds (Å)	0.007
R_{merge} (last shell)[b]	0.098 (0.280)[a]	angles (deg)	1.4
reflections used in refinement (test set)	75977 (3837)	dihedrals (deg)	24.0
sulfate anions[c]	15	impropers (deg)	1.0
solvent atoms (N)[c]	407		

[a] Numbers in parentheses refer to the outer 0.1 Å shell of data. [b] R_{merge} for replicate reflections, $R = \sum|I_h - \langle I_h \rangle|/\sum\langle I_h \rangle$; I_h = intensity measured for reflection *h* and $\langle I_h \rangle$ = average intensity for reflection *h* calculated from replicate data. [c] Per asymmetric unit. [d] Crystallographic *R* factor, $R_{cryst} = \sum||F_o| - |F_c||/\sum|F_o|$ for reflections contained in the working set. Free *R* factor, $R_{free} = \sum||F_o| - |F_c||/\sum|F_o|$ for reflections contained in the test set held aside during refinement (5% of total). $|F_o|$ and $|F_c|$ are the observed and calculated structure factor amplitudes, respectively.

continuously recorded by means of a 20-gauge angiocatheter inserted into the carotid artery and connected to a PT300 pressure transducer (Grass Instruments/Astro-Med, Inc., Warwick, RI). A bipolar platinum wire electrode was applied to the pelvic nerve, and unilateral pelvic nerve stimulation was accomplished with a 30 s train of square waves with 10 V pulse amplitude and 0.8 ms pulse duration. Stimulation frequency was 8 Hz in male and 2 Hz in female rabbits. These parameters have been previously determined to result in submaximal responses.

For assessment of penile erectile function in male rabbits, a 23-gauge needle with PE-50 tubing was filled with normal saline containing 50 U/mL heparin and inserted into one of the cavernosal bodies of the penis. The line was then attached to a PT300 pressure transducer, and intracavernosal pressure was recorded by means of a PI-1-ACDC signal conditioner module and a Grass 7400 physiological recorder (Grass Instruments Div., Astro-Med, Inc., Warwick, RI).

For assessment of genital engorgement in female rabbits, a dual channel laser oximeter (Model 96208; ISS, Inc., Champaign, IL) was utilized, as described previously (*34*). The skin around the labia was shaved to ensure good contact with the optical fibers. The probe was positioned longitudinally, externally over the clitoris, labia, and lower vagina such that the detector fiber was positioned just below the pubic arch. The changes in the concentration of oxyhemoglogin were used as parameters reflecting changes in genital blood flow.

Control responses to pelvic nerve stimulation were determined in rabbits after intracavernosal (male; 0.15 mL) or intravenous (female; 1 mL) administration of vehicle (40% propylene glycol). After 20 min, ABH was administered, and pelvic nerve stimulation was repeated 10 min after drug infusion. The dose of ABH was 150 μg in male rabbits (intracavernosal) and 4 or 6 mg/kg in female rabbits (intravenous).

The amplitude, duration, and area-under-the-curve was determined for each response, and data were expressed as mean ± SEM. For intracavernosal pressure measurements, response duration was defined as the time interval between the initial rise in pressure and the return to baseline. For oximetry data, the change in tissue oxyhemoglobin concentration was determined as the difference between baseline and peak amplitude values. Responses to vehicle and ABH were analyzed by a paired *t* test and determined to be significantly different when the *p* value was less than or equal to 0.05.

RESULTS AND DISCUSSION

Activity, Structure, and Mechanistic Inferences. Truncated human arginase II has a k_{cat} value of 203 s^{-1}, comparable to that of 231 s^{-1} measured for the wild-type enzyme; however, the K_M is 2.4 mM, as compared to that of 0.3 mM for the wild-type enzyme at pH 9.5 (*16*). As with the study of BEC binding to arginase I (*6*), analysis of progress curves for the slow binding inhibition of truncated arginase II by BEC indicates a K_i value of 0.23 μM, which is in good agreement with the value of 0.13 μM estimated from the steady-state velocities (see Supporting Information). These K_i values are approximately 4−8-fold greater than the corresponding values measured with full-length wild-type arginase II, so the C-terminus appears to have a minor effect on ligand binding in the arginase II active site. Nevertheless, these K_i values are approximately 10-fold lower than those measured against arginase I (*6*), so truncated human arginase II retains inhibitor selectivity as compared with arginase I.

The polypeptide fold of human arginase II is topologically identical to the α/β fold of rat arginase I (*21*) and the hexameric arginase from *Bacillus caldovelox* (*35*) as expected from the amino acid sequence identities (62 and 39%, respectively) (Figure 2). The rms deviation is 0.64 Å for 305 C_α atoms between human arginase II and rat arginase I and 2.4 Å for 297 C_α atoms between human arginase II and *B. caldovelox* arginase. The greatest structural differences (~9.5 Å) are observed in the H50-V68 segment of the protein that is variable in sequence among the three isozymes (the rat arginase I numbering scheme is adopted for human arginase II).

Monomer−monomer interactions in the trimer are stabilized by an arginine-rich network of intersubunit salt links, sulfate counterions, and hydrogen bonds more extensive than that observed in rat arginase I (*21*), due in part to a greater number of arginine residues located at the subunit interface of human arginase II (Figure 3). Nevertheless, the conserved R308-D204 salt link remains the central feature of these intermolecular interactions. The side chain of R201 (T in rat arginase I) forms an intermonomer salt link with E263 and also hydrogen bonds with water molecules; the side chain of R205 (K in rat arginase I) forms an intermonomer hydrogen bond with N209 (G in rat arginase I) and also hydrogen bonds with water molecules. Sulfate anions salt link with R201 (T in rat arginase I), R205 (K in rat arginase I), R214 (E in rat arginase I), and R223 (K in rat arginase I).

The binuclear manganese cluster of human arginase II is nearly identical to that of rat liver arginase I in its complex with BEC (Figure 4): Mn^{2+}_A is coordinated by D232 (Oδ2), H101 (Nδ1), D128 (Oδ2), D124 (Oδ2), and boronate hydroxyl groups O1 and O2 with distorted octahedral geometry; Mn^{2+}_B is coordinated by H126 (Nδ1), D124 (Oδ1), D234 (Oδ1 and Oδ2), and boronate hydroxyl group O1 with distorted octahedral geometry. Although D232 (Oδ1) coordinates to Mn^{2+}_B in native arginase I (*21*), the side chain moves in the arginase II−BEC complex such that

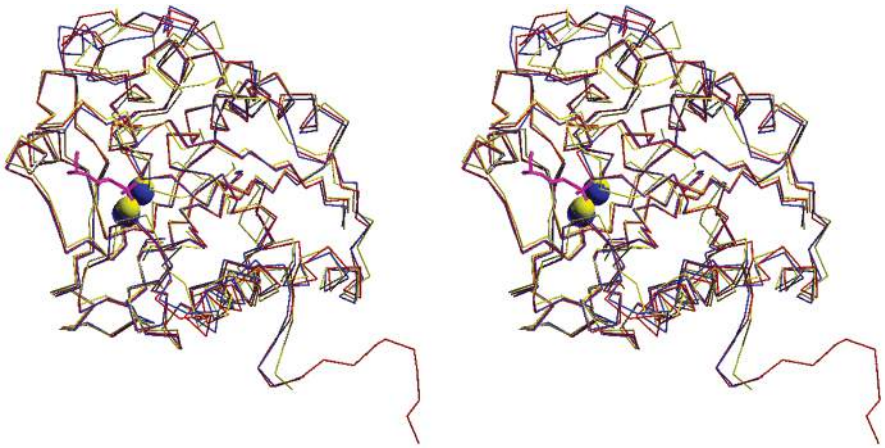

FIGURE 2: Least-squares superposition of monomer C_α traces of rat arginase I (red), human arginase II (blue), and *B. caldovelox* arginase (yellow). Manganese ions appear as spheres; for reference, BEC is shown in magenta.

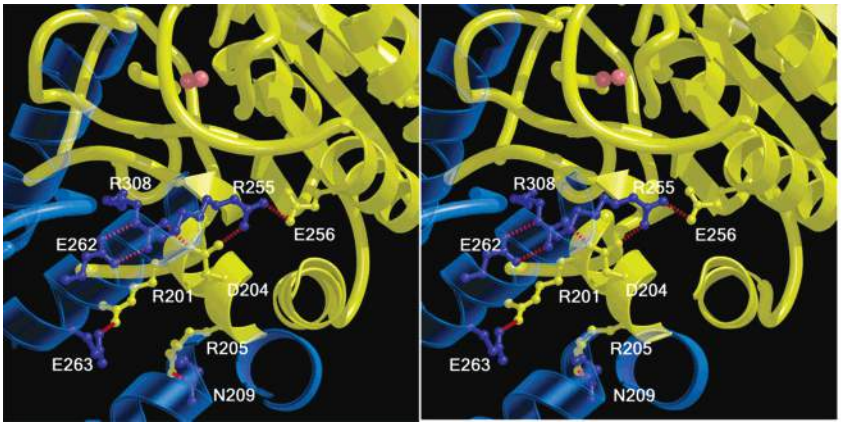

FIGURE 3: Side chains of R201, R205, R255, and R308 nucleate an alternating network of intra- and intersubunit salt-links that stabilize the trimeric quaternary structure. Residues from monomers A and B are blue and yellow, respectively. Manganese ions appear as light-pink spheres. For clarity, intrasubunit hydrogen bonds, water molecules, and sulfate anions are omitted.

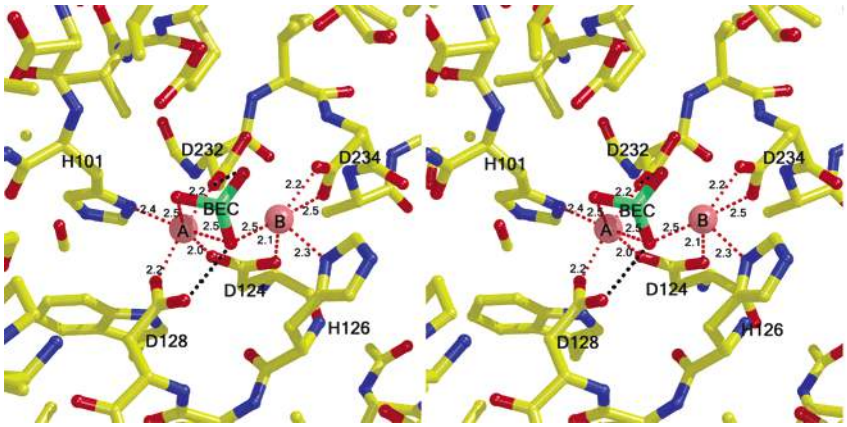

FIGURE 4: Binuclear manganese cluster in the arginase II−BEC complex; for clarity, only the tetrahedral boronate anion of BEC is shown. Atoms are color-coded as follows: C = yellow, O = red, N = blue, S = dark green, and B = pale green; manganese ions appear as light-pink spheres. The corresponding electron density map is found in the Supporting Information.

the D232 (Oδ1)−Mn^{2+}_B separation of 2.6 Å is somewhat long to be considered an inner-sphere coordination interaction. Instead, D232 (Oδ1) is better positioned to accept a hydrogen bond from boronate hydroxyl group O3. Boronate hydroxyl group O1 symmetrically bridges the binuclear manganese cluster, and the Mn^{2+}_A−Mn^{2+}_B separation is 3.3 Å.

An omit electron density map of the arginase II−BEC complex is found in Figure 5. Binding interactions between BEC and the arginase II active site are largely similar, but not identical, to those observed in the arginase I−BEC complex (*6*). The tetrahedral boronate anion of BEC binds in an identical fashion to the binuclear manganese clusters of arginase II and arginase I; additionally, boronate hydroxyl O1 donates a hydrogen bond to D128, and boronate hydroxyl O2 donates a hydrogen bond to the backbone carbonyl of H141. However, boronate hydroxyl O2 donates a hydrogen bond to E277 (O···O separation = 3.0 Å) in the arginase

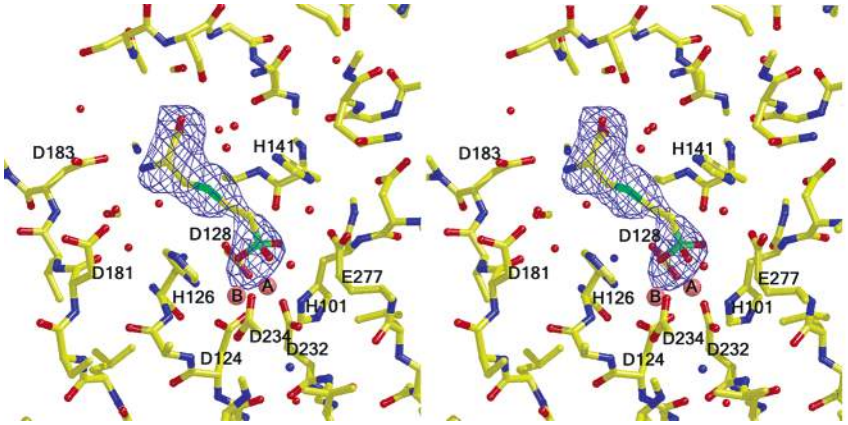

FIGURE 5: Human arginase II−BEC complex. Omit electron density map of BEC in the arginase active site. The map is contoured at 3.8σ, and selected active site residues are indicated. Atoms are color-coded as follows: C = yellow, O = red, N = blue, S = dark green, and B = pale green; manganese ions appear as light-pink spheres, and water molecules appear as red spheres.

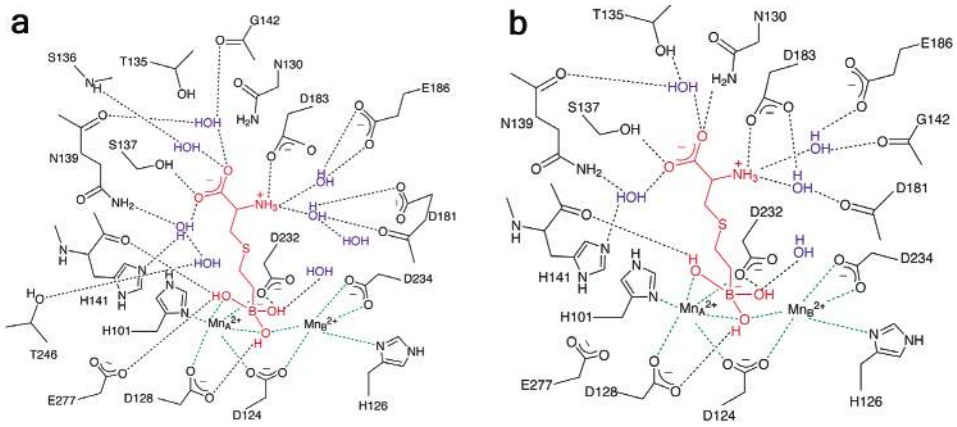

FIGURE 6: (a) Summary of intermolecular interactions in the arginase II−BEC complex. Manganese coordination interactions are designated by green dashed lines, and hydrogen bonds are indicated by black dashed lines. (b) Summary of intermolecular interactions in the arginase I−BEC complex. Manganese coordination interactions are designated by green dashed lines, and hydrogen bonds are indicated by black dashed lines.

II−BEC complex, whereas in the arginase I−BEC complex the corresponding interaction appears slightly weaker (O···O separation = 3.3 Å).

Additional differences are evident in the binding of BEC to arginases I and II. Recognition of the α-carboxylate and α-amino groups of BEC is governed by two direct hydrogen bonds and five water-mediated hydrogen bonds with arginase II residues, whereas in arginase I this recognition is governed by three direct and four water-mediated hydrogen bonds (Figure 6). That more water-mediated enzyme−inhibitor interactions are observed in the arginase II−BEC complex may be due to the larger volume of the arginase II active site cleft. Using CAST (*36*), we calculate the volume of the active site cleft of arginase II to be 554 Å^3 and that of arginase I to be 440 Å^3. The active site cleft of arginase II fits 48 water molecules, while the active site cleft of arginase I fits only 38 water molecules, so perhaps it is not surprising that an additional water-mediated enzyme−inhibitor hydrogen bond interaction is exploited in arginase II.

