Low Intensity Extracorporeal Shock Wave Therapy Improves Erectile Function in a Model of Type II Diabetes Independently of NO/cGMP Pathway

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Abbreviations and Acronyms

ACh = acetylcholine
cGMP = cyclic guanosine monophosphate
CRC = concentration-response curve
ED = erectile dysfunction
EFS = electrical field stimulation
GK = Goto-Kakizaki
ICP = intracavernous pressure
Li-ESWT = low intensity extracorporeal shock wave therapy
MAP = mean arterial pressure during plateau phase
NO = nitric oxide
NOS = NO synthase
PDE5-I = phosphodiesterase type 5 inhibitor
PHE = phenylephrine
SNP = sodium nitroprusside
T2DM = type II diabetes mellitus

Purpose: Erectile dysfunction is highly prevalent in type II diabetes mellitus. Low intensity extracorporeal shock wave therapy improves erectile function in patients with erectile dysfunction of vasculogenic origin, including diabetes. However, its mode of action remains unknown. We investigated the effects of low intensity extracorporeal shock wave therapy compared to or combined with sildenafil on erectile dysfunction in a type II diabetes mellitus model. Our purpose was to test our hypothesis of a mode of action targeting the cavernous nitric oxide/cyclic guanosine monophosphate pathway.

Materials and Methods: GK rats, a validated model of type II diabetes mellitus, and age matched Wistar rats were treated with low intensity extracorporeal shock wave therapy twice weekly for 3 weeks. Treatment was repeated after a 3-week no-treatment interval. The penis was stretched and dipped in a specifically designed water-filled cage. Shock waves were delivered by a calibrated probe yielding a controlled energy flux density (0.09 mJ/mm^2). The probe was attached to an electrohydraulic unit with a focused shock wave source, allowing for accurate extrapolation to humans. Following a 4-week washout period erectile function was assessed as well as endothelium dependent and independent, and nitrergic relaxations of the corpus cavernosum of GK rats.

Results: Low intensity extracorporeal shock wave therapy significantly improved erectile function in GK rats to the same extent as sildenafil. Treatment effects were potentiated when combined with sildenafil. Shock wave effects were not associated with improved cavernous endothelium dependent or independent, or nitrergic reactivity.

Conclusions: Low intensity extracorporeal shock wave therapy improved erectile function in GK rats. Unexpectedly, this was not mediated by a nitric oxide/cyclic guanosine monophosphate dependent mechanism. Sildenafil increased shock wave efficacy. This preclinical paradigm to deliver low intensity extracorporeal

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shock wave therapy to the rat penis should help further exploration of the mode of action of this therapy on erectile tissue.

Key Words: penis, erectile dysfunction, ultrasonic waves, sildenafil citrate, diabetes mellitus

MEN with diabetes, particularly those with T2DM, show a threefold increase in the incidence of ED compared to men without diabetes.[1,2] ED manifests 10 to 15 years earlier in men with diabetes.[1,3] Endothelial dysfunction represents a unifying alteration in the pathogenesis of cardiovascular diseases, diabetes and ED.[4,5]

PDE5-I is first line therapy of ED whatever the etiology.[6] PDE5-I blocks degradation of cGMP in the smooth muscle cells of erectile tissue while cGMP is synthetized in response to the release of NO by endothelial cells and neural terminations in response to sexual stimulation.[7] Consequently, to exert a clinically beneficial effect in patients with ED PDE5-I requires a sufficient NO drive. However, NO release of neuronal and endothelial origin is impaired in diabetes.[8,9] Thus, diabetic patients often show a poor response or become refractory to PDE5-I with time.[6,10] In addition, whatever the PDE5-I dosing regimen used, ie on demand or daily for tadalafil, the effects are symptomatic with no disease modifying effect on the restoration of erectile function.

