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REVIEW

A Systematic Review on Rho-Kinase as a Potential Therapeutic Target for the Treatment of Erectile Dysfunction

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Background: Erectile dysfunction (ED) is a common clinical condition with limited treatment options. The main aim of the present systematic review was to synthesize information on Rho-kinase as a novel therapeutic approach for the treatment of ED.

Methods: We performed a systematic literature study in PubMed, Google Scholar and Scopus. Included studies were original articles studied the role of Rho-kinase in the pathogenesis and/or new treatment approach for ED in animal models and clinical studies, published between 2014 and 2019. Data derived from each study were study design used, interventions applied and main treatment outcomes. The quality of the selected articles was assessed by CAMARADES criteria and data were analyzed using descriptive statistics.

Results: A total of 1067 original articles were retrieved in the given period and eighteen papers met our inclusion criteria. Five articles explain the role of Rho-kinase in ED pathogenesis using different models such as cavernous nerve crush injury, heart failure-induced ED, vasculogenic and post-radical prostatectomy ED, diabetes-induced ED and age-related ED. Other ten papers explain the role of novel drugs evaluated for ED treatment by targeting Rho-kinase as a new approach for ED therapy. The rest three papers discuss the role of plant extracts used by traditional society for the treatment of ED and assess their potential function in targeting Rho-kinase in animal models. The penile erectile functional index has shown that the ratio of intracavernosal pressure to mean arterial pressure (ICP/MAP) was decreased due to age and various chronic diseases. Whilst, ROCK I and ROCK II expression were increased. Western blot findings have also shown that ROCK II and MYPT-1 phosphorylation rates increased in cavernous tissue after ED induction. Besides, compounds which can inhibit the action of Rho-kinase activity showed relaxation of the corpus cavernosum, decrease in corporal fibrosis, and alleviate increased apoptosis and caspase-3 activity in an NO-independent manner. Moreover, histological and molecular dysregulation have been improved by inhibition of Rho-kinase.

Conclusion: Targeting Rho-kinase may be a possible target for the treatment of ED secondary to specific causes, and Rho-kinase inhibitors may be a new drug family for the treatment of ED. However, this requires further studies for in-depth understanding.

Keywords: ROCK, Rho-kinase inhibitors, novel, therapeutic target, erectile dysfunction

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Introduction

Rho Kinase (ROCK)

Rho-associated coil-forming protein kinase (ROCK) is derived from the ACG (cyclic adenosine monophosphate (cAMP)-dependent protein kinase A/protein kinase C/protein kinase G) family of serine/threonine kinases and is activated by

the guanosine triphosphate (GTP)-bound form of RhoA (the small monomeric G-protein).[1,2] ROCK activity is regulated by its upstream regulators, the Rho-GTPases RhoA and RhoC, which belong to the Ras-superfamily.[3] There are two isoforms, ROCK I and II, and are involved in various physiological and pathophysiological signaling pathways.[4,5]

ROCKs have been studied extensively and known as the major downstream effectors of RhoA.[6] It was involved in the generation of actin-myosin contractility and regulation of actin cytoskeleton dynamics.[7] It has also crucial roles in cell proliferation, apoptosis, gene expression and multiple other common cellular functions, such as cell shape, motility, gene expression, secretion and proliferation.[6,8,9] Moreover, it causes a contractile response in vascular smooth muscle cells by increasing Ca^{2+} sensitization.[10]

Rho-binding can modulate the autophosphorylation of the kinase activity of ROCK.[11] However, RhoE (the small G protein) negatively control ROCK I activity by binding in N terminus and prevents RhoA binding to Rho-binding domain (RBD).[7] Phosphoinositide-dependent kinase-1 can counteract the negative regulation produced by RhoE by preventing its binding to the N terminal. Other small G proteins (Rad and Gem) can bind and inhibit ROCK I and II activity by unknown mechanisms.[7,12,13]

Various experiments performed on humans, isolated tissues and genetically modified mouse models have shown that ROCKs have been involved in the pathophysiological pathways of different diseases.[7,14,16] The two ROCK isoforms have been described as a promising and alternative target of inhibitory molecules for the wide variety of human diseases including erectile dysfunction.[17,22]

Erectile Dysfunction

Erectile dysfunction (ED) is the most common chronic illness affecting men and often associated with chronic diseases including hypogonadism, cardiovascular diseases and diabetes mellitus (DM).[23,24] Age was also considered one of the main risk factors for ED.[25]

Different mechanisms have been correlated with ED pathology, which involves physical (organic), emotional and psychological components.[26,27] The most common reported mechanism were physical causes including neuropathies, neurological disorders, endocrinological disorders (hypogonadism or hyperprolactinaemia), vasculogenic ED (hypertension, atherosclerosis, and pelvic irradiation) and medication side effects due to anti-depressants, non-steroidal anti-inflammatory drugs, neuroleptics and antiepileptic medications.[23,28,29] Stressful life events such as daily worries about work, money or other significant occurrences can be associated with emotional and psychological causes of sexual dysfunction.[29] Ageing often associated with changes of the endocrine system (especially in elderly's). At an older age, the testicular Leydig cells do not produce sufficient testosterone and that may lead to ED.[25] Moreover, there is also a decreased in blood supply to the penis.[30]