As proposed for arginase I (*21*), the structure of arginase II is consistent with a metal-activated hydroxide mechanism in which both $Mn^{2+}{}_A$ and $Mn^{2+}{}_B$ activate a bridging hydroxide ion for nucleophilic attack at the substrate guanidinium group. Similar chemistry may be involved in the binding of BEC to arginases I and II, in that the metal-bridging hydroxide ion may attack the trigonal planar boronic acid to yield the tetrahedral boronate anion in the arginase I−BEC complex (*6*) and the arginase II−BEC complex (Figures 6 and 7). An alternate model, in which the preformed tetrahedral boronate binds directly to the enzyme (*19*), cannot be ruled out by the present data.

Insofar that the tetrahedral boronate anion BEC mimics the tetrahedral transition state for L-arginine hydrolysis, the crystal structure of the arginase II−BEC complex provides key inferences on two features of transition state stabilization previously unobserved in arginase I complexes. First, the hydrogen bond between boronate hydroxyl O2 and E277 is consistent with the proposal that E277 hydrogen bonds with the η_1-NH_2 group of the substrate and stabilizes the tetrahedral transition state and intermediate (*21*). Second, the arginase II−BEC structure is the first to show that D232 undergoes changes in $Mn^{2+}{}_B$ coordination to hydrogen bond with boronate hydroxyl group O3; similarly, D232 could move to hydrogen bond with the η_2-NH_2 group of the substrate and tetrahedral intermediate. These changes arise primarily from the slight movement of the D232 carboxylate that aligns a syn-oriented lone electron pair directly toward boronate hydroxyl group O3. These structural insights impact the first step of arginase II catalysis as summarized in Figure 7.

Male and Female Sexual Arousal. The selectivity of inhibitors BEC and ABH toward arginase II and the lack of

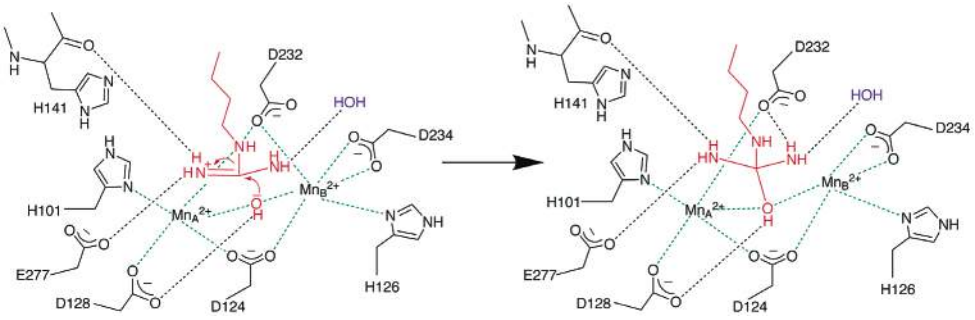

FIGURE 7: Nucleophilic attack of metal-bridging hydroxide at the substrate guanidinium group forms the tetrahedral intermediate stabilized by metal coordination and hydrogen bond interactions with D128, D232, E277, and the backbone carbonyl of H141.

activity against NO synthase (*6*, *12*) allow us to probe the role of arginase II in the regulation of NO-dependent smooth muscle relaxation in male and female genitalia. Previously, we demonstrated that both ABH and BEC enhanced relaxation of these smooth muscle tissues in ex vivo organ bath experiments (*6*, *12*). Given the selectivity of ABH and BEC, it is unlikely that these compounds bind tightly to any other cellular receptor to cause this biological effect. Inhibitors of arginase activity enhance L-arginine concentrations for NO biosynthesis and NO-dependent smooth muscle relaxation in penile corpus cavernosum, so arginase II is strongly implicated in cellular L-arginine trafficking and male erectile function. Notably, the gene expression, protein level, and catalytic activity of arginase II is elevated in diabetic corpus cavernosum, thereby implicating this isozyme in the erectile dysfunction of diabetic men (*13*).

Given the anatomical and physiological homologies between the male and the female genitalia, and given the localization of NO synthase in human clitoral corpus cavernosum (*22*) and vagina (*23*), we hypothesized that arginase II might similarly be a regulator of L-arginine bioavailability in the female genitalia, the implication being that arginase could similarly play a role in regulating female sexual arousal. To this end, conversion of L-arginine into urea was detected in extracts from rabbit vaginal tissue, indicating the presence of arginase activity. Interestingly, arginase activity was significantly higher in the distal vagina (206 nmol of urea/mg of protein) versus the proximal vagina (64 nmol of urea/mg of protein).

Given the presence of arginase activity in vaginal tissue, as well as our previous studies demonstrating the presence of arginase in penile cavernosal tissue, we investigated the effects of ABH on circulation in the genitalia of male and female rabbits in vivo. In the absence of ABH administration, pelvic nerve stimulation caused significant but submaximal increases in intracavernosal pressure and genital tissue oxyhemoglobin concentration in male and female rabbits, respectively. Repeated nerve stimulation did not result in significant changes in either parameter (data not shown). After ABH administration in male rabbits, the rise in intracavernosal pressure was not affected, but the duration of the response increased by ~28%. The overall erectile response, as reflected by the area-under-the-curve, was significantly greater with ABH (Figure 8a). Since the development of pressure within the penile cavernosal bodies is dependent upon increased arterial flow and decreased venous drainage (veno-occlusion), it is interesting to note that the amplitude of the response in male rabbits did not increase when submaximal nerve stimulation was applied

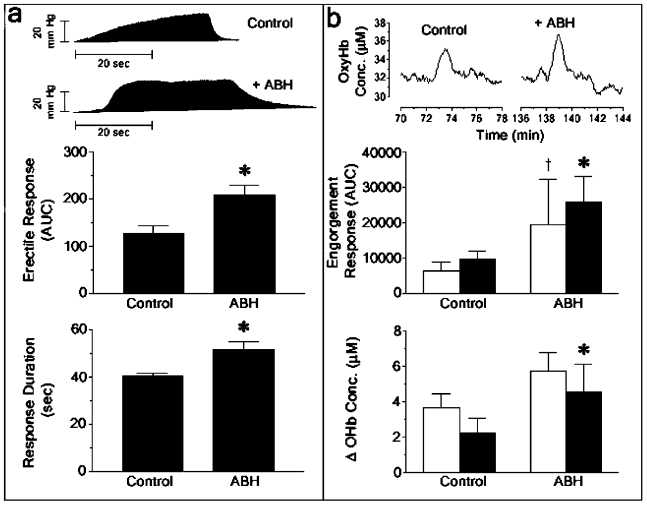

FIGURE 8: Effects of ABH administration on genital hemodynamics. For both male and female rabbits, the amplitude, duration, and area-under-the-curve (AUC) were determined for each response. All data are mean ± SEM. (a) Penile intracavernosal pressure was measured in anesthetized male rabbits to assess erectile function. The pelvic nerve was electrically stimulated 10 min after intracavernosal injection of vehicle (0.15 mL of 40% propylene glycol; control). Nerve stimulation was repeated 10 min after intracavernosal administration of ABH (150 μg). Representative recordings of intracavernosal pressure are shown in top panel. $N = 4$; $^*p < 0.05$. (b) The change in tissue oxyhemoglobin concentration (ΔOHb) was measured using near-infrared spectroscopy in anesthetized female rabbits to assess genital engorgement. The pelvic nerve was electrically stimulated 10 min after intravenous administration of vehicle (1 mL of 40% propylene glycol; control). Nerve stimulation was repeated 10 min after intravenous administration of ABH (4 or 6 mg/kg). Representative oximetry recordings are shown in the top panel. Data for rabbits that were administered the 4 mg/kg dose of ABH are shown as open bars ($N = 5$; $^*p = 0.06$ as compared to control). Data that include three additional rabbits receiving the 6 mg/kg dose of ABH are shown as solid bars ($N = 8$; $^*p < 0.05$ as compared to control).

in the presence of ABH. Thus, at the concentration tested, ABH may prolong the duration of neurogenic penile tumescence without affecting penile rigidity.

After ABH administration in female rabbits, the inhibitor consistently potentiated the rise in genital tissue oxyhemoglobin concentration triggered by pelvic nerve stimulation (Figure 8b). For the group that was administered the 4 mg/kg dose of ABH, this enhancement in genital engorgement approached statistical significance with $p = 0.06$ ($N = 5$). Importantly, the enhancement of genital engorgement reaches statistical significance ($p = 0.02$ for area-under-the-curve; $p = 0.03$ for oxyhemoglobin concentration) if all data ($N = 8$) for rabbits administered either the 4 mg/kg or the 6 mg/

kg dose of ABH are combined. Interestingly, at the doses used, the ~2-fold enhancement in mean genital tissue oxyhemoglobin resulting from ABH treatment is comparable to that resulting from treatment with sildenafil, the active ingredient of Viagra (*34*). This enhancement is consistent with the small, statistically significant increase in vaginal blood flow confirmed by laser Doppler flowmetry (*37*). These results are consistent with the fact that achieving the engorged state in female genital tissues is highly dependent on increased arterial inflow. While we did not measure perfusion within other organs, ABH administration had no discernible effect on systemic arterial blood pressure in either male or female rabbits.

CONCLUSION

The structure of human arginase II is the first of a human arginase and the first of a mammalian type II isozyme. The active site structure is consistent with a metal-activated hydroxide mechanism in catalysis, and the boronic acid inhibitors ABH and BEC (Figure 1b) are the tightest binding inhibitors known to date (*19*). The boronic acid inhibitor BEC binds as an analogue of the tetrahedral intermediate in the arginase II mechanism, and differences in the intermolecular contacts of the α-amino and α-carboxylate groups account for enhanced binding to arginase II relative to arginase I. Interestingly, the binding of BEC to arginases I and II suggests that bridging metal ligand D232 may undergo slight changes in metal coordination interaction to accommodate the binding of the tetrahedral boronate anion and by inference the tetrahedral intermediate and its flanking transition states in catalysis.

That the boronic acid inhibitor ABH enhances erectile function in live male rabbits is consistent with our previous observations using arginase inhibitors in isolated penile cavernosal tissue (*6*, *12*). Additionally, we show for the first time that an arginase inhibitor facilitates genital engorgement in the female. We conclude that arginase inhibition enhances male and female genital blood flow during sexual arousal without affecting systemic arterial blood pressure. Accordingly, arginase II is a potential drug target for the treatment of male and female sexual arousal disorders.

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SUPPORTING INFORMATION AVAILABLE

Inhibition assay and electron density map. This material is available free of charge via the Internet at http://pubs.acs.org.

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Novel nitric oxide signaling mechanisms regulate the erectile response

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Nitric oxide (NO) is a physiologic signal essential to penile erection, and disorders that reduce NO synthesis or release in the erectile tissue are commonly associated with erectile dysfunction. NO synthase (NOS) catalyzes production of NO from L-arginine. While both constitutively expressed neuronal NOS (nNOS) and endothelial NOS (eNOS) isoforms mediate penile erection, nNOS is widely perceived to predominate in this role. Demonstration that blood-flow-dependent generation of NO involves phosphorylative activation of penile eNOS challenges conventional understanding of NO-dependent erectile mechanisms. Regulation of erectile function may not be mediated exclusively by neurally derived NO: Blood-flow-induced fluid shear stress in the penile vasculature stimulates phosphatidyl-inositol 3-kinase to phosphorylate protein kinase B, which in turn phosphorylates eNOS to generate NO. Thus, nNOS may initiate cavernosal tissue relaxation, while activated eNOS may facilitate attainment and maintenance of full erection.
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Keywords: 1-phosphatidylinositol 3-kinase; penile erection; nitric-oxide synthase; endothelium

Introduction

The discovery of nitric oxide (NO) as a major molecular regulator of penile erection about a decade ago has had a profound impact on the field of sexual medicine. The fact that NO signals the biochemical mechanism of corpus cavernosal tissue relaxation established an essential foundation for the development of phosphodiesterase-5 inhibitor therapy for the therapeutic management of erectile dysfunction (ED). Additional translational efforts to harness the potential of this molecule for therapeutic purposes have suggested other possible therapies including pharmacologic NO precursors or actual NO donors,[1–3] NO-based gene therapy,[4,5] derivatives of NO synthase (NOS)-associated regulatory proteins,[6] and compounds targeting other components of the NO-based signal transduction pathway.[7,8]

Ongoing basic scientific investigation of NO biology has revealed new concepts regarding the regulatory roles of this chemical, particularly those pertaining to mechanisms of activating NOS which synthesizes NO. In this brief review, the relatively new science of constitutive NOS activation via protein kinase phosphorylation is discussed, with particular attention given to its role in penile erection and to its possible therapeutic relevance for ED.

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NO signal transduction

Current concepts in erection physiology support the role of NO as the principal mediator of penile erection operating through a specific signal transduction mechanism.[9–11] The molecule has a prominent messenger-signaling function, characterized by its release from nerves and endothelium in the cavernosal tissue and binding to soluble guanylyl cyclase in corporal smooth muscle cells, which results in the activation of the enzyme to produce the potent second messenger molecule, 3′, 5′-cyclic guanosine monophosphate (cGMP). This product then stimulates a cGMP-dependent protein kinase, protein kinase G (PKG), which critically induces the cavernosal tissue relaxant response.

The synthesis of NO depends upon the catalytic action of NOS and is derived from the precursor amino acid, L-arginine. The so-called constitutive NOS isoforms, neuronal NOS (nNOS)

and endothelial NOS (eNOS), account for its controlled formation, which is physiologically relevant for penile erection, distinct from roles attributed to inducible NOS. These constitutive isoforms are regulated by classically known interactions played by the NOS regulatory cofactors, calcium, calcium-binding protein calmodulin, oxygen, and reduced NADPH. They generate NO transiently and in low amounts, consistent with traditional tenets of cell–cell signaling, after a rise in intracellular calcium and calcium–calmodulin binding. This regulatory mechanism describes neural NO release upon neuronal depolarization in nNOS-containing nerve terminals in the penis, which relies on electrical impulses transmitted in response to psychogenic and reflexogenic erogenous stimuli. It also applies to eNOS-containing sinusoidal and penile vascular endothelium, which liberates endothelial NO upon local neurogenic stimulation by acetylcholine, and conceivably other agents such as substance P and bradykinin, transmitted from nerves making contact with the endothelium. Acute increases in 'shear stress,' the term used to indicate the mechanical forces of blood flow on the vascular lining, also supposedly drive rapid, but limited, amounts of NO release from eNOS by related biochemical mechanisms.[12]

Calcium-independent NOS activation

The biology of NO has evolved recently with descriptions of constitutive NOS activation depending less on calcium–calmodulin interactions. New investigative work confirms that physical stimuli and diverse biochemical signals, such as interactions with integrins, G proteins, and protein kinases, are capable of eliciting the long-term production of NO from endothelial sources.[12] An apparent mechanism for this observation subsequently was described in which such influences as chronic shear stress and various growth factors activate phosphatidylinositol-3-kinase (PI3-kinase) and its downstream effector, the serine/threonine protein kinase, protein kinase B (Akt), which in turn phosphorylates eNOS producing an activated state of the enzyme.[13–15] Thus, under some regulatory influences, eNOS is disposed to producing NO in a steady, not merely transient, fashion.

The phosphorylation of eNOS represents an important new area of investigation for the regulation of this enzyme (Figure 1). Several precise sites for phosphorylation of eNOS under the influence of specific protein kinases have been identified. The phosphorylation of eNOS as activated by the PI3-kinase/Akt pathway occurs at the stimulatory site human Ser-1177 (bovine Ser-1179) in the enzyme's C-terminus region, reducing the enzyme's calcium dependence, increasing the rate of electron flux

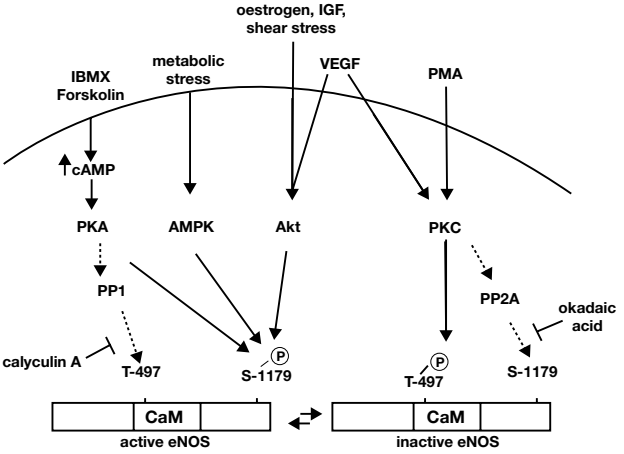

Figure 1 Signaling pathways controlling eNOS phosphorylation at Thr-497 and Ser-1197.[18]

from the reductase domain to the oxygenase domain, and increasing the rate of NO formation.[13,14,16,17] In contrast, the phosphorylation of eNOS at the inhibitory site human Thr-495 (bovine Thr-497) located in the calmodulin-binding sequence leads to deactivation of the enzyme by increasing its calcium–calmodulin dependence.[16,18,19] Importantly, since phosphorylation of eNOS at Ser-1177 is accompanied by dephosphorylation at Thr-495, and *vice versa*, the phosphorylation and dephosphorylation reactions at the two sites are thought to be coordinated[18–20] (Figure 1).