Recently, importance has been given to future treatment strategies that could restore erectile function. Li-ESWT is high pressure acoustic waves that propagate through a medium and can be targeted and focused noninvasively to affect a distant select anatomical region.[11] Pioneer clinical pilot studies suggested that Li-ESWT could improve erectile function and penile hemodynamics in men with ED who are responders to PDE5-I[12–14] or even convert PDE5-I nonresponders to responders.[15,16] While these results are encouraging, the basis for this new treatment modality has been empirical, which could in theory be optimized and better understood if a relevant preclinical model becomes available.

The beneficial use of Li-ESWT has been shown in the penis of rats with streptozotocin induced type I diabetes. Li-ESWT was found to promote the endothelial NOS phosphorylation state and regenerate neuronal NOS positive nerves, endothelium and smooth muscle, thereby suggesting that Li-ESWT targets the NO/cGMP signaling pathway.[17,18] However, to our knowledge it remains to be determined whether these described effects on protein expression have any functional relevance.

Our aim was to further characterize the therapeutic effects and the mechanism of action of Li-ESWT in diabetes associated ED. This study was designed to 1) develop a relevant preclinical methodology by adapting the machine to deliver Li-ESWT and treatment modalities to rat erectile tissue, allowing extrapolation of findings to humans with adequate mimicking of what has been performed in patients with ED,[11–13] 2) provide evidence of the effect of Li-ESWT in GK rats, a robust experimental model of T2DM associated ED in this validated experimental setting,[17,19] and 3) evaluate whether the therapeutic effect of Li-ESWT could exert a functional effect on the NO/cGMP pathway by studying endothelium dependent, independent and nitrergic relaxations of cavernous strips from the same diabetic GK rats.

MATERIALS AND METHODS

Animals and Experimental Design

Six-week-old Wistar rats and age matched GK rats (Metabrain Research, Chilly-Mazarin, France) were randomly distributed into 5 experimental groups, including 1) 13 Wistar control rats without Li-ESWT, 2) 12 GK rats without Li-ESWT, 3) 12 GK rats with Li-ESWT, 4) 12 GK rats with acute sildenafil (0.3 mg/kg) but without Li-ESWT and 5) 12 GK rats with acute sildenafil (0.3 mg/kg) and Li-ESWT. The rats had free access to standard chow and water.

All procedures were performed in compliance with legislation on the use of laboratory animals (National Institutes of Health Publication No. 85-23, 1996) and French Animal Care Regulations (French Ministry of Agriculture, Agreement No. A78-423-01, 2013). The study was reviewed and approved by the local ethics committee under the supervision of the Ministry of Research (Ethics Committee No. 47).

Li-ESWT Treatment Protocol

Animals received 2 sessions of Li-ESWT per week for 3 weeks, which was repeated after a 3-week no treatment interval (fig. 1). Shock waves were delivered by a calibrated probe yielding a controlled energy flux density of 0.09 mJ/mm^2 attached to an Omnispec ED1000™ compact electrohydraulic unit with a focused shock wave source. To facilitate coverage and transmission of shock waves the penis of each anesthetized rat was manually stretched and dipped in a specifically designed water-filled tank. Following a 4-week washout period erectile function was assessed by electrical stimulation of the cavernous nerve in rats under anesthesia (fig. 2).

Erectile Function Evaluation by Cavernous Nerve Electrical Stimulation

Erectile responses were tested using a previously described, well standardized procedure.[20] Rats were