Different biochemical changes were produced in corporal smooth muscles by local and neuronal neurotransmitters through potassium channels, gap junctions and calcium channels.[23,28,30] For example, the neurotransmitter nitric oxide (NO) has a principal role in mediating penile erection. Once synthesized, it diffuses into smooth muscle cells and, binds and stimulates guanyl cyclase, which increases cyclic guanosine monophosphate (cGMP) and activates protein kinase G (PKG). Then after, phosphorylates several intracellular proteins and ion transporters, resulting in hyperpolarization and decreased cytoplasmic $Ca,^{2+}$ causing smooth muscle relaxation.[31] In addition to the reduction of cytosolic Ca^{2+} resulting from the NO cascade, dephosphorylation of myosin light chain (MLC) by myosin light chain phosphatase (MLCP) facilitates the release of myosin from actin and smooth-muscle relaxation in NO-independent manner.[11,31] Any alterations in the ionic channels, gap junctions, cGMP, cAMP and NO pathways affect the corporal smooth muscle contraction and relaxation.[23,28,30]

The prevalence of ED is estimated to be 50–100% in people over 70 years of age[25] and it has been increased in men below 40 years old.[28] Besides, there are limited treatment options for ED and even with the available medications, there are concerns regarding their effectiveness and adverse effects. The quest for alternative ED drugs is necessary in order to address these challenges. Therefore, the purpose of the present review was to synthesis data regarding the role of Rho-kinase in the pathogenesis of ED and the efficacy of Rho-kinase inhibitors in the treatment of ED based on original articles published in peer-reviewed journals.

Methods

Search Strategies

A systematic search of the literature was done in PubMed, Google Scholar and Scopus. A manual search was also done. The database search was carried out using different keywords such as Rho-kinase, ROCK, Rho-kinase

inhibitors, erectile dysfunction and sexual dysfunction, then search terms were combined using either "AND" or "OR" between two or more terms (Table 1).

Inclusion and Exclusion Criteria

Included studies were original articles evaluating the role of Rho-kinase in ED pathogenesis and/or investigating novel ED therapeutic agents by targeting Rho-kinase. Articles were deemed to be eligible for inclusion if the original article was written in the English language and published between 2014 and 2019. Studies that assessed the possible anti-ED effects of Rho-kinase inhibitors using original articles with any kind of study design on animal ED model, ex-vivo model or clinical studies were included. Reviews, opinion pieces, letter to editors, conference abstracts and/or partially accessed (abstract only) articles and commentaries were excluded.

Screening and Eligibility of Studies

Potential articles have been collected from various databases and duplicates have been removed. Two investigators (KAZ and MAA) evaluated title and abstract separately on the basis of predefined inclusion criteria. This process was accompanied by the assessment and retrieval of the full texts of the relevant citations. A third investigator (BAT) was concerned for potential disputes.

Data Extraction and Quality Assessment

The extraction of data from each study was carried out using a predefined form. The two authors (KAZ and DZW) independently performed data extraction. Variables extracted from the included papers were publication year, study design, study population and/or animal model used, sample size, follow-up duration, outcomes of treatment, and risk estimates with their adjusted covariant. The quality of each paper was assessed by DZW and KAZ using the Collaborative Approach to Meta-Analysis and Review of Animal Data from Experimental Studies (CAMARADES) quality assessment tool before data extraction was done.[32] The quality of each paper was expressed in mean ± standard error of mean.

Table 1 Databases Employed and Respective Keywords Used

	Search Arm	Search Terms Used (Free Text or MeSH Terms)
#1	Rho-kinase	Rho-kinase OR Rho-kinase inhibitors OR ROCK
#2	Erectile dysfunction	Erectile dysfunction OR Sexual dysfunction

Notes: The search was made on PubMed, Scopus, Google Scholar and manual search. Search terms within the same arm were combined by "OR" and these search arm #1 and #2 were combined by "AND".

Results

Out of 1067 articles, 18 articles fulfil our inclusion criteria (Figure 1). Among these, 16 were animal-based and the rest were clinical and ex-vivo studies conducted on humans and isolated tissues. In this study, we extracted data on the role of Rho-kinase in the pathogenesis and/or novel therapeutic target for the treatment of ED.

Among the included articles, five articles describe the role of Rho-kinase in ED pathogenesis using various models including cavernous nerve (CN) crush injury, heart failure (HF)-induced ED, Vasculogenic and post-radical prostatectomy (RP) ED, diabetes-induced ED and age-related ED.[17,33,36] Ten papers describe the role of novel drugs which have been tested for the treatment of ED by targeting Rho-kinase as a potential therapy for ED.[19,20,37,45] The remaining three papers describe the role of traditionally claimed plants for the treatment of ED and evaluated for their potential role in targeting Rho-kinase in animal models.[21,46,47] The quality score of the included papers were ranged from 5 to 9 with an average mean articles quality score of 7.39 ± 0.31 (Table 2).