Human Ser-1177 is the target of several protein kinases in addition to Akt, including AMP-activated protein kinase (AMPK), cAMP-dependent protein kinase (protein kinase A), PKG, and calmodulin II protein kinase.[21,22] Phosphorylation at Thr-495 is mediated by protein kinase C.[18] Recent studies have confirmed additional regulatory sites including Ser-617 and Ser-635 (bovine sequences) in bovine aortic endothelial cells, which appear to exert important roles for mediating such eNOS-activating agonists as bradykinin, ATP, and vascular endothelial growth factor.[21]

Model of NOS-regulated erection

The constitutive NOS activation cascade has been further investigated in the regulation of the erectile response. Electrical stimulation of the cavernous nerve or direct injection of the vasorelaxant drug, papaverine, into the corpus cavernosum in rodent animal models results in rapid increases in phosphorylated Akt and eNOS.[23] Such effects, along with the erectile response from electrical stimulation and pharmacostimulation, are blocked by the intracavernosal administration of the PI3-kinase

inhibitors, wortmannin and LY294002.[23] In mice lacking eNOS by targeted gene deletion, only attenuated erectile responses were observed after intracavernosal vasoactive drug administration.[23] Taken together, the results suggested that the PI3-kinase/Akt-induced eNOS phosphorylation mechanism operates under blood flow stimuli in the penis to sustain physiologic penile erection.

Such an NO signaling mechanism fits with the physiologic requirements of penile erection, occurring as a gradually occurring, hemodynamic event. The described molecular mechanism also reconciles the concern that nerve endings in the penis likely discharge NO intermittently and in a finite distribution that is insufficient to exert a wide-ranging, NO-regulated relaxant effect on the corpus cavernosum for producing full erection. Conceivably, the blood flow stimuli occurring at the initiation of erection coordinates a 'feed forward' process of blood flow in progressively more distal vascular tributaries, and expansion of sinusoidal spaces that augments NO release for signaling the widespread relaxation of the cavernosal tissue. Based on these precepts, a proposed model of NO-mediated erectile function fundamentally involves both nNOS and eNOS, whereby the former initiates the erectile response and the latter facilitates full erection (Figures 2 and 3).

Further support for the phosphorylation of eNOS as a determinant of NO release was recently shown in the human corpus cavernosum.[24] Both Akt and the mitogen-activated protein (MAP) kinases 1 and 2 (extracellular signal-regulated protein kinases 1 and 2, (ERK 1/2)), which reduces eNOS activity,[25] are expressed prominently in the penile vascular endothelium and nerves with less pronounced

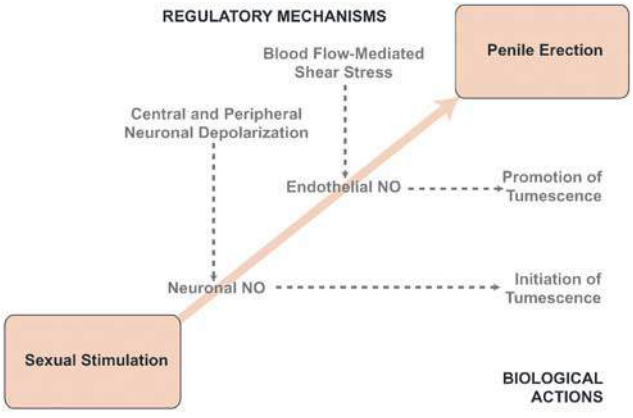

Figure 2 A schematic diagram depicting the integrative NO biology for attaining penile erection. Sexual stimulation such as sexual thoughts (psychogenic stimuli) and genital touch (reflexogenic stimuli) produces penile erection by the combined effects of neuronal and endothelial NO. The former involves central and peripheral nervous system activation, which results in initiation of tumescence. The latter involves intracorporal blood flow mediation, which results in promotion of tumescence.

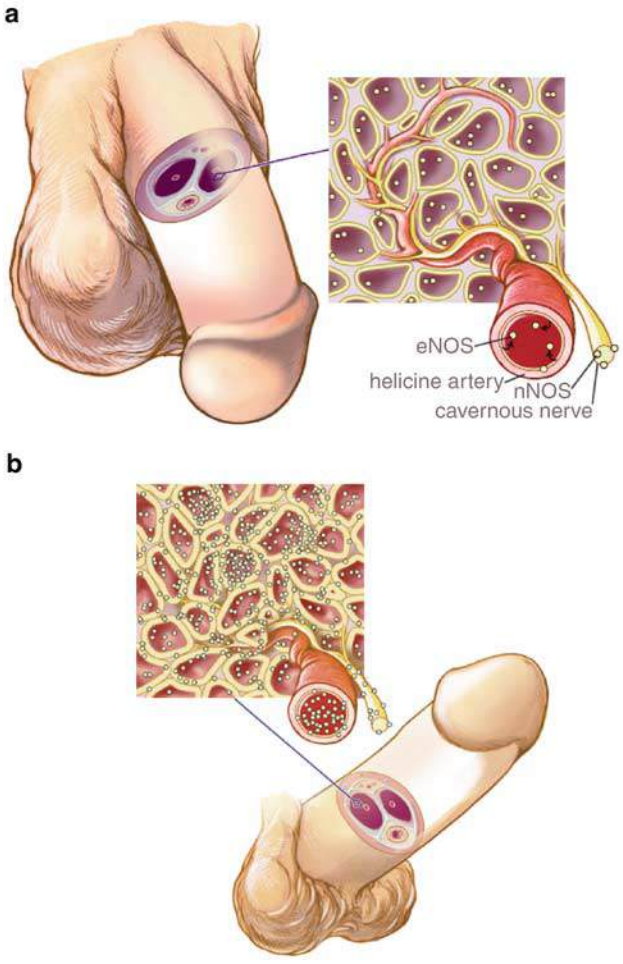

Figure 3 Graphic representation of the integrative NO biology in the penis that mediates penile erection. (a) During flaccidity, a basal release of NO (depicted as vesicle bodies) in the cavernosal tissue is produced by nNOS contained in terminations of the cavernous nerve and eNOS contained in the endothelium lining arterial distributions and sinusoidal spaces. (b) During tumescence, NO release is elevated owing to its increased production to a maximal extent by both nNOS and eNOS catalysis.

localizations to smooth muscle.[24] Differences in immunostaining were identified for MAP kinase 1/2 (ERK 1/2) in the corporal smooth muscle of potent and impotent patients, with only the latter showing substantial expression.[24] The investigators speculated that this finding indicates a negative regulator role for MAP kinase 1/2 (ERK1/2) on eNOS in the penis, operating as a mechanism that contributes to ED. They were unable to identify differences between potent and impotent patients for Akt expression.[24]

The discovery of novel pathways for NO production in the penis further underscores the essential effector role of NO in the physiologic and pharmacotherapeutic stimulation of the erectile response. The NO regulatory pathway influences other molecular mechanisms involved in penile erection, such

as contractile factors of the adrenergic nervous system [26,27] and the RhoA/Rho-kinase pathway.[28] In addition, the efficacy of phosphodiesterase-5 inhibitor therapy for ED relies significantly on upstream regulation by NO. While phosphodiesterase inhibitors prevent the degradation of the potent second messenger molecule cGMP, so that it accumulates to enhance corporal smooth muscle relaxation, NO production and release foremost drives the synthesis of cGMP.[29]

Future therapeutic considerations

The investigation of eNOS phosphorylation regulatory sites may have therapeutic relevance in the treatment of ED. Drugs that phosphorylate or dephosphorylate eNOS at sites leading to its activation (or inhibit eNOS phosphorylation or dephosphorylation at sites associated with its deactivation), or activate select upstream regulatory protein kinases favoring eNOS-dependent erectile responses could be clinically advantageous. Also, methods for gene transfer involving activated eNOS conceivably could offer therapeutic benefit.

The idea that enhanced blood flow in the penile circulation causes NO release advances the possibility that therapies having vasostimulatory effects or angiogenic properties targeting eNOS could indeed be clinically useful to counter atherosclerotic or other vasculopathic derangements involving the penile vascular and cavernosal tissue. For that reason, the role of statins could be investigated beyond simple lipid reduction for the treatment or prevention of ED via eNOS activation in the penis, since these agents have been shown to stimulate Akt phosphorylation of eNOS in isolated vascular preparations.[30]

Further developments in this research area may uncover a sound mechanistic basis to support the use of orally or locally vasoactive therapies for the improvement or restoration of penile health for etiologically obscure erectile disorders, such as those associated with aging and erectile inactivity. For instance, in the context of post-radical prostatectomy ED, the premise that early and repetitively administered pharmacotherapies rapidly recover postoperative erectile function would be supported further by scientific evidence that eNOS is activated with such regimens and revitalizes the cavernosal tissue.

Summary

Concepts of constitutive NOS regulation have evolved recently with the identification of calcium-independent regulatory interactions that determine eNOS activation, by which long-term production of NO occurs. These mechanisms appear to be relevant to the erectile response, with early scientific investigation confirming physiologic roles for phosphorylated constitutive eNOS in erectile function. Based on these precepts, a new understanding of NOS-regulated penile erection is proposed according to which nNOS acts as the neurally directed initiator of penile erection and eNOS subserves a key blood-flow-dependent facilitative role in this physiologic activity. Constitutive NOS activation as a molecular mechanism involved in the regulation of penile erection may be exploited to further innovations in the treatment of ED.

Important questions

(1) Can pharmaceutical research develop a system to drive NO production at the endothelial level, in order to enhance the efficacy of PDE5 inhibitor therapy?

Various options involving the phosphorylated eNOS activation cascade could be explored as possible approaches for this purpose. Specific eNOS-activating agonists, such as bradykinin, ATP, and vascular endothelial growth factor, could be investigated therapeutically while it may also be confirmed that such agonists exert effects on erection physiology through this mechanism.

Other drugs, and even gene therapy that leads to eNOS activation, may eventually be identified and investigated clinically. It is also interesting to consider whether frequent administration of vasoactive therapies for ED, which conceivably induces eNOS phosphorylation by promoting blood flow in the penile circulation, may serve as another nonspecific system to heighten NO production. Further research into such possibilities could then allow PDE5 inhibitor therapy to be used even more effectively than current roles that rely on basal endogenously produced NO.

(2) It appears that a fundamental aspect of eNOS activation is the lowering of calcium that favors the catalytic activity of the enzyme. Are there therapeutic opportunities to drive NO production based on this requirement?

Research findings suggest that calcium-mobilizing agonists promote eNOS enzyme activity by releasing the enzyme from intracellular protein elements, known as caveolae, that are associated with calcium–calmodulin-dependent activity. Release of this tonic inhibition of eNOS by such methods may represent an alternative approach for NO production.

(3) The vascular literature describes laminar shear stress as having a protective effect, such as preventing atherosclerosis. The phosphorylated eNOS activation cascade has been proposed

as the pathway for this effect. In light of the evidence that this cascade operates in the penis, is it possible for treatments based on this cascade to offer long-term benefits on cavernosal tissue health?

Indeed, this cascade offers a pharmacotherapeutic target not just to promote physiologic vessel relaxation but also to inhibit apoptosis and to repress monocyte adhesion among molecular and cellular events promoted by atherogenic factors in the penile circulation.

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Research Article

Clinical Evaluation of the Spermatogenic Activity of the Root Extract of Ashwagandha (*Withania somnifera*) in Oligospermic Males: A Pilot Study

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Ashwagandha (*Withania somnifera*) has been described in traditional Indian Ayurvedic medicine as an aphrodisiac that can be used to treat male sexual dysfunction and infertility. This pilot study was conducted to evaluate the spermatogenic activity of Ashwagandha root extract in oligospermic patients. Forty-six male patients with oligospermia (sperm count < 20 million/mL semen) were enrolled and randomized either to treatment ($n = 21$) with a full-spectrum root extract of Ashwagandha (675 mg/d in three doses for 90 days) or to placebo ($n = 25$) in the same protocol. Semen parameters and serum hormone levels were estimated at the end of 90-day treatment. There was a 167% increase in sperm count ($9.59 \pm 4.37 \times 10^6$/mL to $25.61 \pm 8.6 \times 10^6$/mL; $P < 0.0001$), 53% increase in semen volume (1.74 ± 0.58 mL to 2.76 ± 0.60 mL; $P < 0.0001$), and 57% increase in sperm motility (18.62 ± 6.11% to 29.19 ± 6.31%; $P < 0.0001$) on day 90 from baseline. The improvement in these parameters was minimal in the placebo-treated group. Furthermore, a significantly greater improvement and regulation were observed in serum hormone levels with the Ashwagandha treatment as compared to the placebo. The present study adds to the evidence on the therapeutic value of Ashwagandha (*Withania somnifera*), as attributed in Ayurveda for the treatment of oligospermia leading to infertility.

1. Introduction

Male infertility accounts for about 50% of human infertility. In 40% to 50% of infertile males, the etiology is unknown [1–7]. The pathophysiology of male infertility could be explained by a number of cellular abnormalities manifesting at the molecular and biochemical levels that result in decreased quality and quantity of sperm in the semen [3–5] and an imbalance in the reproductive hormones. Moreover, it has been widely observed that oligospermia is the single most prevalent cause of reduced male fertility [2, 4].

Ayurveda, the traditional system of medicine practiced in India, can be traced back to 6000 Bc [8–11]. For most of this history, Ashwagandha (*Withania somnifera*), also known as "Indian ginseng" due to its rejuvenating effects, has been described in folk medicine as an aphrodisiac and geriatric tonic [12]. It is classified as an "adaptogen," meaning that this herb assists in combating stress and disease, improving physical strength and metabolism without adverse effects [13–16]. Ashwagandha has been used as a "*rasayana*" in Ayurvedic medicine. In particular, the root of Ashwagandha is regarded as a tonic and aphrodisiac. *Ashwagandha* in the Sanskrit language means "horse's smell" (*ashwa*-horse, *gandha*-smell), probably originating from the odor of its root. The species name *somnifera* means "sleep-inducing" in Latin [17].

Ashwagandha is rich in a wide variety of chemical compounds, such as alkaloids, ergostane steroids, amino acids, and neurotransmitters, which explains its numerous

medicinal properties that can directly or indirectly prevent and treat a number of diseases [18–20].

It has been widely documented that, in addition to conventional therapies, many individuals with sexual dysfunction often seek alternative therapies. It is noteworthy that, from ancient times, Ashwagandha has been used by Ayurvedic practitioners as an aphrodisiac to improve on matters related to infertility and sexual activities. Numerous human and animal studies have validated the aphrodisiac and testosterone-enhancing effects of Ashwagandha [8–11, 21–23].

Different investigators have reported that Ashwagandha is beneficial in the treatment of male infertility [5, 21–23]. Experimental studies have shown that treatment with Ashwagandha induced testicular development and spermatogenesis in immature Wistar rats by directly affecting the seminiferous tubules [5, 24, 25], improved prosexual behaviour of sexually sluggish mice, and increased testicular daily sperm production and serum testosterone level [5].

It has been well documented that high levels of reactive oxygen species (ROS) in the semen induce oxidative damage to the sperm and are associated with abnormal sperm parameters leading to infertility [21, 26–30]. Ashwagandha has been found to counteract the formation of ROS in infertile men [21, 22, 27].

Despite numerous studies that report the efficacy of Ashwagandha in the treatment of various diseases, specific double-blind, randomized, placebo-controlled studies assessing the effectiveness of Ashwagandha in treating male infertility are few and, mostly, lacking critical data on safety and tolerability of the therapy.

