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Low Intensity Extracorporeal Shockwave Therapy (Li-ESWT) Improves Erectile Function in a model of Type II Diabetes Independently of NO/cGMP Pathway

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ABSTRACT

Purpose: Erectile dysfunction (ED) is highly prevalent in type II diabetes mellitus (T2DM). Li-ESWT improves erectile function in patients with ED from vasculogenic origin including diabetes, although its mode of action remains unknown. The effects of Li-ESWT on ED in a T2DM model compared or combined to sildenafil were investigated the hypothesis of a mode of action targeting the cavernosal NO/cGMP pathway tested. **Materials and methods:** Goto-Kakizaki (GK), a validated model of T2DM, and age-matched Wistar rats were treated by Li-ESWT twice weekly for 3 weeks, repeated after a 3-week no-treatment interval. The rat penis was stretched and dipped into a specifically designed water-filled cage. Shockwaves were delivered by a calibrated probe yielding a controlled energy flux density ($0.09 mJ/mm^2$) attached to an electrohydraulic unit with a focused shockwave source allowing an accurate extrapolation to humans. Following a 4-week wash-out period, erectile function, endothelium-dependent, –independent and nitrergic relaxations of corpus cavernosum of GK rats were assessed. **Results :** Li-ESWT significantly improved erectile function in GK rats to the same extent as sildenafil. Li-ESWT's effects were potentiated when combined with sildenafil. Li-ESWT's effects were not associated to improved cavernosal endothelium-dependent, -independent or nitrergic reactivity. **Conclusions:** Li-ESWT improves erectile function in GK rats. Unexpectedly, this was not mediated by a NO/cGMP-dependent mechanism. Sildenafil increases Li-ESWT's efficacy. This preclinical paradigm for delivering Li-ESWT to the rat's penis should help further exploration of Li-ESWT mode of action on the erectile tissue.

INTRODUCTION

Men with diabetes, particularly those with type II diabetes mellitus (T2DM), show a threefold increase in incidence of ED when compared to men without diabetes [1,2]. ED is manifest 10–15 years earlier in men with diabetes [1,3]. Endothelial dysfunction constitutes a unifying alteration occurring in the pathogenesis of cardiovascular diseases, diabetes and ED [4,5].

Phosphodiesterase type5 inhibitors (PDE5-Is) are the first-line therapy for the treatment of ED whatever the etiology [6]. PDE5-Is block the degradation of cGMP within the smooth muscle cells of the erectile tissue with cGMP being synthetized in response to the release of nitric oxide (NO) by both the endothelial cells and the neural terminations in response to sexual stimulation [7]. Consequently, to exert a clinically beneficial effect in ED patients, PDE5-Is require a sufficient NO drive. However, NO release from both neuronal and endothelial origin is impaired in diabetes [8,9]. Thus, diabetic patients often show a poor response or become refractory to PDE5-Is over time [6,10]. In addition, whatever the PDE5-Is dosing regimen used ie on-demand or daily for tadalafil, 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 are high pressure acoustic waves that when propagating through a medium, can be targeted and focused noninvasively to affect a distant selected 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-Is [12-14] or even convert PDE5-Is non-responders to responders [15,16]. While these results are very encouraging, the basis for this new treatment modality has been an empirical one which could

in theory be optimized and better understood if a relevant preclinical modelization becomes available.

The beneficial use of Li-ESWT has been shown in the penis of rats with STZ-induced type I diabetes where Li-ESWT was found to promote eNOS phosphorylation state, regeneration of nNOS-positive nerves, endothelium, and smooth muscle, thereby suggesting that Li-ESWT targets the NO/cGMP signalling pathway [17,18]. However, whether these described effects on protein expression have any functional relevance remains yet to be shown.

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

MATERIALS AND METHODS

Animals and experimental design

Wistar rats (6 weeks of age, n=13) and age-matched GK rats (n=12/group, Metabrain Research, France) were randomly distributed into 5 experimental groups where GK rats were treated or not with Li-ESWT, acute sildenafil (0.3 mg/kg) or a combination of both with free access to standard chow and water (Table 1).