RhoA, ROCK I, and ROCK II expression were increased in different scenarios. ROCK II (Rho-kinase) is majorly expressed in the pathogenesis of ED. According to Alves-Lopes et al (2016), high glucose-induced ED secondary to DM increases levels of nitrotyrosine, protein oxidation/carbonylation, and Rho-kinase activity.[34] Ageing has also been reported to increase the risk of developing ED as ICP/MAP ratio decreases with age. A related study showed a marked decline in eNOS protein expression with age, while the Rho-kinase protein level was increased and, in particular, the eNOS/Rho-kinase ratio decreased with age.[35] Involvement of Rho-kinase in ED pathogenesis was also evaluated by animal model with HF.[17] The results showed that the rats developed ED after ligation of the left anterior descending coronary artery, as demonstrated by decreased ICP/MAP responses. In addition, ROCK II and MYPT-1 phosphorylation rates increased in cavernous tissue (Table 3).[17]

Song et al (2015) also suggested that ROCK I has been involved in ED pathogenesis. In this study,[33] effects in LIMK2/cofilin pathway (downstream effectors of ROCK I) following bilateral CN injury in male rats were evaluated. It

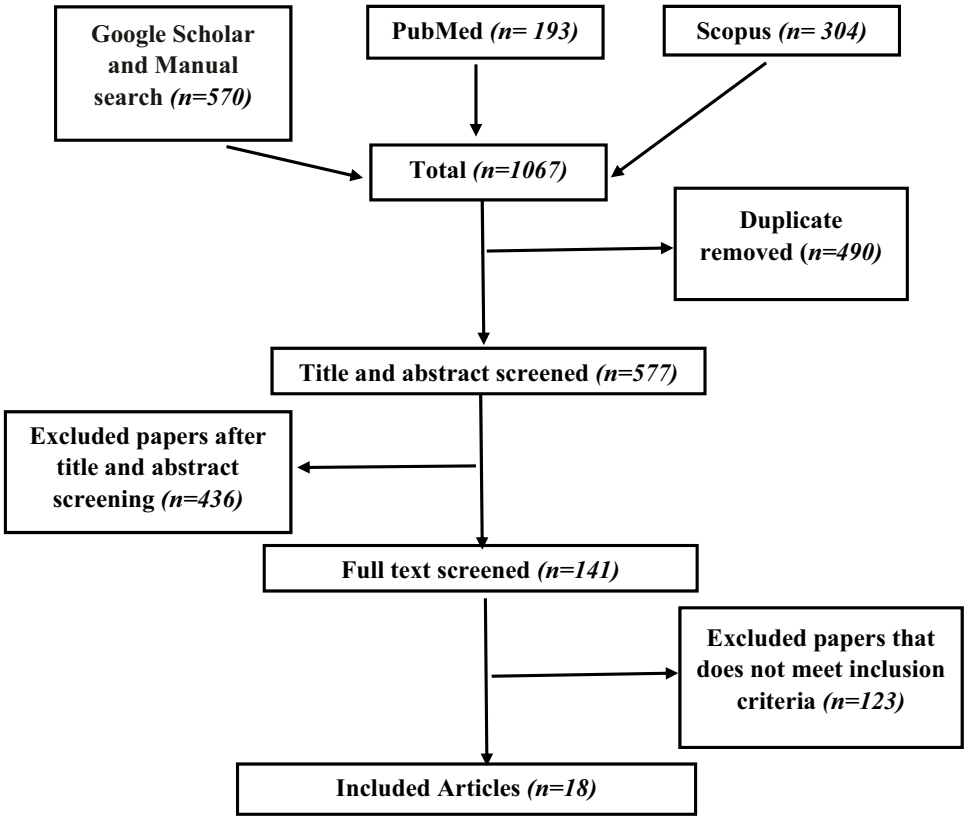

Figure 1 Schematic representation of the data extraction process.

has shown that after one week of CN injury, an upregulation of ROCK I/LIMK II/cofilin pathway was accompanied by impaired erectile response and corporal fibrosis.[33]

Ten novel drugs have been tested for their Rho-kinase inhibitory activity (Table 4). An ex-vivo study conducted by Uvin et al (2017) showed that the additive effects of the Rho-kinase inhibitor Y-27,632 and vardenafil on the relaxation of the corpus cavernosum (CC) tissue of patients with ED and clinical phosphodiesterase type 5 (PDE-5) inhibitor failure were assessed. According to their study, Y-27,632 induces major relaxation of CC in tissue strips of patients with severe ED. Besides, combination therapy has shown that mutual inhibition of Rho-kinase and PDE-5 can be a successful agent in the treatment of severe ED.[37]

Cui et al (2017a) assessed the function of human tissue kallikrein1 (hKLK1) as an inhibitor of Rho-kinase/LIM-kinase/cofilin pathway in aged transgenic rats. According to this study,[41] after ED had tested and validated in older rats, hKLK1 administration has shown to reduce corporal fibrosis by inhibiting Rho-kinase activation and ameliorate age-related ED.[41] Another study was also done to assess the role of fasudil (Rho-kinase inhibitor) in the treatment of ED in the CN crush injured rat model.[42] Results from this study showed increased phosphorylation of myosin phosphatase (MYPT-1) and increased ROCK II expression in the injured groups. Inhibition of Rho-kinase in the injury plus fasudil group restored erectile responses and dynamic infusion cavernosometry parameters by alleviating increased apoptosis, decreased immune histochemical staining of alpha-smooth muscle actin (α-SMA) and increased caspase-3 activity. Histological and molecular dysregulations have been also alleviated by inhibition of Rho-kinase plus fasudil groups.[42]

β_3-adrenoceptor activator (Mirabegron) was also assessed for the treatment of ED by using clinical and animal models.[45] The CC specimens were collected from patients with Peyronie's disease and ED undergoing penile prosthesis. Besides, erectile responses were evaluated in-vivo after intracavernosal injection (ICI) of mirabegron in anaesthetized rats. Studies have shown that mirabegron induces relaxation of phenylephrine-evoked CC contractions in a concentration-dependent manner by activating β_3-adrenoceptors independently of the NO-cGMP pathway and it is hypothesized that there is a near functional link between β_3-adrenoceptors and the RhoA/ROCK pathway.[45]