Hence, the present study aims to investigate the usefulness of a highly concentrated, full-spectrum root extract of Ashwagandha as a suitable herbal supplement in treating male infertility.

2. Subjects and Methods

This two-arm, double-blind, randomized, placebo-controlled, parallel-group study with 1 : 1 random allocation was conducted at five infertility centers in India. The study was conducted in accordance with the good clinical practice guidelines of the Indian Council for Medical Research (ICMR-GCP) and the Declaration of Helsinki and was approved by the "League Health-Independent Ethics Committee."

2.1. Subjects. Sixty-eight infertile males were assessed with regard to eligibility for inclusion in the present study. Forty-six male patients between 22 and 40 years of age with semen factor infertility were enrolled after obtaining informed written consent. All men had a sperm count of 5–20 million/mL, total motility of 10%–30%, with forward motility < 15%, and atypical morphological forms < 70%. All men had a history of regular sexual intercourse over a one-year period with a gynecologically normal female partner with no apparent female infertility.

Men with a total sperm count of <0.5 million/mL or over 20 million/mL were not included. Also, men with primary erectile dysfunction, congenital anomalies, uncontrolled diabetes mellitus, severe hepatic or renal insufficiency, cardiovascular diseases, cerebrovascular accidents, uncontrolled hypertension, or with previous history of cryptorchidism, varicocele and testicular hypertrophy, were excluded from the study. Those with a history of pelvic fractures or prostatectomy or reconstructive or prosthetic surgery on the penis or having total or partly obstructive oligospermia were also excluded. Enrolled patients had not been administered any PDE-V inhibitors (sildenafil, tadalafil, or vardenafil) and glucocorticosteroids within the four weeks prior to enrollment and during the entire course of the study. Patients with known hypersensitivity to Ashwagandha extract were also excluded.

2.2. Randomization and Treatments. The study subjects were randomized to either: (i) the placebo-treated group ($n = 25$) or (ii) the Ashwagandha-treated group ($n = 21$). The study subjects in the Ashwagandha-treated group were administered one capsule (containing 225 mg of a high-concentration full-spectrum root extract of the Ashwagandha plant) orally, thrice daily for a period of 12 weeks, whereas, in the placebo-treated group, capsules containing 225 mg of matching placebo were administered similarly.

The Ashwagandha root extract employed in the present study, KSM-66 Ashwagandha (from Ixoreal Biomed Private Ltd., Hyderabad, India), has been extracted with a unique processing technology producing a broad-spectrum phytopharmaceutical that potentiates the action of Ashwagandha manifold, providing pan-therapeutic effects. It is noteworthy that although various Ashwagandha powders and extracts are available commercially, there are serious shortcomings in standardization and optimization of Ashwagandha extracts. KSM-66 Ashwagandha is standardized to withanolide content of at least 5% as measured by HPLC. It contains the desired quantum of withanolides and alkaloids, short- and long-chain amino acids (threonine, valine, methionine, isoleucine, lysine, aspartic acid, and arginine), complex sugars including oligosaccharides/fructooligosaccharides, vitamin A, calcium, and iron.

2.3. Trial Visits and Assessments. After the screening visit, during the treatment period of 90 days, the subjects were required to present themselves at the trial centers at specified intervals: Visit 1 on Day 30; Visit 2 on Day 60; and Visit 3 on Day 90. The final safety and efficacy assessments were done on Day 90 of the study. Semen analysis and complete physical examination were conducted at baseline and then after 30 days and again after 90 days. Standard manual semen analysis was performed according to WHO guidelines [31, 32]. Hormonal estimations were done for serum testosterone and luteinizing hormone (LH) levels on Day 0 (baseline) and after Day 90 using the chemiluminescence method.

The primary efficacy outcome was the improvement in the semen parameters and serum hormone levels from baseline (Day 0) after 90 days of therapy.

The secondary efficacy outcome was the safety and efficacy of the therapy under investigation. Safety was assessed based on the adverse events recorded during the study. At the end of the study, the four-point Global Assessment Scale

Table 1: Demography and baseline data of the study subjects.

	Placebo (n = 25) mean ± SD	Ashwagandha (n = 21) mean ± SD
Age (yr)	35.28 ± 5.49	32.38 ± 4.31
Height (cm)	167.13 ± 7.53	165.89 ± 8.55
Weight (kg)	74.32 ± 14.52	70.05 ± 11.22
Pulse (per min)	80.04 ± 8.06	79.78 ± 6.96
Respiratory rate (per min)	16.92 ± 2.45	17.39 ± 1.91
Systolic blood pressure (mm Hg)	123.84 ± 9.50	128.00 ± 8.03
Diastolic blood pressure (mm Hg)	80.16 ± 4.28	80.33 ± 3.65
Body temperature (°F)	98.04 ± 0.36	97.83 ± 0.38

for Efficacy (*excellent*, *good*, *satisfactory*, and *poor*) was used for efficacy. The Global Assessment Scale for Tolerability (GATE) was used to assess tolerability to therapy. Compliance was assessed using the tablet count and those who consumed over 80% of tablets were classified as compliant.

2.4. Statistical Methods. In this study being of an exploratory nature, the sample size was not based on any distributional assumptions and power calculations.

Efficacy analysis population included all men who completed the study as per the protocol. Safety analysis was done on the intent-to-treat population. The measurement data were expressed as means with one standard deviation. The two groups were compared for change in the sperm count from the baseline using one-way ANOVA with treatment as a factor. The global assessment scale values for efficacy and tolerability to therapy were compared between the two groups by the Mann-Whitney "U" test. The obtained results were interpreted as insignificant if the P value exceeded 0.05.

3. Results

Sixty-eight infertile males were assessed with regard to eligibility for inclusion in the present study. Forty-six were selected for inclusion. This study presents the data of these 46 oligospermic males randomized in a double-blind protocol to either the placebo-treated group (n = 25) or the high-concentration, full-spectrum Ashwagandha root extract-treated group (n = 21).

The two groups were similar with respect to demographic parameters (Table 1) and all baseline data including semen parameters and serum sexual hormone levels (Table 2).

3.1. Semen Parameters. Treatment with the Ashwagandha root extract resulted in a highly significant ($P < 0.0001$) increase in sperm concentration after 90 days of therapy, as compared to the baseline value on Day 0 of the study period (Table 2). The increase was from 9.59 ± 4.37 × 10^6/mL to 25.61 ± 8.6 × 10^6/mL, corresponding to a percentage increase of 167%. A statistically significant increase was observed in the semen volume (from 1.74 ± 0.58 mL to 2.76 ± 0.60 mL; $P < 0.0001$) and sperm motility (from 18.62 ± 6.11% to 29.19 ± 6.31%; $P < 0.0001$) on Day 90 as compared to the baseline value on Day 0. These corresponded to increases of 53% and 57%, respectively.

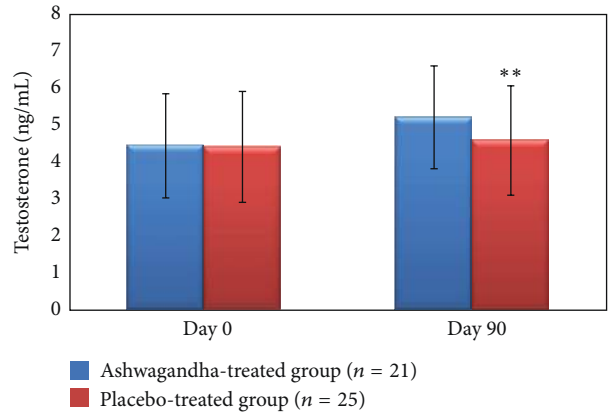

Figure 1: Serum testosterone levels (ng/mL) in the full-spectrum Ashwagandha root extract-treated and placebo-treated study groups including oligospermic males. $^{**}P < 0.0001$ as compared to baseline values on Day 0 of the study duration of 12 weeks. Values are expressed as mean ± SD.

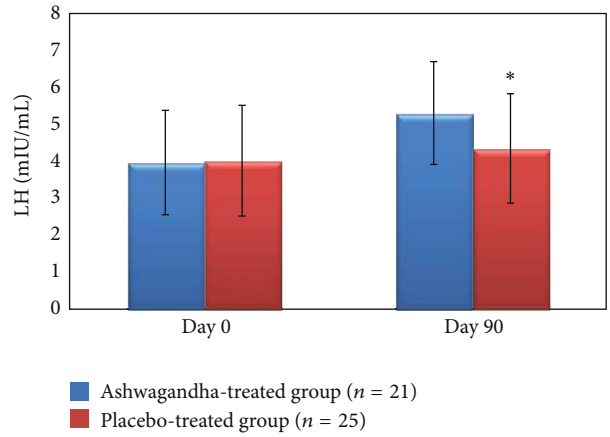

Figure 2: Serum LH (mL IU/mL) in the full-spectrum Ashwagandha root extract-treated and placebo-treated study groups including oligospermic males. $^{*}P < 0.001$ as compared to baseline values on Day 0 of the study duration (12 weeks). Values are expressed as mean ± SD.

3.2. Serum Hormone Levels. Furthermore, a significantly greater improvement and regulation were observed in serum hormone levels with the Ashwagandha root extract treatment as compared to the placebo treatment. Serum testosterone increased significantly by 17% (from 4.45 ± 1.41 ng/mL to 5.22 ± 1.39 ng/mL; $P < 0.01$) and LH by 34% (from 3.97 ± 1.21 mIU/mL to 5.31 ± 1.33 mIU/mL; $P < 0.02$), following treatment with Ashwagandha root extract, as compared to the baseline (Day 0) values of these parameters (Table 2, Figures 1 and 2).

Table 2: Semen profile of the Ashwagandha root extract-treated and placebo-treated oligospermic males.

	Placebo-treated group (n = 25)			Ashwagandha-treated group (n = 21)		
	Day 0	Day 60	Day 90	Day 0	Day 60	Day 90
Sperm concentration ($\times 10^6$/mL)	10.24 ± 2.82	12.42 ± 4.75	13.23 ± 7.74	9.59 ± 4.37	18.8 ± 5.7$^{\#}$	25.61 ± 8.6$^{\#}$
Semen volume (mL)	1.88 ± 0.65	2.3 ± 0.58	2.25 ± 0.41	1.74 ± 0.58	2.56 ± 0.7*	2.76 ± 0.6$^{\#}$
Sperm motility (%)	18.6 ± 5.41	18.87 ± 5.86	20.27 ± 5.97	18.62 ± 6.11	26.04 ± 5.6$^{\#}$	29.19 ± 6.31$^{\#}$
Serum testosterone ng/mL	4.42 ± 1.50	—	4.59 ± 1.48	4.45 ± 1.41	—	5.22 ± 1.39
Serum LH mIU/mL	4.02 ± 1.20	—	4.35 ± 1.28	3.97 ± 1.21	—	5.31 ± 1.33

$^{*}P < 0.05$, $^{\#}P < 0.0001$ as compared to the baseline values on Day 0 of the study duration (12 weeks). Values are expressed as mean ± SD.

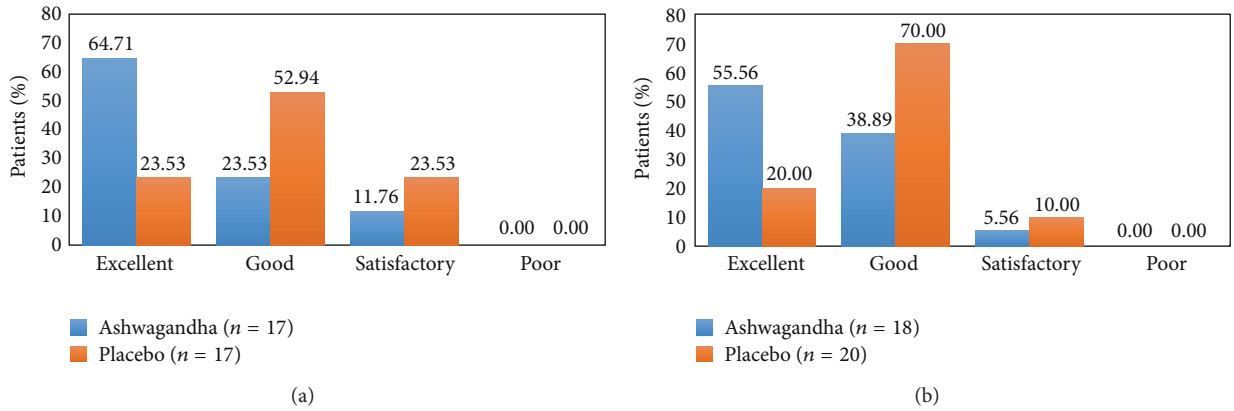

Figure 3: (a) Global Assessment Scale for Tolerability (GATE) by patients. (b) Global Assessment Scale for Tolerability (GATE) by physicians.

Upon evaluation on the Global Assessment Scale for Efficacy (GASE) and Global Assessment Scale for Tolerability (GATE), more patients (68.75%) reported the therapy with Ashwagandha as "excellent" when compared to the placebo (11.76%; Figures 3(a) and 3(b)).

4. Discussion

Male infertility accounts for about 50% of human infertility and in 40% to 50% of infertile males the etiology is unknown [1–7]. Numerous studies have demonstrated that compromised semen quality and sperm output are amongst the important causative factors of male infertility [3–5]. Moreover, it has been widely observed that oligospermia is the single most prevalent cause of reduced male fertility [2, 4].

Infertility is defined as the failure to conceive after 12 months of unprotected intercourse with the same partner. Twelve months are the lower reference limit for time to pregnancy by the World Health Organization [6, 31].

Ashwagandha (*Withania somnifera*) is an important medicinal plant that has been used in Ayurvedic medicine for over 6,000 years. In view of its varied and effective therapeutic potential, Ashwagandha has been the subject of considerable modern scientific investigation [8–12]. Ashwagandha has been used for centuries as a "*rasayana*" in Ayurvedic medicine. The root of Ashwagandha is specially regarded as a tonic and aphrodisiac [12–16]. Ashwagandha is often called "Indian ginseng" due to its rejuvenating effects [12, 13]. Nonetheless, the specific effects are not similar to ginseng. Rather than providing restless energy as does ginseng, Ashwagandha often causes relaxation.

In Ayurveda, certain herbal formulas are considered to be rejuvenating [9, 13–15, 18]. These formulas are called "*rasayana*" tonics, taken as a remedy for general weakness and exhaustion, as well as for their stress-relieving qualities.

Chris Kilham, a renowned author, educator and the founder of Medicine Hunter Inc., in accordance with the Indian Materia Medica, emphasized the use of Ashwagandha for general debility, impotence, brain fatigue, low sperm count, nervous exhaustion, and in situations in which general vigor must be restored, as Ashwagandha builds strength from within.

The present study employed a high-concentration, full-spectrum root extract of Ashwagandha, which retains and potentiates the synergism in the whole root.

Extensive clinical and experimental research has been carried out to address possible therapeutic modalities for the treatment of oligospermia utilizing various natural sources of plant and mineral origin as mentioned in Ayurveda and other classical traditional medical texts throughout the world [21–25].

In the present study, treatment with a high-concentration, full-spectrum root extract of Ashwagandha resulted in significantly improved semen parameters in concert with improved and regulated sexual hormone levels in oligospermic males. The analyses of our data indicated significantly increased sperm concentration and overall motility, which are regarded as the most important criteria for normal fertilizing ability of the spermatozoa. Our study outcome showed significant

enhancement of the semen volume in the Ashwagandha-treated infertile males.

Our data are in agreement with many investigations reporting improved sperm parameters including sperm concentrations and sperm motility [21–23]. The study by Mahdi et al. [22] compared the effects of Withania in smokers and those with psychological stress, whereas the study by Ahmad et al. [21] focused on the oxidative biomarkers. The observations reported in these studies and the current findings reinforce the beneficial effects of Ashwagandha in maintaining good sperm health and treating "male factor infertility."

In the present study, treatment with the Ashwagandha root extract resulted in a higher level of testosterone and a concomitant increase in serum levels of LH among infertile men having suboptimal testosterone levels before therapy. Apart from spermatogenesis, testosterone also controls the functional competence of the accessory sex organs, as adequate seminal fluid is necessary for the survival and motility of spermatozoa. Thus, it is postulated that the probable reasons of the increased sperm concentration and motility in the present findings lie in the higher levels of testosterone. These observations have been reported by other workers investigating the fertility-enhancement potential of Ashwagandha and other herbs and minerals [21–23].