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

Li-ESWT treatment protocol

Animals received 2 sessions of Li-ESWT per week for 3 weeks, repeated after a 3-week no treatment interval (Figure 1). Shockwaves were delivered by a calibrated probe yielding a controlled energy flux density of 0.09mJ/mm2 attached to a compact electrohydraulic unit with a focused shockwave source (Omnispec ED1000, Medispec, USA). To facilitate coverage and transmission of the shockwaves, the penis of each anesthetized rat was manually stretched and dipped into a specifically designed water-filled tank. Following a 4-week wash-out period, erectile function was assessed by electrical stimulation of the cavernous nerve in rats under anaesthesia (Figure 2).

Erectile function evaluation: electrical stimulation of the cavernous nerve (ES CN)

Erectile responses were tested using a previously described and well-standardized procedure [20]. Rats were anesthetized (urethane 1.2 mg/kg), tracheotomised and their temperature

maintained at 37°C. Catheters were inserted into the carotid artery and corpus cavernosum to record blood pressure via pressure transducers (Elcomatic 750, UK). The cavernous nerve (CN) 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 electrical stimulator (AMS 2100, 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 min thereafter, the CN was stimulated (6 V, 1 ms for 45 s) at different frequencies (0, 2.5, 5, 7.5, 10, 12.5, and 15 Hz) at 3-min intervals in a randomized manner to assess the erectile responses. Each ES CN was repeated twice in view of establishing a frequency-response curve for each animal. Erectile responses to ES CN were expressed as a ratio of ΔICP (mmHg) / MAP (mmHg) x 100, ΔICP being the difference between the intracavernosal pressure (ICP) in the flaccid state, i.e. before stimulation and ICP during the plateau phase of the erectile response, and MAP, the mean arterial pressure during the plateau phase, and as the ratio of AUC_{tot} / MAP and AUC_{45} / MAP, AUC_{tot} and AUC_{45} being the area under de curve measured during the entire erectile response or during the first 45 s after the beginning of the electrical stimulation, respectively [21].

Organ bath ex vivo experiments on isolated cavernosal strips

At the end of erectile function evaluation, **blood samples were taken and** rats were euthanized with an overdose of urethane. Rat cavernosal strips were obtained and placed in organ chambers (5ml) filled with oxygenated physiological salt solution (PSS: NaCl 118mM, KCl 4.6mM, $CaCl_2$ 2.5mM, KH_2PO_4 1.2mM, $MgSO_4$ 1.2mM, $NaHCO_3$ 25mM and glucose 11.1mM) at 37°C for isometric tension recording. After equilibration, the cavernosal strips were precontracted by phenylephrine (PHE.10^{-6} M for Wistar and 10^{-5} M for GK) in order to attain comparable levels of precontraction. Concentration-response curves (CRC) to acetylcholine, ACh, were performed by cumulative addition of 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. Then, frequency response curves (FRC) elicited by electrical field stimulations (EFS) were performed on precontracted cavernosal strips using a stimulator delivering increasing single square-wave pulses (1 ms - 10 s - 300 mA, 1, 2, 4, 8, 16 and 32 Hz). Then, after washings, CRC to sodium nitroprusside, SNP, were performed on precontracted cavernosal strips by cumulative addition of increasing drug concentrations (SNP 10^{-9} to 10^{-5} M) to the baths in log increments.

Drugs and chemicals

Sildenafil citrate was purchased from Sequoia Research (UK). All other drugs and chemicals were purchased from Sigma-Aldrich (France).

Statistical analysis

All results are presented as mean ± SEM. Statistical comparisons were performed using a two-way ANOVA test or Student's t-test where applicable using GraphPad Prism® 5.04. P values<0.05 were considered significant.

RESULTS

When compared with Wistar rats, GK rats displayed significant elevated plasma glucose levels (10.52 ± 0.24 mmol/L versus 18.24 ± 0.74 mmol/l, respectively, $P < 0.001$) and insulin levels (331 ± 33 pmol/L versus 428 ± 41 pmol/L, $p<0.05$).