Table 2 The Quality Assessment of Individual Study Obtained According to Modified CAMARADES Checklist Items

References	I	II	III	IV	V	*VI*	VII	VIII	IX	X	Total (of 10)
33	1	0	1	1	1	0	1	1	1	1	8
17	1	0	1	1	0	0	1	1	1	1	7
34	1	0	1	1	0	0	0	1	1	1	6
35	1	1	1	1	0	0	1	1	0	1	7
36	1	1	0	1	0	0	1	1	0	1	6
37	1	1	0	1	0	0	1	1	1	1	7
41	1	1	1	1	0	0	1	1	1	1	8
19	1	1	0	1	0	0	0	1	1	1	6
44	1	1	1	1	1	0	1	1	0	1	8
40	1	1	1	1	1	0	1	1	1	1	9
38	1	1	1	1	1	0	1	1	1	1	9
42	1	1	1	1	1	0	1	1	1	1	9
43	1	1	0	1	1	0	1	1	1	1	8
20	1	1	1	1	1	0	1	1	1	1	9
45	1	0	1	1	0	0	1	1	1	1	6
46	1	0	1	1	0	0	1	1	0	1	6
47	1	1	1	1	1	0	1	1	1	1	9
21	1	0	0	1	0	0	1	1	0	1	5

Notes: I: Publication in a peer-reviewed journal, II: Number of experimental and control groups, III: Housing and Husbandry Conditions, IV: Details of intervention/exposure group procedures, V: Random allocation to groups, VI: Concealment of allocation, VII: Blinded assessment of outcomes, VIII: Biochemical evaluations, IX: Histopathological evaluations, X: Statistical analysis 1: Criterion is satisfied, 0: Insufficiently described or not explained at all; the mean quality score is expressed as mean ± standard error of the mean (SEM) and (minimum and maximum score).

Various plant extracts have been evaluated for the treatment of ED and their function in inhibiting Rho-kinase in animal models (Table 5). Eupatilin (the main compound present in *Artemisia* species) was assessed for its relaxant effect in-vitro and protein expression. The finding indicates Eupatilin effectively relaxed the phenylephrine-induced tone in the rabbit CC strips in a concentration-dependent manner. The relaxing effect of Eupatilin on CC smooth muscle cells may be due to activation of BK_{Ca+2} channels and inhibition of RhoA/Rho-kinase.[46] Ye et al (2019) have also reported that the effect of HongJing I (HJI), a traditional Chinese herbal remedy, in ED. Once ED was induced by bilateral cavernous nerve injury (BCNI) in rats, ICP was recorded, and histological examination was done. Results showed that RhoA, ROCK I, and ROCK II expression levels were increased with BCNI-ED induction, while HJI successfully inhibited cavernosum fibrosis and RhoA/ROCK II signal activation.[47]

Discussion

ED has proved to be a significant health issue for elderly's and persons with chronic diseases. It is prevalent in people older than 40 years of age and affects their quality of life.[23,26] Penile erection is a complex process in which flaccidity/rigidity within it is regulated by a balance between contractile and relaxing effects.[48] Erection requires neutrally mediated relaxation of arteriolar smooth muscle and engorgement of cavernosal tissues. Current medical therapies for ED mainly perform by maximizing endogenous NO signaling. However, certain etiologies, including diabetes, are difficult to treat with current modalities.[49] In addition, they are also related to increased side effects and cost.[24] Therefore, the search for a new target and/or potentially effective new drugs with decreased side effects are important. Recently, research has demonstrated the importance of ROCK signaling in maintaining a flaccid penile state, and inhibition of ROCK signaling potentiates smooth-muscle relaxation in a NO-independent manner.

Rho-Kinase Signaling on the Pathophysiology of Erectile Dysfunction

The Rho/Rho-kinase pathway plays an important role in pathophysiology and progression of various diseases.[10] Altered RhoA/ROCK activity in the penis is a pathogenic factor contributing to ED development.[50] As shown in Figure 2, RhoA and its effector Rho-kinase have been noticed for facilitating vasoconstriction activity in the penis through inhibition of MLCP, thereby increases MLC phosphorylation and increase Ca^{2+} sensitivity.[18,31,33,48] Ca^{2+} influx into cells increases intracellular Ca^{2+} that is available to bind to calmodulin. This binding causes a conformational change and enable