Complementing our study, a recent study [22] conducted to assess the effect of Ashwagandha root on semen variables, oxidative biomarkers, and hormone levels among infertile young men aged 25–40 years in India demonstrated increased testosterone and LH among infertile men having suboptimal testosterone levels, compared with the control.

A decrease in testosterone and sperm counts indicates qualitative impairment of spermatogenesis and perhaps defects in sertoli and Leydig cell function [33], pointing toward severe infertility causing reproductive impairment. Thus, testosterone is imperative in aiding the production of sperm. Plant testosterone is much safer than taking an artificial form of testosterone, of which many pharmaceutical products contain [34].

There are reports that gonadal and sexual dysfunction are associated with elevated circulating cortisol levels. Cortisol levels in circulation rise sharply in response to stress followed by a significant drop in testosterone secretion [25]. There are reports that elevated psychological stress is associated with increased oxidative stress that may enhance the generation of reactive oxygen species (ROS).

The hypothalamic-pituitary-gonadal (HPG) axis is known to be involved in stress response and controls spermatogenesis. Hence, disruption of the HPG axis on account of stress results in the failure of the testes to produce adequate levels of testosterone and a normal number of sperms. It is diagnosed by a low sperm count or low serum testosterone levels and reduction in fertility and libido.

Ashwagandha is an effective herbal remedy for stress and infertility. It improves blood circulation throughout the body and enhances sperm quality naturally. Apart from curing sperm problems, intake of Ashwagandha helps in improving the overall health and wellbeing of a person. It relaxes the nerve cells and reduces the occurrence of various health disorders.

The most consistent positive finding of the present study was that decreased fertility in males was ameliorated by Ashwagandha root extract as evidenced by an increase in sperm concentration, ejaculate volume, and motile sperm count and an increase in the serum levels of testosterone.

The use of traditional or complementary/alternative medicine (CAM) for health care has been increasingly described in medical and science reports [35]. There has been an effort in recent years to evaluate the pharmacological properties of Ashwagandha, which has resulted in a better understanding of its therapeutic potential.

Nonetheless, if medical professionals are to prescribe herbal remedies for male infertility or any other medical condition, previous rigorous scientific investigations documenting their safety and efficacy from a Western scientific perspective are required. The outcomes of the present study provide evidence for the safety, efficacy, and tolerability of therapy with Ashwagandha root extract.

The present study suggests potential role of high-concentration, full-spectrum root extract of Ashwagandha in treating male infertility, which needs further exploration.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Clinical Evaluation of the Spermatogenic Activity of the Root Extract of Ashwagandha (*Withania somnifera*) in Oligospermic Males: A Pilot Study

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Ashwagandha (*Withania somnifera*) has been described in traditional Indian Ayurvedic medicine as an aphrodisiac that can be used to treat male sexual dysfunction and infertility. This pilot study was conducted to evaluate the spermatogenic activity of Ashwagandha root extract in oligospermic patients. Forty-six male patients with oligospermia (sperm count < 20 million/mL semen) were enrolled and randomized either to treatment ($n = 21$) with a full-spectrum root extract of Ashwagandha (675 mg/d in three doses for 90 days) or to placebo ($n = 25$) in the same protocol. Semen parameters and serum hormone levels were estimated at the end of 90-day treatment. There was a 167% increase in sperm count ($9.59 \pm 4.37 \times 10^6$/mL to $25.61 \pm 8.6 \times 10^6$/mL; $P < 0.0001$), 53% increase in semen volume (1.74 ± 0.58 mL to 2.76 ± 0.60 mL; $P < 0.0001$), and 57% increase in sperm motility (18.62 ± 6.11% to 29.19 ± 6.31%; $P < 0.0001$) on day 90 from baseline. The improvement in these parameters was minimal in the placebo-treated group. Furthermore, a significantly greater improvement and regulation were observed in serum hormone levels with the Ashwagandha treatment as compared to the placebo. The present study adds to the evidence on the therapeutic value of Ashwagandha (*Withania somnifera*), as attributed in Ayurveda for the treatment of oligospermia leading to infertility.

1. Introduction

Male infertility accounts for about 50% of human infertility. In 40% to 50% of infertile males, the etiology is unknown [1–7]. The pathophysiology of male infertility could be explained by a number of cellular abnormalities manifesting at the molecular and biochemical levels that result in decreased quality and quantity of sperm in the semen [3–5] and an imbalance in the reproductive hormones. Moreover, it has been widely observed that oligospermia is the single most prevalent cause of reduced male fertility [2, 4].

Ayurveda, the traditional system of medicine practiced in India, can be traced back to 6000 Bc [8–11]. For most of this history, Ashwagandha (*Withania somnifera*), also known as "Indian ginseng" due to its rejuvenating effects, has been described in folk medicine as an aphrodisiac and geriatric tonic [12]. It is classified as an "adaptogen," meaning that this herb assists in combating stress and disease, improving physical strength and metabolism without adverse effects [13–16]. Ashwagandha has been used as a "*rasayana*" in Ayurvedic medicine. In particular, the root of Ashwagandha is regarded as a tonic and aphrodisiac. *Ashwagandha* in the Sanskrit language means "horse's smell" (*ashwa*-horse, *gandha*-smell), probably originating from the odor of its root. The species name *somnifera* means "sleep-inducing" in Latin [17].

Ashwagandha is rich in a wide variety of chemical compounds, such as alkaloids, ergostane steroids, amino acids, and neurotransmitters, which explains its numerous

medicinal properties that can directly or indirectly prevent and treat a number of diseases [18–20].

It has been widely documented that, in addition to conventional therapies, many individuals with sexual dysfunction often seek alternative therapies. It is noteworthy that, from ancient times, Ashwagandha has been used by Ayurvedic practitioners as an aphrodisiac to improve on matters related to infertility and sexual activities. Numerous human and animal studies have validated the aphrodisiac and testosterone-enhancing effects of Ashwagandha [8–11, 21–23].

Different investigators have reported that Ashwagandha is beneficial in the treatment of male infertility [5, 21–23]. Experimental studies have shown that treatment with Ashwagandha induced testicular development and spermatogenesis in immature Wistar rats by directly affecting the seminiferous tubules [5, 24, 25], improved prosexual behaviour of sexually sluggish mice, and increased testicular daily sperm production and serum testosterone level [5].

It has been well documented that high levels of reactive oxygen species (ROS) in the semen induce oxidative damage to the sperm and are associated with abnormal sperm parameters leading to infertility [21, 26–30]. Ashwagandha has been found to counteract the formation of ROS in infertile men [21, 22, 27].

Despite numerous studies that report the efficacy of Ashwagandha in the treatment of various diseases, specific double-blind, randomized, placebo-controlled studies assessing the effectiveness of Ashwagandha in treating male infertility are few and, mostly, lacking critical data on safety and tolerability of the therapy.

Hence, the present study aims to investigate the usefulness of a highly concentrated, full-spectrum root extract of Ashwagandha as a suitable herbal supplement in treating male infertility.

2. Subjects and Methods

This two-arm, double-blind, randomized, placebo-controlled, parallel-group study with 1 : 1 random allocation was conducted at five infertility centers in India. The study was conducted in accordance with the good clinical practice guidelines of the Indian Council for Medical Research (ICMR-GCP) and the Declaration of Helsinki and was approved by the "League Health-Independent Ethics Committee."

2.1. Subjects. Sixty-eight infertile males were assessed with regard to eligibility for inclusion in the present study. Forty-six male patients between 22 and 40 years of age with semen factor infertility were enrolled after obtaining informed written consent. All men had a sperm count of 5–20 million/mL, total motility of 10%–30%, with forward motility < 15%, and atypical morphological forms < 70%. All men had a history of regular sexual intercourse over a one-year period with a gynecologically normal female partner with no apparent female infertility.

Men with a total sperm count of <0.5 million/mL or over 20 million/mL were not included. Also, men with primary erectile dysfunction, congenital anomalies, uncontrolled diabetes mellitus, severe hepatic or renal insufficiency, cardiovascular diseases, cerebrovascular accidents, uncontrolled hypertension, or with previous history of cryptorchidism, varicocele and testicular hypertrophy, were excluded from the study. Those with a history of pelvic fractures or prostatectomy or reconstructive or prosthetic surgery on the penis or having total or partly obstructive oligospermia were also excluded. Enrolled patients had not been administered any PDE-V inhibitors (sildenafil, tadalafil, or vardenafil) and glucocorticosteroids within the four weeks prior to enrollment and during the entire course of the study. Patients with known hypersensitivity to Ashwagandha extract were also excluded.

2.2. Randomization and Treatments. The study subjects were randomized to either: (i) the placebo-treated group ($n = 25$) or (ii) the Ashwagandha-treated group ($n = 21$). The study subjects in the Ashwagandha-treated group were administered one capsule (containing 225 mg of a high-concentration full-spectrum root extract of the Ashwagandha plant) orally, thrice daily for a period of 12 weeks, whereas, in the placebo-treated group, capsules containing 225 mg of matching placebo were administered similarly.

The Ashwagandha root extract employed in the present study, KSM-66 Ashwagandha (from Ixoreal Biomed Private Ltd., Hyderabad, India), has been extracted with a unique processing technology producing a broad-spectrum phytopharmaceutical that potentiates the action of Ashwagandha manifold, providing pan-therapeutic effects. It is noteworthy that although various Ashwagandha powders and extracts are available commercially, there are serious shortcomings in standardization and optimization of Ashwagandha extracts. KSM-66 Ashwagandha is standardized to withanolide content of at least 5% as measured by HPLC. It contains the desired quantum of withanolides and alkaloids, short- and long-chain amino acids (threonine, valine, methionine, isoleucine, lysine, aspartic acid, and arginine), complex sugars including oligosaccharides/fructooligosaccharides, vitamin A, calcium, and iron.

2.3. Trial Visits and Assessments. After the screening visit, during the treatment period of 90 days, the subjects were required to present themselves at the trial centers at specified intervals: Visit 1 on Day 30; Visit 2 on Day 60; and Visit 3 on Day 90. The final safety and efficacy assessments were done on Day 90 of the study. Semen analysis and complete physical examination were conducted at baseline and then after 30 days and again after 90 days. Standard manual semen analysis was performed according to WHO guidelines [31, 32]. Hormonal estimations were done for serum testosterone and luteinizing hormone (LH) levels on Day 0 (baseline) and after Day 90 using the chemiluminescence method.

The primary efficacy outcome was the improvement in the semen parameters and serum hormone levels from baseline (Day 0) after 90 days of therapy.

The secondary efficacy outcome was the safety and efficacy of the therapy under investigation. Safety was assessed based on the adverse events recorded during the study. At the end of the study, the four-point Global Assessment Scale

Table 1: Demography and baseline data of the study subjects.

	Placebo (n = 25) mean ± SD	Ashwagandha (n = 21) mean ± SD
Age (yr)	35.28 ± 5.49	32.38 ± 4.31
Height (cm)	167.13 ± 7.53	165.89 ± 8.55
Weight (kg)	74.32 ± 14.52	70.05 ± 11.22
Pulse (per min)	80.04 ± 8.06	79.78 ± 6.96
Respiratory rate (per min)	16.92 ± 2.45	17.39 ± 1.91
Systolic blood pressure (mm Hg)	123.84 ± 9.50	128.00 ± 8.03
Diastolic blood pressure (mm Hg)	80.16 ± 4.28	80.33 ± 3.65
Body temperature (°F)	98.04 ± 0.36	97.83 ± 0.38

for Efficacy (*excellent*, *good*, *satisfactory*, and *poor*) was used for efficacy. The Global Assessment Scale for Tolerability (GATE) was used to assess tolerability to therapy. Compliance was assessed using the tablet count and those who consumed over 80% of tablets were classified as compliant.

2.4. Statistical Methods. In this study being of an exploratory nature, the sample size was not based on any distributional assumptions and power calculations.

Efficacy analysis population included all men who completed the study as per the protocol. Safety analysis was done on the intent-to-treat population. The measurement data were expressed as means with one standard deviation. The two groups were compared for change in the sperm count from the baseline using one-way ANOVA with treatment as a factor. The global assessment scale values for efficacy and tolerability to therapy were compared between the two groups by the Mann-Whitney "U" test. The obtained results were interpreted as insignificant if the P value exceeded 0.05.

3. Results

Sixty-eight infertile males were assessed with regard to eligibility for inclusion in the present study. Forty-six were selected for inclusion. This study presents the data of these 46 oligospermic males randomized in a double-blind protocol to either the placebo-treated group (n = 25) or the high-concentration, full-spectrum Ashwagandha root extract-treated group (n = 21).

The two groups were similar with respect to demographic parameters (Table 1) and all baseline data including semen parameters and serum sexual hormone levels (Table 2).

3.1. Semen Parameters. Treatment with the Ashwagandha root extract resulted in a highly significant ($P < 0.0001$) increase in sperm concentration after 90 days of therapy, as compared to the baseline value on Day 0 of the study period (Table 2). The increase was from 9.59 ± 4.37 × 10^6/mL to 25.61 ± 8.6 × 10^6/mL, corresponding to a percentage increase of 167%. A statistically significant increase was observed in the semen volume (from 1.74 ± 0.58 mL to 2.76 ± 0.60 mL; $P < 0.0001$) and sperm motility (from 18.62 ± 6.11% to 29.19 ± 6.31%; $P < 0.0001$) on Day 90 as compared to the baseline value on Day 0. These corresponded to increases of 53% and 57%, respectively.

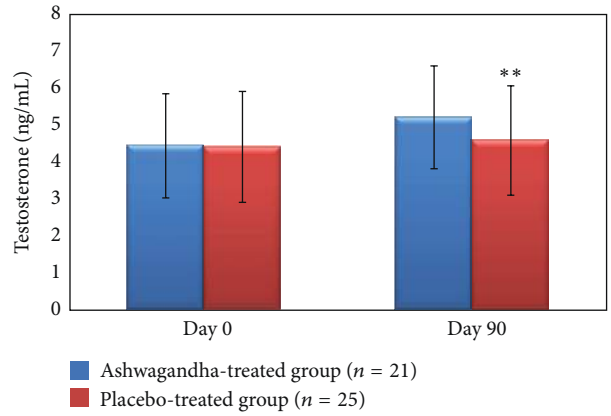

Figure 1: Serum testosterone levels (ng/mL) in the full-spectrum Ashwagandha root extract-treated and placebo-treated study groups including oligospermic males. $^{**}P < 0.0001$ as compared to baseline values on Day 0 of the study duration of 12 weeks. Values are expressed as mean ± SD.

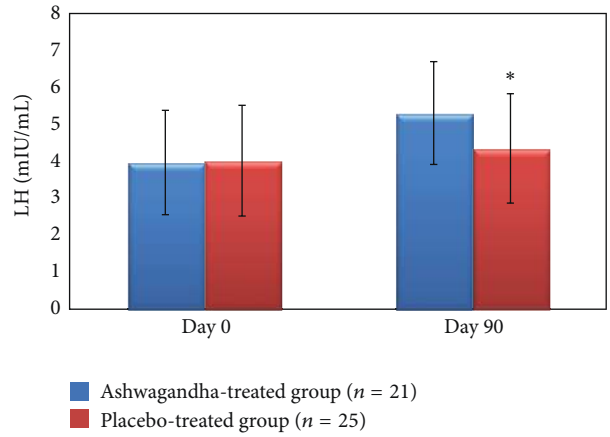

Figure 2: Serum LH (mL IU/mL) in the full-spectrum Ashwagandha root extract-treated and placebo-treated study groups including oligospermic males. $^{*}P < 0.001$ as compared to baseline values on Day 0 of the study duration (12 weeks). Values are expressed as mean ± SD.

3.2. Serum Hormone Levels. Furthermore, a significantly greater improvement and regulation were observed in serum hormone levels with the Ashwagandha root extract treatment as compared to the placebo treatment. Serum testosterone increased significantly by 17% (from 4.45 ± 1.41 ng/mL to 5.22 ± 1.39 ng/mL; $P < 0.01$) and LH by 34% (from 3.97 ± 1.21 mIU/mL to 5.31 ± 1.33 mIU/mL; $P < 0.02$), following treatment with Ashwagandha root extract, as compared to the baseline (Day 0) values of these parameters (Table 2, Figures 1 and 2).