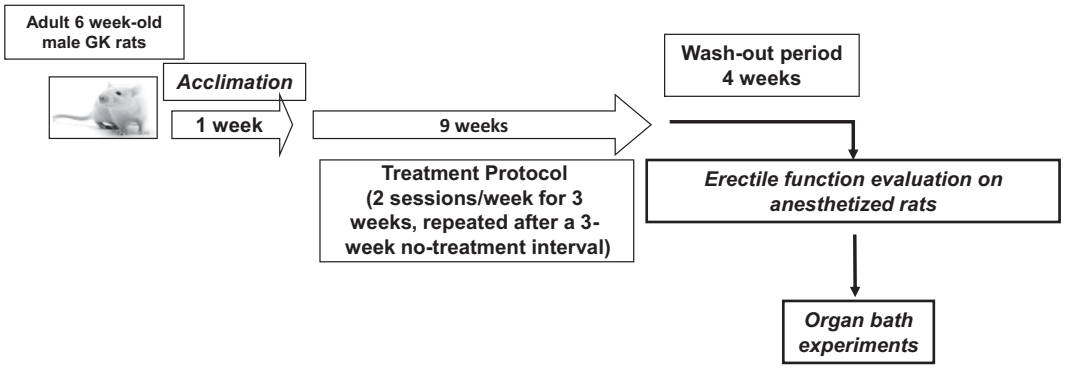

Figure 1. Treatment protocol design. At 1 week of acclimation type II diabetic GK rats were treated with Li-ESWT twice weekly for 3 weeks. Treatment was repeated once after 3 weeks. In vivo erectile function experiments were done in anesthetized rats after 4-week washout after last Li-ESWT session. Rats were sacrificed for further ex vivo experiments.

anesthetized (urethane 1.2 mg/kg), tracheotomy was performed and temperature was maintained at 37C. Catheters were inserted in the carotid artery and the corpus cavernosum to record blood pressure via an Elcomatic 750 pressure transducer (Elcomatic, Irvine, United Kingdom). The cavernous nerve was exposed at the lateral aspect of the prostate with the aid of a dissecting microscope and mounted on a bipolar platinum electrode connected to an AMS 2100 electrical stimulator (Phymep, Paris, France). Sildenafil (0.3 mg/kg), a previously determined dose known to improve erectile responses without introducing a confounding blood lowering effect,[19] or vehicle was then intravenously injected. Exactly 4 minutes later the cavernous nerve was stimulated (6 V and 1 millisecond to 45 seconds) at different frequencies (0, 2.5, 5, 7.5, 10, 12.5 and 15 Hz) at 3-minute intervals in a randomized manner to assess erectile responses. Each cavernous nerve electrical stimulation was repeated twice to establish a frequency-response curve for each animal.

Erectile responses to cavernous nerve electrical stimulation are expressed as the ratio, ΔICP in mm Hg/MAP in mm Hg × 100 with ΔICP representing the difference between ICP in the flaccid state, ie before stimulation, and ICP during the plateau phase of the erectile response, and MAP representing MAP during the plateau phase. In the ratios AUC_{tot}/MAP and AUC_{45}/MAP AUC_{tot} and AUC_{45} represent the AUC measured during the entire erectile response and during the first 45 seconds after the beginning of electrical stimulation, respectively.[21]