Table 3 Role of Rho Kinases (ROCK) in the Pathogenesis of Erectile Dysfunction

Model and Method Used	Intervention and Process	Treatment Outcomes and Conclusion	References
Bilateral CN crush injury in 10-weeks old male Sprague Dawley (SD) rats.	Electro-stimulation was performed to assess erectile function. Penile tissue was processed for Masson's trichrome staining, Western blot and double immunofluorescent staining.	A significantly lower percent of ratio of maximal ICP/MAP and areas under the ICP curve to MAP (AUC/MAP). A significantly higher expression of ROCK I/LIMK II in injured groups as compared with controls. Conclusion: The ROCK I/LIMK2/cofilin pathway may be involved in the ED.	[33]
Ligation of the left anterior descending coronary artery (HF) induced ED in rats	Electro-stimulation was performed to assess erectile function. RhoA, Rho-kinase II (ROCK II) and myosin phosphatase target protein 1 (MYPT-1) protein expression and phosphorylation levels were determined by Western blot analysis.	HF rats display impaired erectile function represented by decreased ICP/MAP responses. HF decreased RhoA protein expression, but increased ROCK II and MYPT-1 phosphorylation levels in cavernous tissue. Conclusion: Increased responses in cavernosal tissue, suggesting the involvement of ROCK signaling pathway in ED genesis.	[17]
Streptozotocin (STZ) induced DM on C57BL/6 and NOX1 knockout mice.	DM was induced by STZ. Functional properties of internal pudendal arteries (IPA) were assessed using a myograph, protein expression and peroxiredoxin oxidation by Western blot RNA expression by a polymerase chain reaction.	IPA from diabetic mice displayed increased contractions to phenylephrine. High glucose increased ROS generation in IPA vascular smooth muscle cell. High glucose increased levels of nitrotyrosine, protein oxidation/carbonylation, and Rho kinase activity Conclusion: Rho-kinase activation, via NOX1-derived ROS and downregulation of Nrf2 system, impairs IPA function in DM.	[34]
Age-associated ED was assessed in SD rats.	Erectile response measurements were performed by ICP/MAP ratio. Detection of Rho-kinase and eNOS protein was done by Western blot analysis. Finally, correlation analyses of the association between ICP/MAP and Rho-kinase, eNOS, or eNOS/Rho-kinase, as well as between age and eNOS or Rho-kinase, were performed.	The functional index ICP/MAP decreased with age in SD rats. The expression of eNOS protein decreased, while Rho-kinase expression increased. (eNOS/Rho-kinase) ratio decreased with age. eNOS was found to be significantly negatively correlated with age, whereas Rho-kinase was positively correlated with age. Conclusion: Age-associated ED was therefore correlated with decreased eNOS and increased Rho-kinase.	[35]
Vasculogenic and post-radical prostatectomy induced ED in human subjects	Samples were collected from individuals who undergo penile surgery for Peyronie's disease and from men with ED who underwent penile prosthesis implantation. ED was categorized into vasculogenic and post-RP subtypes. Penile erectile tissue samples were collected for molecular analyses of protein expressions of nNOS and eNOS, (Ser-1412, (Ser-1177), (Ser-473), PDE5, a-smooth muscle actin phosphomyosin phosphatase target subunit 1, RhoA/ROCK-I/ROCK-II by Western blot.	Vasculogenic ED was characterized by decreased eNOS protein expression and eNOS and eNOS phosphorylation on their activatory sites (Ser-1177 and Ser-1412, respectively), uncoupled eNOS, upregulated PDE5 protein expression, increased ROCK activity, and increased oxidative stress in erectile tissue. Post-RP ED was characterized by decreased eNOS protein expression, increased eNOS phosphorylation on its activator site (Ser-1412), uncoupled eNOS, down-regulated PDE5 protein expression, and increased oxidative stress in erectile tissue.	[36]

Abbreviations: CN, cavernous nerve; DM, diabetes mellitus; ED, erectile dysfunction; eNOS, endothelial nitric-oxide synthase; ICP, intracavernosal pressure; MAP, mean arterial pressure; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; ROCK, Rho-associated coiled-coil-forming protein kinase.

Table 4 Role of Rho Kinases (ROCK) Inhibitors as a Novel Therapeutic Agent for the Treatment of Erectile Dysfunction

Model/Method	Intervention and Process	Treatment Outcomes and Conclusion	References
Relaxation of CC tissue on patients with ED after penile prosthesis implantation.	Human CC samples were obtained from individuals undergoing penile prosthesis implantation. qPCR was performed for the expression of RhoA and ROCK subtypes 1 and 2. Immunohistochemistry staining against ROCK and α smooth muscle actin (α-SMA) was performed on CC of an ED patient. Effect of Y-27,632 and vardenafil were assessed.	The expression of ROCK I was unchanged, while ROCK II was significantly upregulated in ED patients. After incubation with DMSO, Y-27,632 relaxed pre contracted tissues with 85.9 ± 10.3% ($p = 0.0016$ when compared to vehicle). Additive effects on the relaxation of human CC were seen after pre-incubation with 1μM vardenafil.	[37]
DM-induced ED (DMED) in rats	Type 1 DM was induced by STZ. Eight weeks later, the erectile function of rats was assessed with an Apomorphine test. Role of FTY720 was assessed Metabolic parameters; erectile function; sphingosine-1-phosphate receptor 3 (S1P3), protein kinase B (Akt), nitric oxide (NO), and cGMP signaling pathway; corporal fibrosis; apoptosis level; and Smad and non-Smad signaling pathways were assessed.	Erectile function in the DMED group was significantly impaired but significantly, improved in the DMED+ FTY720 group. The DMED group showed inhibited activity of the S1P3-Akt-NO-cGMP signalling pathway, and the inhibition was partly reversed in the DMED + FTY720 group. The DMED group showed serious corporal fibrosis, higher apoptosis level, a higher level of Rho-kinase, LIM domain kinase 2, and cofilin. FTY720 supplementation partly inhibited the activity of Rho-kinase, LIM domain kinase 2 (the Smad and non-Smad pathways).	[38]
DM-induced ED (DMED) in rats	After grouping DM was induced in rats via STZ Eight weeks after DM induction, rats with ED were selected via an Apomorphine test. JTE-013 was administered intraperitoneally for treatment groups. The rest were fed under the same condition. Erectile function was measured by CN electro stimulation. The expression levels of related signaling pathways were evaluated using Western blotting, real-time PCR and immunohistochemistry.	Erectile function was significantly impaired in the DMED group and was partially improved in the DMED + JTE-013 group. The expression RhoA/ROCK/p-MYPT1 pathway proteins were higher in the DMED group and JTE-013 treatment significantly reduced the expression/activity of these proteins. Conclusion: JTE-013 supplementation significantly ameliorated these pathological changes	[19]
Clinical and DM-induced ED (DMED) in rats	DM was induced by administering STZ. Apomorphine was used to assess erection. Role of microRNA-141 was assessed Hematoxylin-eosin (HE) staining and Masson staining, Immunohistochemistry, Quantitative real-time polymerase chain reaction qRT-PCR, cell culture and western bloating	The mRNA and protein expressions of RhoA and ROCK II were significantly increased while the expression of microRNA-141 was decreased in the penile tissues. The microRNA-141 expression in the microRNA-141 inhibitors + siRNA-Rho group was significantly decreased. MicroRNA-141 specifically bound to Rho-3′-UTR and down-regulated the expression of the Rho gene at the post-transcriptional level.	[20]
Partial bladder outlet obstruction (PBOO) in a rat model.	After animals were grouped, PBOO was induced by ligation of the urethra for 6 weeks. In vivo, erectile responses were monitored by evaluating ratios of ICP/MAP ratio. Organ-bath studies were performed on CC strips. Penises were assessed at baseline for protein expression of eNOS and ROCK-II by Western blot.	The ratio of ICP/MAP was significantly decreased in obstructed rats, which was restored after treatment eNOS expression in the obstructed group decreased, which was improved by treatment. However, there was no significant difference in the protein levels of ROCK II between groups.	[40]