Table 2: Semen profile of the Ashwagandha root extract-treated and placebo-treated oligospermic males.

	Placebo-treated group (n = 25)			Ashwagandha-treated group (n = 21)		
	Day 0	Day 60	Day 90	Day 0	Day 60	Day 90
Sperm concentration ($\times 10^6$/mL)	10.24 ± 2.82	12.42 ± 4.75	13.23 ± 7.74	9.59 ± 4.37	18.8 ± 5.7$^{\#}$	25.61 ± 8.6$^{\#}$
Semen volume (mL)	1.88 ± 0.65	2.3 ± 0.58	2.25 ± 0.41	1.74 ± 0.58	2.56 ± 0.7*	2.76 ± 0.6$^{\#}$
Sperm motility (%)	18.6 ± 5.41	18.87 ± 5.86	20.27 ± 5.97	18.62 ± 6.11	26.04 ± 5.6$^{\#}$	29.19 ± 6.31$^{\#}$
Serum testosterone ng/mL	4.42 ± 1.50	—	4.59 ± 1.48	4.45 ± 1.41	—	5.22 ± 1.39
Serum LH mIU/mL	4.02 ± 1.20	—	4.35 ± 1.28	3.97 ± 1.21	—	5.31 ± 1.33

$^{*}P < 0.05$, $^{\#}P < 0.0001$ as compared to the baseline values on Day 0 of the study duration (12 weeks). Values are expressed as mean ± SD.

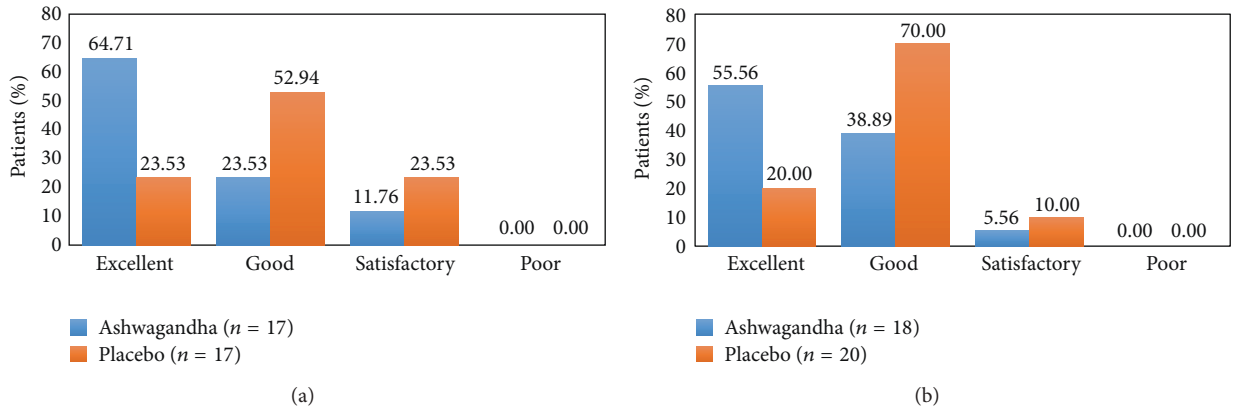

Figure 3: (a) Global Assessment Scale for Tolerability (GATE) by patients. (b) Global Assessment Scale for Tolerability (GATE) by physicians.

Upon evaluation on the Global Assessment Scale for Efficacy (GASE) and Global Assessment Scale for Tolerability (GATE), more patients (68.75%) reported the therapy with Ashwagandha as "excellent" when compared to the placebo (11.76%; Figures 3(a) and 3(b)).

4. Discussion

Male infertility accounts for about 50% of human infertility and in 40% to 50% of infertile males the etiology is unknown [1–7]. Numerous studies have demonstrated that compromised semen quality and sperm output are amongst the important causative factors of male infertility [3–5]. Moreover, it has been widely observed that oligospermia is the single most prevalent cause of reduced male fertility [2, 4].

Infertility is defined as the failure to conceive after 12 months of unprotected intercourse with the same partner. Twelve months are the lower reference limit for time to pregnancy by the World Health Organization [6, 31].

Ashwagandha (*Withania somnifera*) is an important medicinal plant that has been used in Ayurvedic medicine for over 6,000 years. In view of its varied and effective therapeutic potential, Ashwagandha has been the subject of considerable modern scientific investigation [8–12]. Ashwagandha has been used for centuries as a "*rasayana*" in Ayurvedic medicine. The root of Ashwagandha is specially regarded as a tonic and aphrodisiac [12–16]. Ashwagandha is often called "Indian ginseng" due to its rejuvenating effects [12, 13]. Nonetheless, the specific effects are not similar to ginseng. Rather than providing restless energy as does ginseng, Ashwagandha often causes relaxation.

In Ayurveda, certain herbal formulas are considered to be rejuvenating [9, 13–15, 18]. These formulas are called "*rasayana*" tonics, taken as a remedy for general weakness and exhaustion, as well as for their stress-relieving qualities.

Chris Kilham, a renowned author, educator and the founder of Medicine Hunter Inc., in accordance with the Indian Materia Medica, emphasized the use of Ashwagandha for general debility, impotence, brain fatigue, low sperm count, nervous exhaustion, and in situations in which general vigor must be restored, as Ashwagandha builds strength from within.

The present study employed a high-concentration, full-spectrum root extract of Ashwagandha, which retains and potentiates the synergism in the whole root.

Extensive clinical and experimental research has been carried out to address possible therapeutic modalities for the treatment of oligospermia utilizing various natural sources of plant and mineral origin as mentioned in Ayurveda and other classical traditional medical texts throughout the world [21–25].

In the present study, treatment with a high-concentration, full-spectrum root extract of Ashwagandha resulted in significantly improved semen parameters in concert with improved and regulated sexual hormone levels in oligospermic males. The analyses of our data indicated significantly increased sperm concentration and overall motility, which are regarded as the most important criteria for normal fertilizing ability of the spermatozoa. Our study outcome showed significant

enhancement of the semen volume in the Ashwagandha-treated infertile males.

Our data are in agreement with many investigations reporting improved sperm parameters including sperm concentrations and sperm motility [21–23]. The study by Mahdi et al. [22] compared the effects of Withania in smokers and those with psychological stress, whereas the study by Ahmad et al. [21] focused on the oxidative biomarkers. The observations reported in these studies and the current findings reinforce the beneficial effects of Ashwagandha in maintaining good sperm health and treating "male factor infertility."

In the present study, treatment with the Ashwagandha root extract resulted in a higher level of testosterone and a concomitant increase in serum levels of LH among infertile men having suboptimal testosterone levels before therapy. Apart from spermatogenesis, testosterone also controls the functional competence of the accessory sex organs, as adequate seminal fluid is necessary for the survival and motility of spermatozoa. Thus, it is postulated that the probable reasons of the increased sperm concentration and motility in the present findings lie in the higher levels of testosterone. These observations have been reported by other workers investigating the fertility-enhancement potential of Ashwagandha and other herbs and minerals [21–23].

Complementing our study, a recent study [22] conducted to assess the effect of Ashwagandha root on semen variables, oxidative biomarkers, and hormone levels among infertile young men aged 25–40 years in India demonstrated increased testosterone and LH among infertile men having suboptimal testosterone levels, compared with the control.

A decrease in testosterone and sperm counts indicates qualitative impairment of spermatogenesis and perhaps defects in sertoli and Leydig cell function [33], pointing toward severe infertility causing reproductive impairment. Thus, testosterone is imperative in aiding the production of sperm. Plant testosterone is much safer than taking an artificial form of testosterone, of which many pharmaceutical products contain [34].

There are reports that gonadal and sexual dysfunction are associated with elevated circulating cortisol levels. Cortisol levels in circulation rise sharply in response to stress followed by a significant drop in testosterone secretion [25]. There are reports that elevated psychological stress is associated with increased oxidative stress that may enhance the generation of reactive oxygen species (ROS).

The hypothalamic-pituitary-gonadal (HPG) axis is known to be involved in stress response and controls spermatogenesis. Hence, disruption of the HPG axis on account of stress results in the failure of the testes to produce adequate levels of testosterone and a normal number of sperms. It is diagnosed by a low sperm count or low serum testosterone levels and reduction in fertility and libido.

Ashwagandha is an effective herbal remedy for stress and infertility. It improves blood circulation throughout the body and enhances sperm quality naturally. Apart from curing sperm problems, intake of Ashwagandha helps in improving the overall health and wellbeing of a person. It relaxes the nerve cells and reduces the occurrence of various health disorders.

The most consistent positive finding of the present study was that decreased fertility in males was ameliorated by Ashwagandha root extract as evidenced by an increase in sperm concentration, ejaculate volume, and motile sperm count and an increase in the serum levels of testosterone.

The use of traditional or complementary/alternative medicine (CAM) for health care has been increasingly described in medical and science reports [35]. There has been an effort in recent years to evaluate the pharmacological properties of Ashwagandha, which has resulted in a better understanding of its therapeutic potential.

Nonetheless, if medical professionals are to prescribe herbal remedies for male infertility or any other medical condition, previous rigorous scientific investigations documenting their safety and efficacy from a Western scientific perspective are required. The outcomes of the present study provide evidence for the safety, efficacy, and tolerability of therapy with Ashwagandha root extract.

The present study suggests potential role of high-concentration, full-spectrum root extract of Ashwagandha in treating male infertility, which needs further exploration.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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RESEARCH ARTICLE Open Access

Examining the effect of *Withania somnifera* supplementation on muscle strength and recovery: a randomized controlled trial

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Abstract

Background: *Withania somnifera* (*ashwagandha*) is a prominent herb in Ayurveda. This study was conducted to examine the possible effects of ashwagandha root extract consumption on muscle mass and strength in healthy young men engaged in resistance training.

Methods: In this 8-week, randomized, prospective, double-blind, placebo-controlled clinical study, 57 young male subjects (18–50 years old) with little experience in resistance training were randomized into treatment (29 subjects) and placebo (28 subjects) groups. Subjects in the treatment group consumed 300 mg of ashwagandha root extract twice daily, while the control group consumed starch placebos. Following baseline measurements, both groups of subjects underwent resistance training for 8 weeks and measurements were repeated at the end of week 8. The primary efficacy measure was muscle strength. The secondary efficacy measures were muscle size, body composition, serum testosterone levels and muscle recovery. Muscle strength was evaluated using the 1-RM load for the bench press and leg extension exercises. Muscle recovery was evaluated by using serum creatine kinase level as a marker of muscle injury from the effects of exercise.

Results: Compared to the placebo subjects, the group treated with ashwagandha had significantly greater increases in muscle strength on the bench-press exercise (Placebo: 26.4 kg, 95 % CI, 19.5, 33.3 vs. Ashwagandha: 46.0 kg, 95 % CI 36.6, 55.5; $p = 0.001$) and the leg-extension exercise (Placebo: 9.8 kg, 95 % CI, 7.2,12.3 vs. Ashwagandha: 14.5 kg, 95 % CI, 10.8,18.2; $p = 0.04$), and significantly greater muscle size increase at the arms (Placebo: 5.3 cm^2, 95 % CI, 3.3,7.2 vs. Ashwagandha: 8.6 cm^2, 95 % CI, 6.9,10.8; $p = 0.01$) and chest (Placebo: 1.4 cm, 95 % CI, 0.8, 2.0 vs. Ashwagandha: 3.3 cm, 95 % CI, 2.6, 4.1; $p < 0.001$). Compared to the placebo subjects, the subjects receiving ashwagandha also had significantly greater reduction of exercise-induced muscle damage as indicated by the stabilization of serum creatine kinase (Placebo: 1307.5 U/L, 95 % CI, 1202.8, 1412.1, vs. Ashwagandha: 1462.6 U/L, 95 % CI, 1366.2, 1559.1; $p = 0.03$), significantly greater increase in testosterone level (Placebo: 18.0 ng/dL, 95 % CI, -15.8, 51.8 vs. Ashwagandha: 96.2 ng/dL, 95 % CI, 54.7, 137.5; $p = 0.004$), and a significantly greater decrease in body fat percentage (Placebo: 1.5 %, 95 % CI, 0.4 %, 2.6 % vs. Ashwagandha: 3.5 %, 95 % CI, 2.0 %, 4.9 %; $p = 0.03$).

Conclusion: This study reports that ashwagandha supplementation is associated with significant increases in muscle mass and strength and suggests that ashwagandha supplementation may be useful in conjunction with a resistance training program.

Keywords: Ashwagandha, Adaptogen herbs, Muscle, Muscle strength, Muscle mass, Testosterone, Body fat, Creatine kinase

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Background

Both the modern medical literature and traditional Ayurveda writings report many potential health benefits of the Ashwagandha herb (*Withania somnifera*, also known as Indian Ginseng or Winter Cherry) under the rubrics of anti-stress effects, neuroprotective effects, immunomodulatory effects, and rejuvenating effects, via the herb's interplay with the nervous system, the endocrine system, the cardiopulmonary system, the energy production system and the immune system including analgesic, antimicrobial, anti-inflammatory, anti-tumor, anti-stress, anti-diabetic, neuroprotective, immunoprotective and cardioprotective effects [1–7]. This paper focuses on ashwagandha as an ergogenic aid and is the first to present the results of a randomized, double-blind, placebo-controlled clinical study on ashwagandha's effects, as an adjuvant to a resistance training program, multifariously on muscle strength, muscle hypertrophy, muscle recovery and body composition. We add to the broader literature on ashwagandha's effect on physical performance. This literature has only a small set of published papers [8–10], which is surprising because traditional Ayurveda explicitly advocates the use of ashwagandha toward "bala", which means "strength" in the Sanskrit language [11] .

Ashwagandha is a member of the family of herbs referred to as "adaptogens". The term "adaptogen" is applied to a herb with phytonutrients that regulate metabolism when a body is perturbed by physical or mental stress, and help the body adapt by (a) normalizing system functions, (b) developing resistance to future such stress, and (c) elevating the body's functioning to a higher level of performance [12]. The adaptogen family of herbs has many members, noteworthy among them being ashwagandha, rhodiola, ginseng, schisandra and maca [12]. Adaptogens are used commonly for stress relief, brain health, adrenal health and for ameliorating HPA-axis dysfunction. More recently, adaptogens have started to be used in sports supplements that aim to enhance physical fitness. Recent research has found adaptogens to be promising in this application domain [13–15]. However, the results in this literature are mixed and therefore more research is needed so that we have a better understanding of adaptogens as ergogenic aids [14]. This present study attempts to make a small step towards such an understanding.

A resistance training program consists of exercises that cause skeletal muscles to contract against external resistance. The body often responds to such programs with increased strength and correlated adaptations [16–19]. The present research work was motivated by the hypothesis that ashwagandha supplementation can increase some of these adaptations and gains, thereby serving as a useful adjuvant to a resistance training program. There are several rationales underlying this hypothesis: Studies in healthy normal adults demonstrated that ashwagandha improves muscular strength/coordination, and cardiorespiratory endurance [8–10]. Ashwagandha's roots are classified as a "rasayana" (rejuvenator), and have been used toward promoting health and longevity, slowing the aging process, revitalizing the body and generally creating a sense of well-being [4, 20]. Ashwagandha has a wide range of pharmacological effects: it has anxiolytic, hypotensive, sedative, central nervous system, immunomodulatory, analgesic, anti-inflammatory, anti-tumor, anabolic, cardiopulmonary and antioxidant effects [4, 9, 21–27]. It also stimulates respiratory function, causing smooth muscle relaxation, and stimulates thyroid activity [3]. Studies in humans show that ashwagandha is well tolerated and is associated with decreases in cortisol [28], and increases in testosterone [29]. Research suggests that ashwagandha may reduce increases of blood urea nitrogen, lactic acid, corticosterone in response to stress [4], and also reduce the tendency of dopamine receptors in the brain to activate under stress [3, 5, 21]. Ashwagandha contains several active components, which may account for the various mechanisms of action by which it exerts its effects. These include steroidal lactones (withanolides, withaferins), saponins and alkaloids like isopelletierine and anaferine [5, 30].