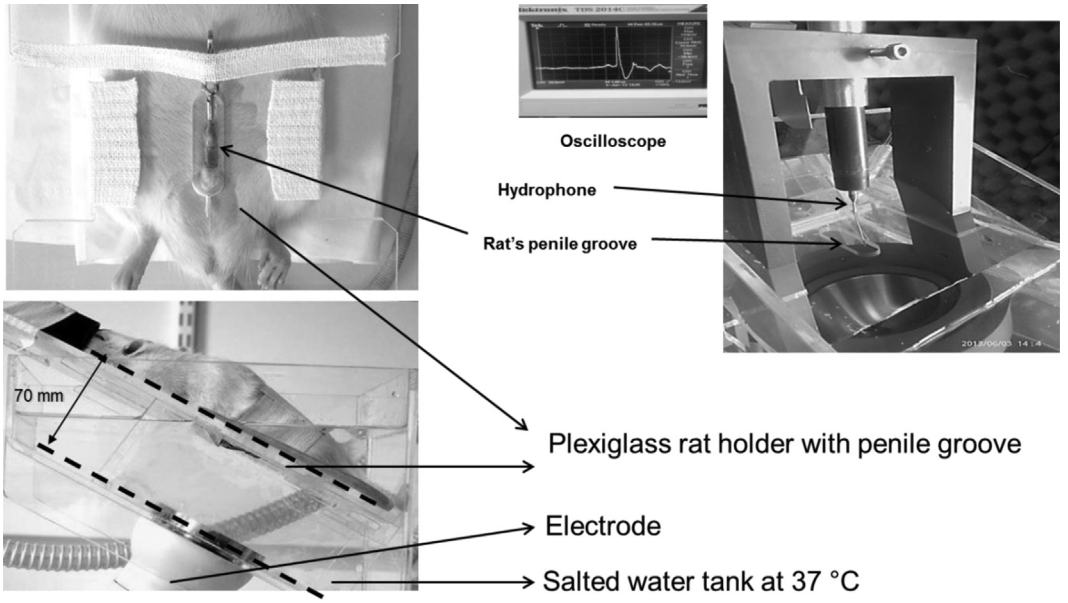

Figure 2. Shock wave device calibration and setup of rodent adapted Li-ESWT. Shock waves were delivered by probe attached to electrohydraulic unit with focused shock wave source. Electrode-penis distance was adjusted by hydrophone connected to oscilloscope. Anesthetized rat penis was dipped in tank and shock waves were delivered simultaneously to whole penis. Li-ESWT session comprised 300 shocks at 0.09 mJ/mm^2 controlled energy flux density and 2 Hz frequency.

Isolated Cavernous Strip Organ Bath Ex Vivo Experiments

At the end of erectile function evaluation blood samples were taken and rats were sacrificed by an overdose of urethane. Rat cavernous strips were obtained and placed in organ chambers (5 ml) filled with oxygenated physiological salt solution composed of 118 mM NaCl, 4.6 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$ and 11.1 mM glucose at 37C for isometric tension recording. After equilibration the cavernous strips were precontracted by PHE (10^{-6} M in Wistar and 10^{-5} M in GK rats) to attain comparable levels of precontraction. CRCs to ACh were performed by cumulatively adding increasing drug concentrations (ACh 10^{-9} to 10^{-4} M) to the baths in semi-log increments. After washings guanethidine (5 μmol/l) and atropine (1 μmol/l) were added to the organ chambers. Frequency-response curves elicited by EFS were then performed on precontracted cavernous strips using a stimulator delivering increasing single square wave pulses (1 millisecond to 10 seconds at 300 mA, and 1, 2, 4, 8, 16 and 32 Hz). After washings CRC to SNP were performed on precontracted cavernous strips by cumulatively adding increasing drug concentrations (SNP 10^{-9} to 10^{-5} M) to the baths in log increments.

Drugs and Chemicals

Sildenafil citrate was obtained from Sequoia Research, Pangbourne, United Kingdom. All other drugs and chemicals were obtained from Sigma-Aldrich®.

Statistical Analysis

All results are presented as the mean ± SEM. Statistical comparisons were performed using 2-way ANOVA or the Student t-test as applicable using Prism®, version 5.04 with $p < 0.05$ considered significant.

RESULTS

Compared to Wistar rats GK rats showed significantly elevated levels of plasma glucose (mean 10.52 ± 0.24 vs 18.24 ± 0.74 mmol/l, $p < 0.001$) and insulin (331 ± 33 vs 428 ± 41 pmol/l, $p < 0.05$).

Li-ESWT Device Development

The treatment protocol used in this study was based on the previously described paradigm performed in patients with ED.[11–13] Some modifications were made to account for anatomical differences between rat and human penises.

To warrant equivalent release of energy in type II diabetic GK rats compared to that delivered in men the Li-ESWT device was thoroughly adapted to the rat scale and its anatomy. The distance from probe to penis was carefully adjusted using a hydrophone to release the desired energy at the site of the rat penis. Several positions were tested (ie the distance from the probe) and electrical signals were received and measured by an oscilloscope coupled to the hydrophone. The precise location where desired energy was recorded was retained (Medispec, personal communication). To facilitate shock wave coverage and transmission the penis of each anesthetized rat was manually stretched and dipped in a salt water-filled tank so that shock waves were delivered at once to the whole penis. A Li-ESWT session comprised 300 shocks at a frequency of 2Hz (fig. 2).