(*Continued*)

Table 4 (Continued).

Model/Method	Intervention and Process	Treatment Outcomes and Conclusion	References
Age-associated corporal fibrosis in SD rats.	Effect of human tissue kallikrein 1 (hKLK1) on TGR harboring the hKLK1 gene was fed to 4- or 18-month-old rats and divided into three groups: young WTR (yWTR) as the control, aged WTR (aWTR), and aged TGR (aTGR). The erectile function of all rats was assessed by CN electrostimulation method. Masson's trichrome staining and Western blotting were used to evaluate corporal fibrosis in the CC.	The erectile function of rats in the aWTR group was significantly lower than the other two groups. Immunohistochemistry and Western blotting showed that expression of transforming growth factor-β 1 (TGF-β1), RhoA, ROCK I, p-MYPT1, p-LIMK2, and p-cofilin were higher in the aWTR group and improved in hKLK1 treated groups. Conclusion: hKLK1 may reduce this corporal fibrosis by inhibiting the activation of Rho-kinase.	41
CN-crush injury in male SD rats.	Animals were grouped into 3 groups, including sham surgery, CN crush injury and CN- crush injury treated with fasudil. Effect of Fasudil was assessed Electro stimulation and dynamic infusion cavernosometry were performed postoperatively. Penile tissue was processed for immunohistochemistry, double immune fluorescent and Masson trichrome staining, TUNEL, caspase-3 activity assay and Western blot.	The CN crush injury group showed significantly lower ICP/MAP. Rho-kinase inhibition in the injury plus fasudil group restored erectile responses. Conclusion: Rho-kinase inhibition in the injury plus fasudil group alleviated the histological and molecular dysregulation.	42
Methylglyoxal (MGO) administered male rats.	The effect of exendin-4 (Ex-4) treatment on CC dysfunction was assessed. Animals were grouped into four groups as control, MGO, MGO + low-dose Ex-4 and MGO + high-dose Ex-4. Endothelial nitric oxide synthase (eNOS), phosphorylated eNOS (p-eNOS), NADPH oxidase subunit gp91phox (NOX2), and Rho kinase (ROCK II) expressions in CC were investigated by immunohistochemistry.	In MGO administered rats, both endothelium-dependent and neurogenic CC relaxations were significantly impaired. The diminished response was significantly improved by exendin-4 treatment. Conclusion: Exendin-4 treatment improves NO-mediated CC relaxations in MGO administered rats probably by inhibiting NADPH oxidase.	43
DM induced ED in a rat model.	Role of angiotensin (Ang) II inhibitor (short hairpin RNA (shRNA)) was assessed. ICP/MAP was measured after electrical stimulation. Western blotting and quantitative RT-PCR were applied to measure the expressions of RhoA, ROCK-I and II. Radioimmunoassay was applied to detect the levels of Ang II.	Rats with DMED had worse ICP and MAP than Ang-II-silenced rats. Prolonged erectile time had shown in Ang-II-silenced rats. The contraction ability was markedly improved and relaxation ability was decreased in Ang-II-silenced rats. The mRNA and proteins of RhoA and ROCK II were expressed in a similar way. Conclusion: Ang-II silencing improves ED via down-regulating the RhoA/Rho-kinase signaling pathway.	44
Patients who had overactive bladder (OAB) and ED and Mirabegron induced CC relaxation in animal models	CC specimens were obtained from patients with ED and Peyronie's disease undergoing penile prosthesis implantation. Erectile responses were evaluated in vivo after intracavernosal injection (ICI) of mirabegron in anaesthetized rats. Mirabegron-elicited relaxation responses on phenylephrine-induced contraction were seen in human CC (HCC) and rat CC strips in isolated organ bath studies. The effects of inhibitors, namely L-NAME, ODQ, methylene blue, SR59230A and fasudil on mirabegron-induced relaxation, responses were evaluated. Immunohistochemistry was used to localize b3-adrenoceptors and ROCK in CC smooth muscle cells.	Mirabegron resulted in a relaxation of phenylephrine evoked CC and SR59230A antagonized the mirabegron induced relaxations in HCC and rat CC. Mirabegron relaxation was enhanced by fasudil (ROCK inhibitor) in rat but not in HCC strips. Immunohistochemistry data showed b3-adrenoceptors localized in the smooth muscle cells of the HCC and rat CC.	45