The adaptogenic properties of ashwagandha raise the possibility of it being an effective ergogenic aid because the strain from exercise can be viewed as a form of stress, with enhanced human physical performance as the corresponding stress response upon ashwagandha supplementation. This study seeks to examine the hypothesis that ashwagandha supplementation may moderate the body's adaptation in response to resistance training. While this hypothesis is rooted in traditional Ayurvedic medicine and previous studies, well designed clinical trials are clearly needed to test and characterize the effects. The present double-blind, randomized, placebo-controlled study in healthy adults will, it is hoped, help toward expanding scientific understanding in this domain.

Methods

The study design, recruitment and methods were approved by all the authors' institutes' review boards on human subjects' studies and followed the guidelines of the Declaration of Helsinki and Tokyo for humans.

Subjects selection, incentives and participation

Healthy male subjects (18–50 years old) were recruited by use of fliers circulated in the vicinity of the gymnasium which served as the site of the training program. Subject enrollment, allocation, attrition, and analysis is summarized in Fig. 1. The purpose and protocol for the study was described to the subjects. The subjects had to

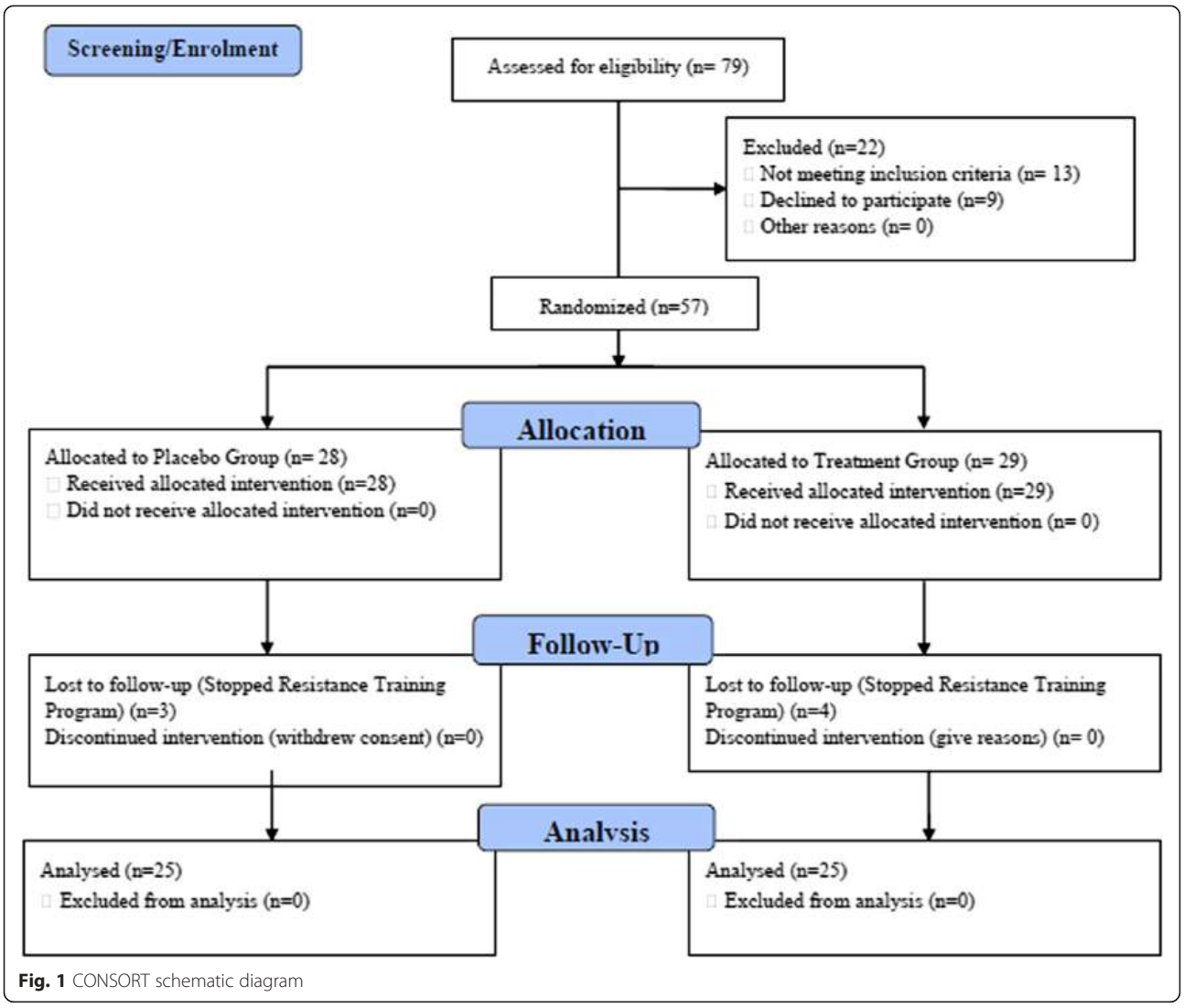

Fig. 1 CONSORT schematic diagram

sign a written consent for the study, agree to refrain from alcohol and tobacco during the study, and had to receive permission of their physician to participate. Subjects were excluded from the study if they met any of the following criteria: 1) taking any medication or steroids to enhance physical performance, 2) weight loss of >5 kg in the previous 3 months, 3) any history of drug abuse, smoking 10+ cigarettes day or consuming more than 14 grams of alcohol daily, 4) hypersensitivity to ashwagandha, 5) history of any orthopedic injury or surgery within the previous 6 months, 6) participation in other clinical studies during the previous 3 months, 7) any other conditions which the investigators judged problematic for participation in the study. Subjects were requested to refrain from using anti-inflammatory agents during the study and to report any ill effects from consuming the ashwagandha/ placebo.

Because participation in the study would require a significant amount of time allocation from the subjects, the recruiters offered as compensation one year of paid membership to the gym and three months of professional trainer support. As Fig. 1 indicates, 57 subjects were initially recruited for the study and 50 completed the study, including 25 in the ashwagandha group and 25 in the placebo group. For the ashwagandha treatment group and for the placebo group, the mean age ± standard deviation were 28 ± 8 years and 29 ± 9 years, respectively.

Study design

This study was a prospective, double-blind, placebo-controlled parallel group study to measure the possible effects of ashwagandha extract on muscle strength/size, muscle recovery, testosterone level and body fat percentage in

young males undergoing weight training. Adverse events were assessed by patient/ researcher reporting and the PGATT (Physicians Global Assessment of Tolerability to Therapy) form.

The resistance training program

The resistance training program consisted of sets of exercises over major muscle groups in both the upper body and the lower body. Directions for the resistance training were obtained from publications of the National Strength and Conditioning Association (NSCA) [31–33]. Each subject in both groups was asked to come to a training session every other day, with one rest day per week, for three days per week. Every session began with a warm up consisting of five minutes of low-intensity aerobic exercise.

The subjects were instructed to perform, for each set, as many repetitions as they could until failure. The subjects were asked to go through the full range of motion and were demonstrated the proper technique for safe and effective weight lifting.

Exercise selection

The specific exercises and the number of sets in each session were as follows. For the first week, the subjects were asked to perform the barbell squat (2 sets), the leg extension (1 set), the seated leg curl (2 sets), the machine chest press (1 set), the barbell chest press (2 sets), the seated machine row (1 set), the one-arm dumbbell row (2 sets), the machine biceps curl (1 set), the dumbbell biceps curl (2 sets), the cable triceps press-down (2 sets), the dumbbell shoulder press (2 sets), and the straight-arm pull-down (2 sets). For the second week, the subjects were asked to perform the leg extension (1 set), the barbell squat (2 sets), the barbell chest press (3 sets), the seated leg curl (2 sets), the seated cable row (3 sets), dumbbell biceps curl (3 sets), the cable triceps press-down (3 sets), the dumbbell shoulder press (3 sets), and the straight-arm pull-down or lat pull-down (3 sets). After this two-week acclimatization phase, for the rest of the study, the subjects were asked to perform the barbell squat (3 sets), the leg extension (3 sets), the leg curl (2 sets), one chest exercise (flat, incline or decline press or fly, cable crossover, 3 sets), one back exercise (rows, pull up, pull down or seated cable row, 3 sets), another chest exercise (3 sets), another back exercise (3 sets), one biceps exercise or one triceps exercise (curls or extensions, 3 sets), and one shoulder exercise (raises or presses, 3 sets).

How the target number of repetitions was chosen

The number of repetitions for the initial two weeks was set to be 15, which is a moderately high number (implying correspondingly lower weights), chosen to allow a subject's body and neural system to get accustomed to strength training [31, 33]. The subsequent 6 weeks had a varying number of repetitions, akin to a rudimentary non-linear periodization programs, because these have been shown to induce greater adaptation and more gains in muscle strength and size [33]. The number of repetitions specified for each of the days in the training program is given in Table 1.

How the load was chosen

The load was chosen to be such that the subject would reach the failure-point upon performing approximately the target number of repetitions (chosen as described in the preceding paragraph) when lifting that load. The corresponding load for an exercise was estimated on the basis of the 1RM prediction equations of Epley, Wathan and others [34].

Treatments and dosing

The researchers engaged a local laboratory to fill cellulose-based vegetarian capsules with either 300 mg of starch or 300 mg of a high-concentration ashwagandha root extract, KSM-66, manufactured by Ixoreal BioMed, Los Angeles, California, USA. This extract was produced using a water-based process using no alcohol or solvents and is standardized to a 5 % concentration of withanolides as measured by HPLC. The two capsules were identical in appearance, weight, and texture. Both the control and ashwagandha groups received a bottle of 60 pills at start of study and at 4 weeks. The pill count at 4 weeks allowed for a compliance check. The subjects were instructed to store the capsules between 18 and 32 °C and to take the capsules twice a day, once shortly after awakening and again shortly before bed, for the 8 weeks of the study.

Primary efficacy endpoint

The primary efficacy endpoint was muscle strength. Muscle strength is often measured by 1RM, the "one-repetition maximum", which is specific to a certain person and a certain exercise movement and refers to the maximal load that a subject can lift for one movement cycle of the exercise [31, 35]. Measurements were made at the first day of training and again 2 days after the 8 week training ended. The equipment used machine models DPL0802 (bench press) and DSL0605 (leg extension),

Table 1 The targeted number of repetitions over the course of the resistance training program

	Weeks 1–2	Weeks 3–4	Weeks 5–6	Weeks 7–8
Day 1	15	13	5	9
Day 2	15	9	13	5
Day 3	15	13	5	9
Day 4	15	9	13	5

manufactured by Precor (Woodinville, Washington, USA The 1-RM measurement was done using a variant of the widely used Baechle-Earle-Wathen protocol, employing the multiple RM method [36–40]. To reduce measurement error in strength assessment, we were careful to ensure consistency in the range of motion and that each movement on the chest press and the leg extension was complete and in accordance with the guidelines of the NSCA.

Secondary efficacy endpoints

The secondary efficacy endpoints related to serum testosterone level, muscle recovery and anthropometric factors capturing muscle size and body fat percentage.

Anthropometry

Muscle size: Muscle size was measured at 3 sites: the arm (flexed mid upper arm), chest (sternum at mid-tidal volume) and upper thigh (just inferior to gluteal fold). Measurements were done on the first day of the training period and 2 days after the last day of training. For the chest, we measured the girth, taken at the level of the middle of the sternum, with the tape passing under the arms and at the end of a normal expiration. For the thigh and arm, we assessed the maximal cross-sectional area (CSA) using the method of Moritani-DeVries, which is based on girth and skin-fold measurements [41–43]. The literature shows that the muscle CSA measures obtained by the Moritani-DeVries method are highly correlated with measures obtained by computer tomography or muscle biopsy, the gold standards for muscle CSA measurement [41–43]. Because of this high correlation, the across-time (Day 0 versus Day 56) or across-group (treatment versus placebo) comparisons on the basis of the Moritani-DeVries method are strongly indicative of the directionality and strength of the corresponding comparisons on the basis of the computer tomography. We chose to use the Moritani-DeVries method because it is less time-consuming and invasive than computer tomography or biopsy, and therefore less likely to discourage study participation.

Body fat percentage

Body fat percentage was calculated with a bioelectrical impedance method using machine with electrodes placed at the hand, wrist, foot, and ankle [44, 45]. Because bioelectrical impedance analysis based measurement of fat composition is known to be affected by extraneous factors like hydration level and temperature, we tried to maintain consistency in these factors by instructing the subjects to: 1) abstain from eating or drinking for 4 h before measurements, 2) urinate 30 min prior, 3) not engaged in exercise for 12 h prior and 4) not consume alcohol of caffeinated products [35, 45]. Body composition was measured two days after the first day of resistance training and again two days after the last day of resistance training.

Testosterone

Total blood testosterone serum levels were measured twice: once 2 days after the study commenced and again 2 days after the study ended. The blood draw was timed to be between two hours and three hours of each subject's regular waking time, and prior to any substantial physical activity, in order to minimize the effects of the natural diurnal variation in testosterone level. The 20 ml blood draws were from an antecubital vein, punctured with a 20-gauge disposable needle connected to a Vacutainer tube. The blood serum samples were analyzed by an ELISA (enzyme-linked immunosorbent assay).

Muscle recovery

Resistance training frequently damages skeletal muscle tissue. Such damage can result in decreased muscle force production and performance in subsequent training sessions, thereby possibly reducing the extent of adaptation and gains from resistance training [46]. Muscle recovery refers to the reduction in exercise-induced muscle damage over time. The level of creatine kinase, a protein, in the blood is a commonly used measure of muscle damage because this protein is specific to muscle tissue [47]. When muscles are overexerted, the muscle filaments are damaged and become necrotic, thereby causing soluble proteins like creatine kinase to migrate from muscle tissue into the blood stream [48]. The body on its own repairs such damage over 1 to 10 days and serum creatine kinase returns to baseline levels [48]. A bout of exercise tends to produce less damage in muscle tissue when repeated in subsequent training sessions after the body gets accustomed to the exercise. This is because of adaptation and strengthening of the muscle tissue. Serum creatine kinase was measured at 24 h and at 48 h after the end of the first exercise session, and also at 24 h and at 48 h after the end of the last exercise session approximately 8 weeks later, from 20 ml blood draws using a 20-gauge disposable needle and a Vacutainer setup. The creatine kinase level was determined in a commercial laboratory using enzymatic analysis tracking nicotinamide adenine diphesphopyridine (NADPH). The increase in creatine kinase from the 24-h point to the 48-h point can be taken as a biomarker of recovery in that a smaller increase corresponds to faster stabilization of creatine kinase level and hence faster recovery of muscle tissue from exercise-induced damage.

Tolerability

The subjects were asked to report any adverse events experienced at any point in the study. We used the

Physicians Global Assessment of Tolerability to Therapy (PGATT) form [49–51]. Subjects used a five-point scale to assess tolerability from "worst tolerability" (which corresponds to patients' not being able to tolerate the drug at all) to "excellent tolerability" (which corresponds to no adverse effects and the patient being able to tolerate the drug excellently).

Statistical analysis

Assessment of statistical significance of continuous treatment effects was done using ANOVA with group identity (treatment versus placebo) as a factor. We used the Mann-Whitney test if the data were found to be not normally distributed. Frequencies of the tolerability scale values were compared using the chi-square test for contingency tables. The accepted level of significance was $\alpha = 0.05$.

Results

Tables 2, 3, 4, 5 and 6 compare the treatment group and the placebo group at baseline, at the start of the study and at the end of the 8 week study for the following 5 factors: testosterone level (ng/dL), muscle strength on the bench press 1-RM (Kg), muscle strength on the leg extension 1-RM (Kg), muscle size at thighs (cm^2), muscle size at arms (cm^2), muscle size at chest (cm), body fat percentage, muscle recovery in terms of creatine kinase levels change (U/L),

Primary efficacy measure: muscle strength

There was a significant increase in muscle strength and muscle size in both the ashwagandha group and the placebo group, for both the upper and lower body. This is unsurprising because both group engaged in resistance training. The focal question is whether the adaptation is greater under ashwagandha supplementation. Table 2 and Fig. 2 show that the increases in muscle strength were statistically significantly greater in the ashwagandha group than in the placebo group, for the upper body (Placebo: 26.42 kg, 95 % CI, 19.52, 33.32 vs. Ashwagandha: 46.05 kg, 95 % CI 36.56, 55.54; $p = 0.001$) and the lower body (Placebo: 9.77 kg, 95 % CI, 7.18, 12.35 vs. Ashwagandha: 14.50 kg, 95 % 10.76, 18.23; $p = 0.04$).