Type II Diabetic GK Rats with ED

Li-ESWT Improved Erectile Response. Erectile responses elicited by cavernous nerve electrical stimulation were markedly decreased in all GK rats compared to age matched Wistar rats (at 15 Hz ΔICP/MAP −47%, AUC_{45}/MAP −35% and AUC_{tot}/MAP −40%, each $p < 0.001$, fig. 3).

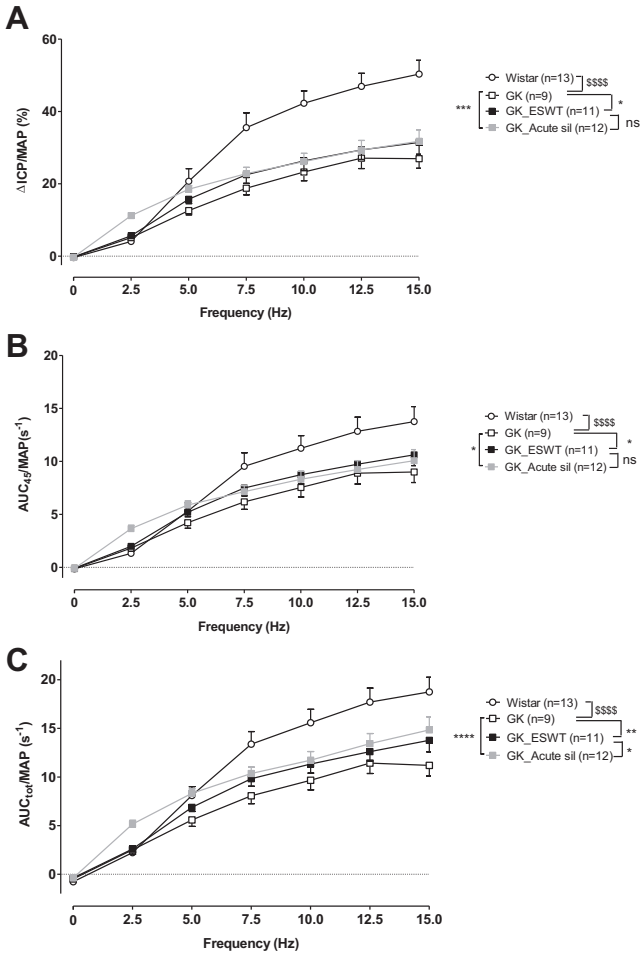

Figure 3. Mean ± SEM Li-ESWT efficacy in type II diabetic GK rats with ED. Erectile responses were elicited by cavernous nerve electrical stimulation at increasing frequencies in 13 anesthetized control Wistar rats (circles) and 9 GK controls (open boxes). Acute intravenous injection of sildenafil and Li-ESWT were done in 12 (gray boxes) and 11 (black boxes) GK rats, respectively. *A*, ΔICP/MAP. *B*, AUC_{45}/MAP. *C*, AUC_{tot}/MAP. Two-way ANOVA p values. *ns*, not significant. Single asterisk indicates $p < 0.05$. Double asterisks indicate $p < 0.01$. Triple asterisks indicate $p < 0.001$. Quadruple asterisks indicate $p < 0.0001$. Dollar signs indicate $p < 0.0001$.

GK rats treated with Li-ESWT showed increased erectile responses compared to control GK rats (at 15 Hz ΔICP/MAP 17%, AUC_{45}/MAP 18% and AUC_{tot}/MAP 23%, p <0.05, <0.05 and <0.01, respectively, fig. 3). Similarly, when acute sildenafil was administered, at 15 Hz ΔICP/MAP, AUC_{45}/MAP and AUC_{tot}/MAP increased by 18%, 11% and 32% (p <0.001, <0.05 and <0.001, respectively). However, neither acute sildenafil nor Li-ESWT restored erectile responses to the level in Wistar rats.