Abbreviations: CN, cavernous nerve; CC, corpus cavernosum; DM, diabetes mellitus; ED, erectile dysfunction; eNOS, endothelial nitric-oxide synthase; ICP, intracavernosal pressure; MAP, mean arterial pressure; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; ROCK, Rho-associated coiled-coil-forming protein kinase; STZ, streptozotocin.

Table 5 Traditional Plants Used as Rho Kinases (ROCK) Inhibitors as a Therapeutic Agent for the Treatment of Erectile Dysfunction

Model/Method	Intervention and Process	Treatment Outcomes/Conclusion	References
The ex-vivo activity was done on the isolated CC of rabbits.	Isolated rabbit CC strips were mounted in an organ bath system. Effect of Eupatilin (the main compound present in *Artemisia* species) A conventional whole-cell patch-clamp technique was used to measure activation of calcium-sensitive K +-channel currents in human corpus cavernously smooth muscle (CCSM) cells. The relaxation effect of Eupatilin was evaluated by cumulative addition to CC strips precontracted with phenylephrine. Western blotting analysis was performed to measure MYPT1 and protein kinase C and to evaluate the effect of Eupatilin on the RhoA/Rho-kinase pathway.	Eupatilin effectively relaxed the phenylephrine-induced tone in the rabbit CC. Eupatilin reduced the phosphorylation level of MYPT1 at Thr853 of MLCP and CPI-17 at Thr38. Eupatilin-induced relaxation of the CCSM cells via NO-independent pathways. Conclusion: The relaxation effects of eupatilin on CCSM cells were partially due to activation of Ca^{+2} activated K^{+} (BK_{Ca+2}) channels and inhibition of RhoA/Rho-kinase	[46]
Bilateral CN injury (BCNI) induced ED in a rat model.	Effect of HongJing I (HJI) was assessed Rats were divided into five groups: normal control (NC), BCNI-induced ED model (M), M + low-dose HJI (HL), M + medium-dose HJI (HM), and M + high-dose HJI (HH). All groups were treated with normal saline or the relevant drug for 28 days after inducing BCNI-ED. Finally, ICP was recorded, Immunofluorescence staining and Western blotting were applied to detect the changes in fibrosis protein and RhoA, ROCK I, and ROCK II expression.	In addition, RhoA, ROCK1, and ROCK2 expression levels were increased upon BCNI-ED induction. HJI effectively improved the ICP in the treatment groups. HJI successfully inhibited cavernosum fibrosis and the activation of RhoA/ROCK2 signaling. Overall, these results suggest that the effects of HJI in attenuating ED may be caused, at least in part, by the suppression of RhoA/ROCK II signaling and alleviation of fibrosis.	[47]
In vitro, ROCK II Kinase assay was done.	Effect of *Eurycoma longifolia* (EL) Jack and Y- 27,632 [(+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4 pyridyl) cyclohexane carboxamide dihydrochloride] was used as standard drug. Luminescence was recorded using Topotecan, USA, Spark 10 M, multimode microplate reader. Standard curve for ROCK-II enzyme was done. Serial dilution and IC 50 of the AE was performed in triplicate	EL and Y-27,632 as a standard showed a significant inhibition for ROCK-II activity. The inhibition of ROCK-II activity at $P < 0.001$. IC 50 in ROCK-II inhibition assay of EL (651.1 ± 32.9 ng/mL). The traditional use of *E. longifolia* as aphrodisiac and for male sexual disorders might be partially due to the ROCK-II inhibitory activity.	[21]

Abbreviations: CCSM, corpus cavernously smooth muscle; CN, cavernous nerve; DM, diabetes mellitus; ED, erectile dysfunction; ICP, intracavernosal pressure; MAP, mean arterial pressure; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; ROCK, Rho-associated coiled-coil-forming protein kinase.

complex with MLC kinase. After subsequent phosphorylation, the myosin–actin complex resulting smooth muscle contraction and a flaccid penis.[31,48] In addition, upregulation of RhoA/ROCK suppresses endothelial nitric oxide synthase (eNOS) activity and it is the expression in the penis (Figure 2). Proteomics analysis on Western blot has shown that the ratio of eNOS/Rho-kinase decreased significantly due to ageing, DM and various chronic diseases.[33,35,51]

Effect of Rho-Kinase Inhibition on Erectile Dysfunction

The potential effect of RhoA/Rho-kinase mediated Ca^{2+} sensitization pathway in ED pathogenesis provided a logical pharmacological target for clinical application.[15] Studies have demonstrated that inhibition of tonic contraction of corporal smooth muscle by intracavernosal injection or topical application of Rho-kinase inhibitors to the penis results in increased blood flow into erectile tissue and causing an erection.[31] Rho-kinase inhibition restored erectile responses and dynamic infusion cavernosometry parameters by alleviating increased apoptosis, decreased immune histochemical staining of α-SMA and increased caspase-3 activity in a NO-independent manner. Moreover, histological and molecular dysregulation were alleviated by Rho-kinase inhibition,[14,19,41,42] which might improve its efficacy as compared with the currently available anti-ED drugs. However,