Secondary efficacy measures

Anthropometry

Muscle Size: For muscle size (Table 3; Fig. 2), the increases are significantly greater in the ashwagandha group than the placebo group in the arm (Placebo: 5.30 cm^2, 95 % CI, 3.34,7.25 vs. Ashwagandha: 8.89 cm^2, 95 % 6.95,10.84; $p = 0.01$) and chest (Placebo: 1.43 cm, 95 % CI, 0.83, 2.02 vs. Ashwagandha: 3.37 cm, 95 % CI, 2.59, 4.15; $p < 0.001$) but not in the thighs (Placebo: 6.22 cm^2, 95 % CI, 2.61, 9.84 vs. Ashwagandha: 8.71 cm^2, 95 % CI, 4.56, 12.87; $p = 0.36$).

Body composition: Table 4 shows that body fat percentages declined in both groups over the 8 week study, with the fat percentage decrease being significantly greater among subjects in the ashwagandha group as compared to the placebo group (Placebo: 1.52 %, 95 % CI, 0.46, 2.59, vs. Ashwagandha: 3.47 %, 95 % CI, 1.99, 4.95; $p = 0.03$).

Serum testosterone

Over the eight weeks, there was a significant increase in testosterone level in the ashwagandha treatment group relative to the placebo group (Table 5; Fig. 3). The increase in testosterone level was significantly greater with ashwagandha supplementation than with the placebo (Placebo: 18.00 ng/dL, 95 % CI, -15.83, 51.82 vs. Ashwagandha: 96.19 ng/dL, 95 % CI, 54.86, 137.53; $p = 0.004$). While the mean post-intervention level was notably higher in the ashwagandha group than in the placebo group (726 versus 693), the numbers are not detectable as statistically significantly different, very likely because the across-subject variance is high.

Muscle recovery

Recall that the level of recovery from exercise-induced muscle damage is assessed through the increase in level of serum creatine kinase from Hour 24 to Hour 48 after the end of the resistance training session. A smaller increase in this muscle protein in the blood stream

Table 2 Muscle strength

		Treatment group	Placebo group	Between group comparison
		Mean (SD)	Mean (SD)	(*p*-values)
	Sample size (n)	$n = 25$	$n = 25$	
Bench Press 1RM (Kg)	Pre intervention	33.21 (8.50)	31.35 (7.97)	0.44
	Post intervention	79.26 (25.90)	57.77 (16.48)	0.001
	Change	46.05 (23.00); 95 % CI: 36.56, 55.54**	26.42 (16.72); 95 % CI: 19.52, 33.32**	0.001
Leg Extension 1RM (Kg)	Pre intervention	27.89 (4.24)	25.22 (7.03)	0.11
	Post intervention	42.38 (10.80)**	34.98 (10.54)**	0.02
	Change	14.50 (9.04); 95 % CI: 10.76, 18.23**	9.77 (6.27); 95 % CI: 7.18, 12.35**	0.04

** = $p < 0.001$ within group comparison

Table 3 Muscle size

		Treatment group mean (SD)	Placebo group mean (SD)	Between group comparison (*p*-values)
	Sample size (n)	*n* = 25	*n* = 25	
Thigh (cm^2)	Pre intervention	107.84 (24.61)	111.18 (17.15)	0.58
	Post intervention	116.56 (26.04)	117.40 (19.96)	0.9
	Change	8.71 (10.06); 95 % CI: 4.56, 12.87**	6.22 (8.76); 95 % CI: 2.61, 9.84*	0.36
Arm (cm^2)	Pre intervention	51.96 (10.88)	53.13 (14.84)	0.75
	Post intervention	60.85 (13.23)	58.43 (17.66)	0.59
	Change	8.89 (4.71); 95 % CI: 6.95, 10.84**	5.30 (4.74); 95 % CI: 3.34, 7.25**	0.01
Chest (cm)	Pre intervention	101.40 (11.22)	101.16 (8.93)	0.93
	Post intervention	104.77 (11.09)	102.58 (8.76)	0.44
	Change	3.37 (1.89); 95 % CI: 2.59, 4.15**	1.43 (1.45); 95 % CI: 0.83, 2.02**	0.0002

* = $p < 0.01$; ** = $p < 0.001$ within group comparison

corresponds to faster muscle tissue repair, which in turn corresponds to greater recovery. Table 6 and Fig. 3 show how this metric varied across groups and over time. It is important to keep in mind that smaller numbers are to be interpreted as better recovery. What is striking is that recovery was dramatically better after 8 weeks of resistance training, in both the ashwagandha group and the placebo group, likely because of muscle tissue getting accustomed to the training regimen and developing greater integrity to resist any damage. Comparing the ashwagandha group and the placebo group, the results showed that recovery is substantially higher in the ashwagandha group than in the placebo group (Placebo: 1307.48 U/L, 95 % CI, -1202.82, 1412.14 vs. Ashwagandha: 1462.68 U/L, 95 % CI, 1366.27, 1559.09; $p = 0.03$).

Tolerability

No serious side effects were reported by subjects in either group. All subjects rated tolerability as either "good" or "excellent" on the PGATT form. There was no statistically significant difference in PGATT scores between the 2 groups.

Table 4 Body fat percentage

	Treatment group	Placebo	Between group comparison
	Mean (SD)	Group mean (SD)	(*p*-values)
Sample size (n)	*n* = 25	*n* = 25	
Pre intervention	21.60 (3.91)	22.01 (3.37)	0.7
Post intervention	18.13 (3.13)**	20.48 (1.85)*	0.003
Change	−3.47 (3.58); 95 % CI: -4.95, -1.99**	−1.52 (2.58); 95 % CI: -2.59, -0.46*	0.03

* = $p < 0.01$; ** = $p < 0.001$ within group comparison

Discussion

This is the first research paper that we know of that studies ashwagandha as an adjuvant to resistance training programs. Because subjects in both the ashwagandha group and the placebo group engaged in resistance training, we would expect to see a substantial degree of improvement in muscle-related parameters in both groups, and indeed we did. These findings are consistent with numerous studies which have measured adaptation to strength training programs in the absence of any supplementation [33]. The focal question that we sought to examine in this research is related to whether ashwagandha supplementation would magnify these adaptations. The adaptations were found to be statistically significantly greater, at a *p*-value threshold of 0.05, with ashwagandha supplementation than under placebo for all parameters (muscle strength, muscle size and body fat percentage, testosterone, and muscle recovery) except for thigh muscle size, though some effects were marginal. Increased recovery from muscle damage has the practical implication that it allows one to resume resistance training more quickly, thereby increase the volume of training per unit time and thereby potentially achieve

Table 5 Serum testosterone level (ng/dL)

	Treatment group	Placebo group	Between group comparison
	Mean (SD)	Mean (SD)	(*p*-values)
Sample size (n)	*n* = 25	*n* = 25	
Pre intervention	630.45 (231.88)	675.12 (157.02)	0.43
Post intervention	726.64 (171.55)**	693.12 (115.04)	0.42
Change	96.19 (100.14); 95 % CI: 54.86, 137.53**	18.00 (81.94); 95 % CI: -15.83, 51.82	0.004

** = $p < 0.001$ within group comparison

Table 6 Muscle recovery: increase of serum creatine kinase from hour 24 to hour 48 after end of exercising (U/L)

	Treatment group	Placebo group	Between group comparison
	Mean (SD)	Mean (SD)	(*p*-values)
Sample size (n)	$n = 25$	$n = 25$	
Pre intervention	1478.88 (239.60)	1406.52 (264.45)	0.31
Post intervention	16.20 (9.47)**	99.04 (16.77)**	<0.0001
Change	−1462.68 (233.57); 95 % CI: -1559.09,-1366.27**	−1307.48 (253.54); 95 % CI: -1412.14, -1202.82**	0.03

** = $p < 0.001$ within group comparison

greater gains per unit time. Our basic results are consistent with the findings of previous human studies with ashwagandha [8, 9, 28, 29, 52] in demonstrating gains in muscle strength, body composition and testosterone, though these other studies do not follow these parameters all in a single clinical trial or in conjunction with a resistance training program. The ashwagandha extract was tolerated very well at the study dose with no side effects reported. This good safety profile of ashwagandha is consistent with reports from previous studies [8, 9, 28, 29, 52].

There are also studies considering the use of other adaptogenic herbs like *Rhodiola rosea*, *Eleutherococcus senticosus*, *Schisandra chinensis* and ginseng toward physical performance. One study [53] gave evidence that *Rhodiola rosea* supplementation can improve endurance and reduce time to exhaustion. A review of Russian research [15] identifies human studies that show improved physical and mental performance from Schisandra supplementation. It is suggested that [15] Schisandra supplementation can help elite athletes adapt to high physical intensities. More study of the use of adaptogen herbs in the aid of muscle strength and recovery is needed.

There are several mechanisms of action that could have contributed to the positive effects of ashwagandha supplementation on resistance training and performance improvements in this study. These can be viewed from two perspectives: muscle development and muscle recovery.

Muscle development

The ability to lift weights is a function of (a) muscle size, (b) energy production and (c) the nervous system's

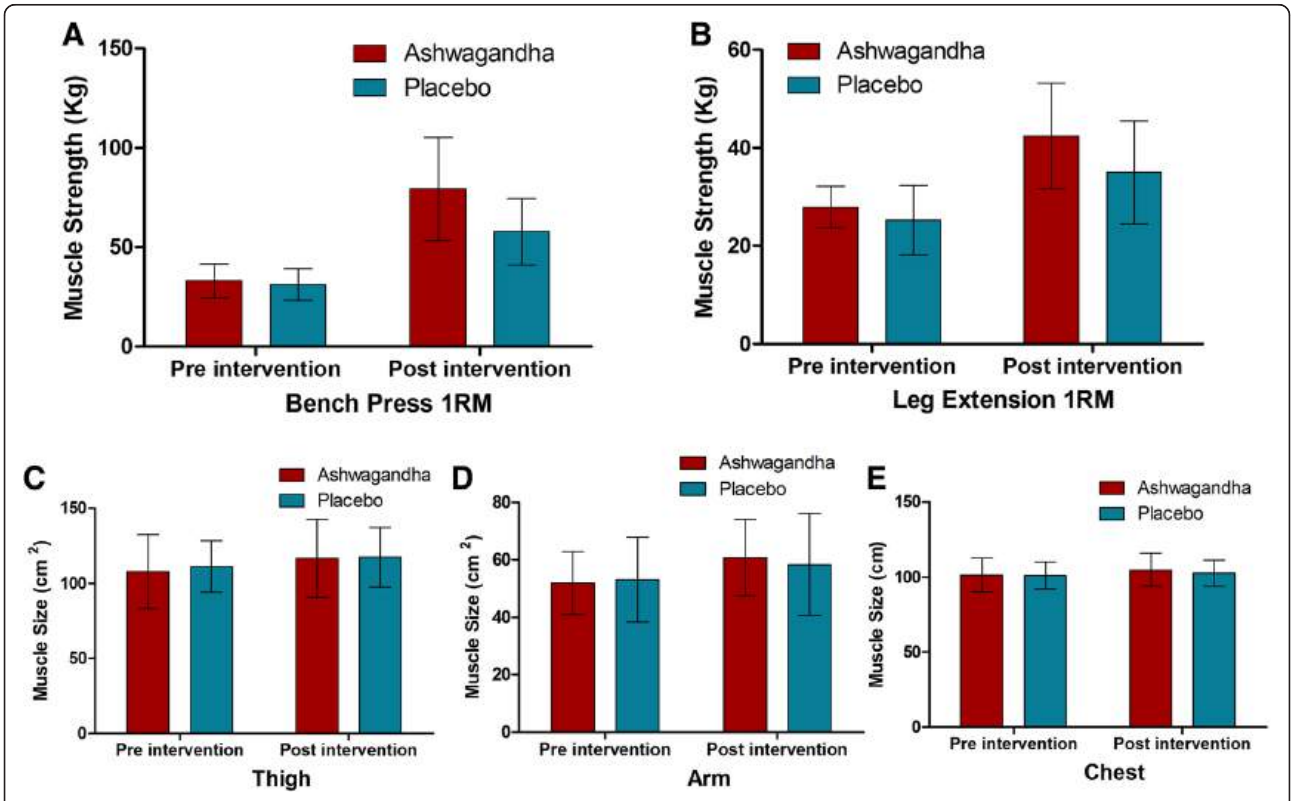

Fig. 2 The effect of Ashwagandha treatment in muscle strength [**a** bench press 1 RM, **b** leg extension 1RM] and muscle size in **c** thigh, **d** arm and **e** Chest

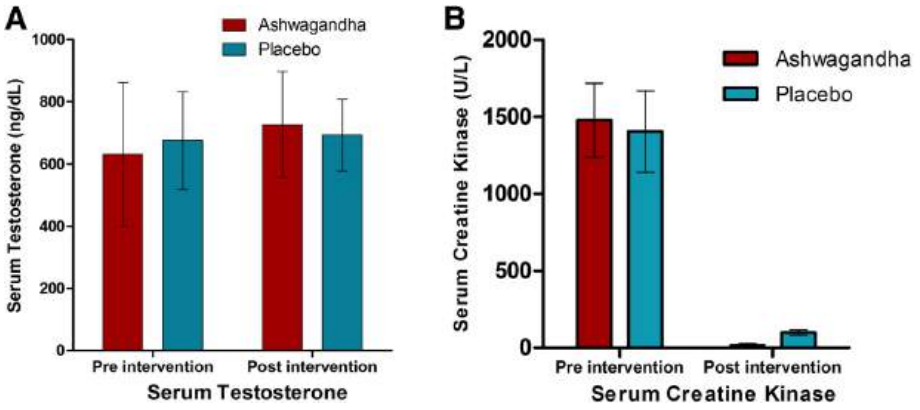

Fig. 3 The effect of Ashwagandha treatment in **a** serum testosterone and **b** serum creatine kinase level

ability to recruit muscles and coordinate them to generate the required force. Muscle size is a function of muscle growth, which is affected by two of ashwagandha's effects: (i) increase in testosterone, which leads to muscle growth and (ii) decrease in the levels of cortisol, which as a catabolic agent detracts from muscle mass. In terms of energy production, ashwagandha (i) can have beneficial effects on mitochondrial energy levels and functioning and reduce the activity of the Mg2 + -dependent ATPase enzyme responsible for the breakdown of ATP [54], and (ii) can increase creatine levels that can in turn lead to ATP generation [8]. Finally, the effects of ashwagandha on the nervous system as anti-anxiety agent and in promoting focus and concentration [28] may translate to better coordination and recruitment of muscles. The reason for the lack of an effect on thigh size is not clear. Longer term studies are needed to shed light on this, as are studies looking at markers in the local environment of these muscles to rule out any biochemical anomalies as contributing factors.

Muscle recovery

In the present study, more rapid recovery from muscle damage under supplementation with ashwagandha was demonstrated by monitoring creatine kinase. The faster recovery could be due to a number of mechanisms, or more likely their synergistic effects, as mediated by the various extract components, such as antioxidant effects to combat free radical damage both at the muscle and central nervous system levels, anti-inflammatory and analgesic effects and reduction in lactic acid and blood urea nitrogen [4–6]. In that vein, muscle soreness is a common occurrence following exercise for those less accustomed to physical activity. Delayed onset muscle soreness (DOMS) presents between 24 and 48 h after exercise as tenderness to palpation, and/or movement accompanied by decreases in flexibility and maximal voluntary force production. This soreness can inhibit full and proper exercise. Thus, a reduction in DOMS as a consequence of ashwagandha's effect on reduced muscle injury would counteract this negative consequence.

Conclusion

This study confirms previous data regarding the adaptogenic properties of ashwagandha and suggests it might be a useful adjunct to strength training. This study has the following limitations which should lead us to interpret the findings with some caution: the subjects are untrained and moderately young, the sample size of 50 is not large and the study period is of duration only 8 weeks. Research studying the possible beneficial effects of ashwagandha needs to be conducted for longer periods of time and for different populations including females and older adults of both genders.

Competing interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' contributions

SW and DL developed the clinical trial design and the resisitance training protocol. They carried out the data collection in collaboration with KJ and SS. SW, DL, KJ and SS oversaw the data treatment and data analysis. SB contributed to the writing, the presentation, the bibliography and managed correspondence. All authors were involved in the writing and drafting, and all read and approved the final manuscript.

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