Li-ESWT device development

The treatment protocol used in this study is based on the previously described paradigm performed in ED patients by Vardi *et al*. [11-13] with some modifications to take into account the anatomical differences between rat's and human penises.

In order 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 the probe to the penis was carefully adjusted using a hydrophone in order to release the desired energy at the site of the rat's penis. Several positions were tested (i.e. 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). In order to facilitate coverage and transmission of the shockwaves, the penis of each anesthetized rat was manually stretched and dipped into a salt water-filled tank so shockwaves were delivered at once to the whole penis. A Li-ESWT session comprised 300 shocks at a frequency of 2Hz (Figure 2).

Improvement of erectile response by Li-ESWT in type II diabetic GK rats with ED

Erectile responses elicited by ES CN were markedly diminished in **all** GK compared to age-matched Wistar rats (at 15Hz, ΔICP/MAP: -47%, $p<0.001$; AUC_{45}/MAP: -35%, $p <0.001$ and AUC_{tot}/MAP: -40%, $p <0.001$) (Figure 3A-C).

GK rats treated with Li-ESWT showed increased erectile responses compared to control GK rats (at 15 Hz, ΔICP/MAP: +17%, $p<0.05$; AUC_{45}/MAP: +18%, $p<0.05$; AUC_{tot}/MAP: +23%,

p<0.01) (figure 3A-C). Similarly, ΔICP/MAP, AUC_{45}/MAP and AUC_{tot}/MAP were increased by 18% (p<0.001), 11% (p<0.05) and 32% (p<0.001), respectively at 15Hz when acute sildenafil was administered. However, neither acute sildenafil nor Li-ESWT was able to restore erectile responses to the level of Wistar rats.

Potentiation of the pro-erectile effect of Li-EWT when combined with acute sildenafil in type II diabetic GK rats with ED

Pro-erectile effect of Li-ESWT was potentiated when combined with acute administration of sildenafil (figure 4A-C) with an increase of +33% (p <0.01) in ΔICP/MAP, +28% (p <0.05) in AUC_{45}/MAP and +39% (p <0.001) in AUC_{tot}/MAP at 15 Hz compared to control GK rats. Similarly, erectile responses were slightly increased in rats who received the combination treatment compared to GK rats who received sildenafil alone although this increase did not reach statistical significance (p=0.0538; p=0.0581 and p=0.2 for ΔICP/MAP, AUC_{45}/MAP and AUC_{tot}/MAP respectively, figure 4A-C).

Ruling out the functional upregulation of the cavernosal NO/cGMP pathway in type II diabetic GK rats treated with Li-ESWT

ACh-induced relaxations of cavernosal strips from GK rats, CRC to SNP or FRC elicited by EFS were significantly impaired compared to Wistar rats (p<0.001, Figure 5A-C). Li-ESWT did not improve the altered endothelium-dependent (figure 5A), -independent (figure 5C) and nitrergic relaxations (figure 5B) observed in GK rats.

DISCUSSION

In the present 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 the 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], and suggest that this model is responsive to Li-ESWT, thus allowing further exploration of the efficacy and mechanisms of action by which Li-ESWT exerts its effects. The present study provides an evaluation of the functional reactivity of the isolated erectile tissue following Li-ESWT which had never been studied to date. Unexpectedly, we have not evidenced any beneficial effect of Li-ESWT on endothelium-dependent, -independent or nitrergic relaxations of the corpus cavernosum. Nonetheless, the combination of Li-ESWT with an acute administration of sildenafil further improved erectile responses of type II diabetic GK rats in vivo compared to rats treated with Li-ESWT alone.