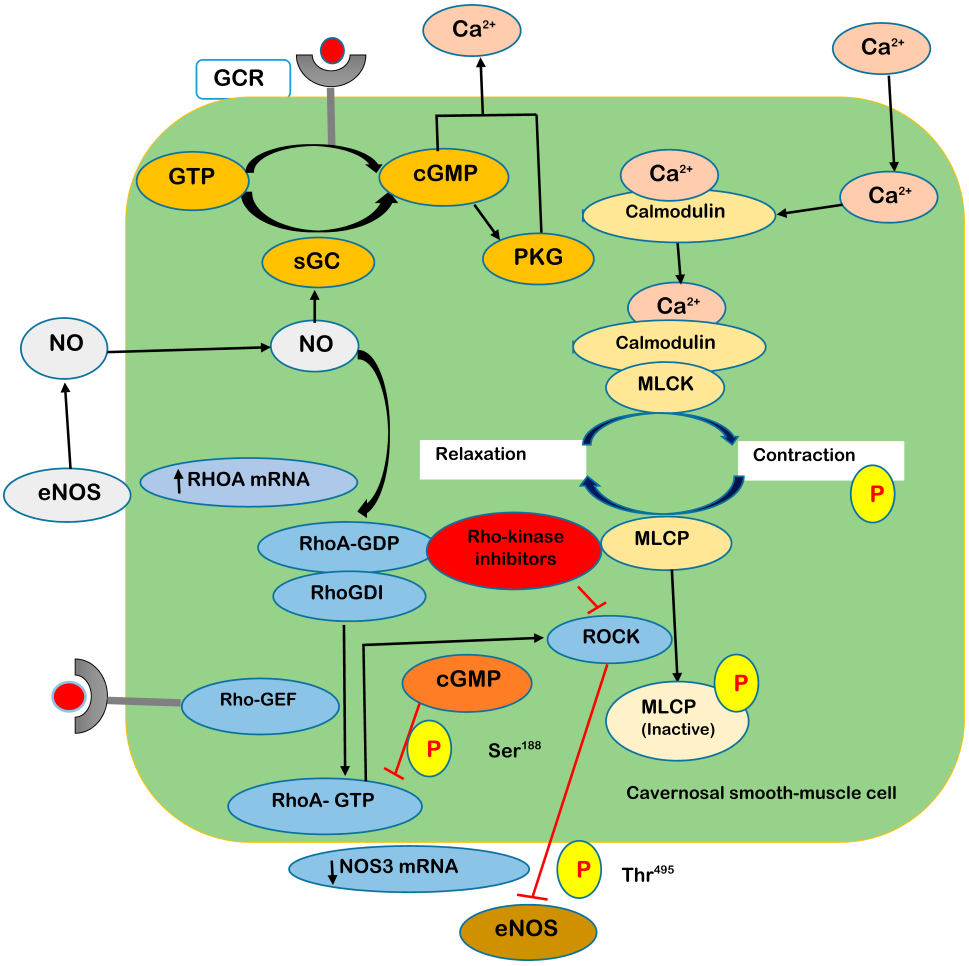

Figure 2 RhoA/Rho-kinase signaling pathway for Ca^{2+} sensitization in cavernosal smooth-muscle cells.

some findings showed that the vasorelaxant effect induced by the administered drugs was not mainly by the involvement of Rho-kinase inhibition, rather, by improving NO-mediated CC relaxations probably by inhibiting NADPH oxidase.[43] Likewise, similar changes were noticed in rats with CN injury by involving TGF-β- mediated fibrosis.[19]

Strengths and Limitations

To the best of our knowledge, this is the first systematic review that gives insight for an alternative treatment option, targeting Rho-kinase as a potential therapeutic option in the treatment of ED. However, our review did not conduct statistical analysis due to the heterogeneous nature of the included studies. Moreover, most of the reviewed papers are animal studies, which makes it difficult to conclude the models have been addressed all the pathogenic mechanisms for ED. So, further in-depth studies will be required to investigate their role in ED pathogenesis and to elucidate mechanisms for Rho-kinase inhibitors.

Conclusion

Overall, our study indicates Rho-kinase can be a potential target for the treatment of ED secondary to different causes. Recently, animal and clinical studies proved that there is an up-regulation of Rho-kinase (especially ROCK II) in ED. Rho-kinase inhibitors improve erectile function in a NO-independent manner and might be a new drug family for the treatment of ED. However, more information and further detailed understanding are needed to show the ultimate effects on health outcome.

Abbreviations

α-SMA, alpha-smooth muscle actin; CC, corpus cavernosum; CN, cavernous nerve; DM, diabetes mellitus; ED, erectile dysfunction; eNOS, endothelial nitric oxide synthase;

hKLK1, human tissue kallikrein1; HF, heart failure; ICP, intracavernosal pressure; NO, nitric oxide; MAP, mean arterial pressure; MLC, myosin light chain; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; MYPT, myosin phosphatase regulatory; ROCK, Rho-associated coiled-coil-forming protein kinase; STZ, streptozotocin.

Author Contributions

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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