Pro-Erectile Effect of Li-ESWT Potentiation Combined with Acute Sildenafil. The pro-erectile effect of Li-ESWT was potentiated when combined with acute administration of sildenafil (fig. 4). There was an increase of 33% in ΔICP/MAP, 28% in AUC_{45}/MAP and 39% in AUC_{tot}/MAP at 15 Hz compared to control GK rats (p <0.01, <0.05 and <0.001, respectively). Similarly, erectile responses were slightly increased in rats that received combination treatment compared to GK rats that received sildenafil alone, although this increase did not reach statistical significance for ΔICP/MAP, AUC_{45}/MAP and AUC_{tot}/MAP (p = 0.0538, 0.0581 and 0.2, respectively, fig. 4).

Ruling Out Functional Up-Regulation of Cavernous NO/cGMP Pathway after Li-ESWT. ACh induced relaxation of cavernous strips from GK rats, CRCs to SNP and frequency-response curves elicited by

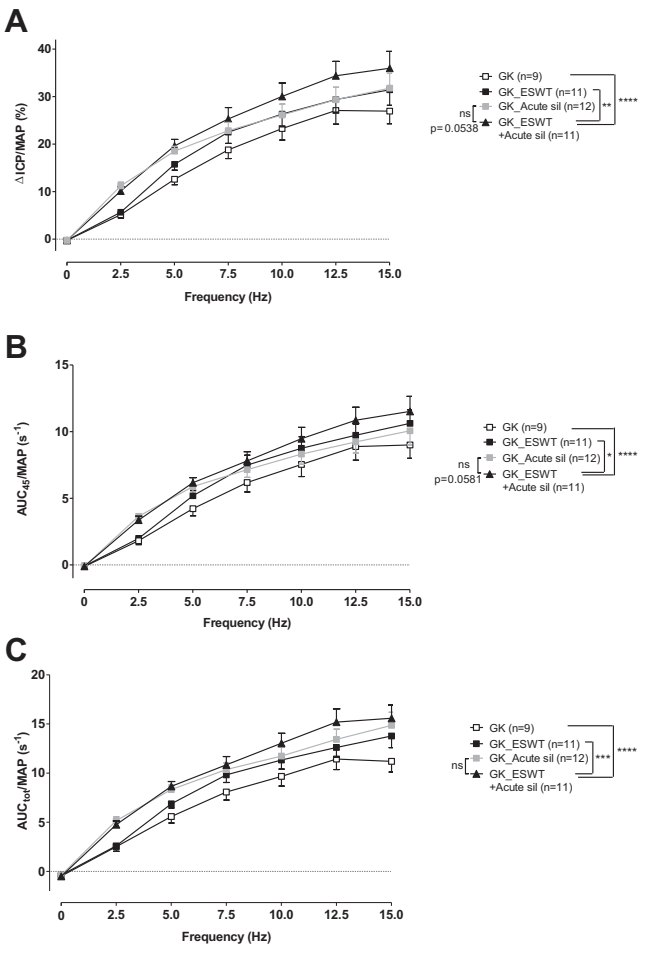

Figure 4. Mean values ± SEM effect of acute sildenafil and Li-ESWT combination in type II diabetic GK rats with ED. Erectile responses were elicited by cavernous nerve electrical stimulation at increasing stimulation frequencies in 9 anaesthetized control GK rats and 12 GK rats with acute intravenous injection of sildenafil, 11 with Li-ESWT, and 11 with combined acute sildenafil and Li-ESWT. *A*, ΔICP/MAP. *B*, AUC_{45}/MAP. *C*, AUC_{tot}/MAP. *ns*, not significant. Single asterisk indicates p <0.05. Double asterisks indicate p <0.01. Triple asterisks indicate p <0.001. Quadruple asterisks indicate p <0.0001.

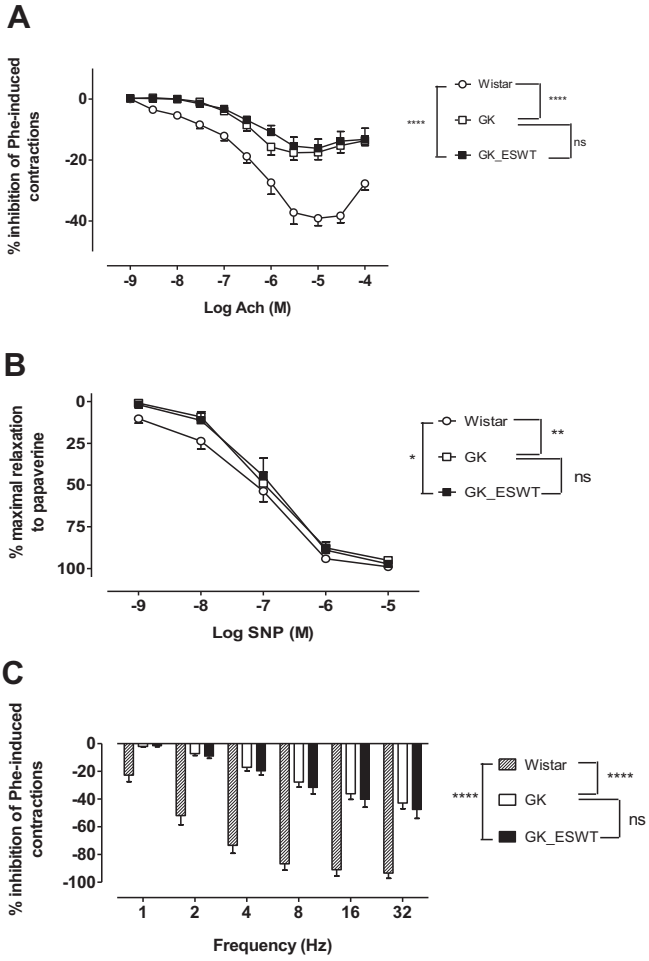

Figure 5. Mean ± SEM Li-ESWT effect on endothelium dependent and independent, and nitrergic relaxation of PHE precontracted cavernous strips from 11 Wistar controls, 11 GK type II diabetic controls and 9 GK type II diabetic rats treated with Li-ESWT. *A*, CRCs of endothelium dependent relaxant responses to ACh. *B*, CRCs of endothelium independent relaxant responses to SNP. *C*, frequency-response curves of nitrergic relaxant responses induced by EFS. Two-way ANOVA p values. *ns*, not significant. Single asterisk indicates p <0.05. Double asterisks indicate p <0.01. Quadruple asterisks indicate p<0.0001.

EFS were significantly impaired compared to those in Wistar rats ($p < 0.001$, fig. 5). Li-ESWT did not improve the altered endothelium dependent and independent, and nitrergic relaxations observed in GK rats (fig. 5).

DISCUSSION

In the current study the beneficial pro-erectile effect of Li-ESWT in type II diabetic GK rats was evidenced after carefully adapting the treatment paradigm used in men to rat penis scale and anatomy to guarantee equivalent release of energy to the erectile tissue. These results confirm GK rats as a suitable T2DM associated ED model.[19] Results also suggest that this model is responsive to Li-ESWT, allowing for further exploration of the efficacy and mechanisms of action by which Li-ESWT exerts effects.

The current series provides an evaluation of the functional reactivity of isolated erectile tissue after Li-ESWT, which to our knowledge has never before been studied. Unexpectedly, we found no evidence of any beneficial effect of Li-ESWT on endothelium dependent or independent, or nitrergic relaxations of the corpus cavernosum. Nonetheless, the combination of Li-ESWT with acute administration of sildenafil further improved the erectile responses of type II diabetic GK rats in vivo compared to that of rats treated with Li-ESWT alone.

The beneficial effect of Li-ESWT on erectile responses in diabetic rats has already been reported in 3 studies.[18,22,23] In those series a chemically induced model of type I diabetes was used. In contrast, our study was done using one of the best characterized spontaneous model of T2DM. The technologies differed in the manner in which shock waves were produced, controlled and focused, resulting in variations in how shock waves penetrated and in the energy intensity applied. In the current study accurate calibration of the quantity of delivered energy and measurement of the precise distance from the electrode to the rat penis were performed to exactly mimic the treatment paradigm in human. This procedure provides a suitable preclinical treatment paradigm in accordance with the clinical setting, enabling further exploration of optimal treatment modalities in future studies.

An additive pro-erectile effect was found when combining acute sildenafil (0.3 mg/kg) with Li-ESWT. Several lines of evidence suggest that Li-ESWT is an emerging treatment strategy for ED, not only by improving erectile function in patients who respond to PDE5-I[12–14] but also by converting PDE5-I nonresponders to responders.[15,16] However, preclinical studies of the mechanism of action of Li-ESWT are still scarce.

One hypothesis to explain these effects is that Li-ESWT results in an increase in blood flow due to up-regulation of the NO/cGMP signaling pathway. It has been reported that the NO/cGMP pathway is impaired in cavernous tissue from type II diabetic patients[8] and rats,[7,9] consistent with the current study. This impairment leads to reduced NO production, bioavailability and/or sensitivity at the smooth muscle level and subsequently to decreased cGMP synthesis.[24] Based on previous studies that Li-ESWT enhanced NO production in cells[25] and increased endothelial and neuronal NOS expression in the corpus cavernosum of rats receiving Li-ESWT[18,22,23] we evaluated whether Li-ESWT treatment could functionally stimulate the NO/cGMP signaling pathway. Endothelium dependent and independent, and nitrergic mediated relaxation of cavernous strips from GK rats were not improved after Li-ESWT, suggesting that protein expression does not preclude function. These results may indicate that the pro-erectile effect of Li-ESWT could be mediated by a NO/cGMP independent mechanism. This was confirmed by the additive effect of Li-ESWT when combined with sildenafil, which recruits the NO/cGMP pathway.

On the other hand, the improved hemodynamics after Li-ESWT might also have resulted from an angiogenesis promoting effect. Indeed, studies in animal models of ischemic myocardial dysfunction[26] or hind limb ischemia[27] evoked the potential effect of Li-ESWT in promoting neovascularization.[28] Li-ESWT could exert a local mechanical stress (shear stress), which could trigger different intracellular signaling pathways that up-regulate the expression of angiogenic related growth factors such as VEGF (vascular endothelial growth factor) or PCNA (proliferating cell nuclear antigen) as previously reported.[18,22,23] Since decreased arterial perfusion has been considered one of the underlying pathophysiological mechanisms responsible for ED, it is conceivable that Li-ESWT could exert its beneficial effect by promoting cavernous angiogenesis. Further studies should focus on mapping penile microvascularization to corroborate this hypothesis.

CONCLUSIONS

This study presents the development of a standardized preclinical procedure by which Li-ESWT application to the rat penis simulates the treatment performed in patients with ED. Its usefulness for studying Li-ESWT as ED treatment was demonstrated in GK rats, a preclinical model of ED associated with T2DM. These results support Li-ESWT not only as an effective, alternative,

noninvasive therapeutic option for ED in diabetic patients but also its use in combination with PDE5-Is. The beneficial effect of Li-ESWT in improving erectile function in GK rats did not depend on functional up-regulation of the NO/cGMP pathway. This preclinical paradigm should help in further exploring and understanding the mechanism of action of Li-ESWT on erectile tissue, thus, better defining the patients who might benefit from it as well as assisting with research for optimized treatment modalities.

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