The beneficial effect of Li-ESWT on erectile responses in diabetic rats has already been reported in three papers [18,22,23]. In these reports, a chemically-induced model of type I diabetes has been used, while this study has been conducted using one of the best characterized spontaneous model of T2DM. The technologies used differ in the manner in which the shockwaves were produced, controlled and focused, resulting in variations in which the shockwaves can penetrate as well as variations in energy intensity applied. In the current study, an accurate calibration of the quantity of delivered energy and the measurement of the precise distance from the electrode to the rat penis were performed in order to mimic exactly the treatment paradigm in human. This procedure provides a suitable preclinical treatment paradigm in accordance with the clinical setting allowing further exploration of optimal treatment modalities in future studies.

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

One hypothesis to explain these effects is that Li-ESWT results in an increase in blood flow due to the upregulation of the NO/cGMP signaling pathway. It has been reported that the NO/cGMP pathway is impaired in cavernosal tissue from type II diabetic patients [8] and rats [7,9], consistent with the present study. This impairment leads to a reduced NO production, bioavailability and/or sensitivity at the smooth muscle level and subsequent reduced cGMP synthesis [24]. Based on previous studies reporting that Li-ESWT enhances NO production in cells [25] and increases eNOS and nNOS expression in corpus cavernosum of rats receiving Li-ESWT [18,22,23], we aimed to evaluate whether Li-ESWT treatment could functionally stimulate the NO/cGMP signaling pathway. Endothelium-dependent, -independent and nitrergic mediated relaxations from cavernosal strips of GK rats were not improved after Li-ESWT treatment, 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 is confirmed by the additive effect of Li-ESWT when combined to sildenafil which recruits the NO/cGMP pathway.

On the other hand, the improved hemodynamics following Li-ESWT might also result from an angiogenesis-promoting effect. Indeed, studies performed in animal models of ischaemic myocardial dysfunction [26] or hindlimb ischaemia [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 up-regulating 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 a diminished arterial perfusion has been considered as one of the underlying pathohysiological mechanism responsible for ED, it is conceivable that Li-ESWT treatment could exert its beneficial effect by promoting cavernosal angiogenesis. Further studies should focus on mapping penile microvascularization to corroborate this hypothesis.

In summary, this study presents the development of a standardized preclinical procedure by which Li-ESWT application to the rat's penis simulates the treatment performed in ED patients. Its utility for the study of Li-ESWT in the treatment of ED has been demonstrated using GK rats, a pre-clinical model of ED associated to T2DM. These results not only support Li-ESWT as an effective alternative non-invasive therapeutic option for ED in diabetic patients, but also support its use in combination with PDE5-Is. The beneficial effect of Li-ESWT in the improvement of erectile function in GK rats is not dependent on the functional upregulation of the NO/cGMP pathway. This preclinical paradigm should help further explore and understand the mechanism of action of Li-ESWT on the erectile tissue, thus better defining the patients that might benefit from it, as well as assist in researching for optimized treatment modalities.

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LEGENDS TO THE FIGURES

Figure 1. **Design of the treatment protocol**. After 1 week of acclimation, type II diabetic GK rats were treated with Li-ESWT twice a week for 3 weeks. Treatment was repeated once after a 3-week rest period. *In vivo* erectile function experiments were carried out in anaesthetized rats after a 4-week of wash-out period following the last Li-ESWT session, then rats were euthanized for further *ex vivo* experiments.

Figure 2. **Calibration and setting up of the shockwave device for rodents' adapted Li-ESWT treatment**. Shockwaves were delivered by a special probe yielding a controlled energy flux density of 0.09mJ/mm2 attached to a compact electrohydraulic unit with a focused shockwave source (Omnispec ED1000, Medispec Ltd, USA). The distance from the electrode to the penis was adjusted using a hydrophone connected to an oscilloscope. The penis of each anesthetized rat was dipped into a salt water-filled tank and shockwaves were delivered simultaneously to the whole penis. Li-ESWT session comprised 300 shocks at an energy flux density of 0.09 mJ/mm2 and a frequency of 2Hz

Figure 3. **Efficacy of Li-ESWT treatment in type II diabetic GK rats with ED** Erectile responses elicited by ES CN **(**6 V, 1 ms for 45 s) at increasing stimulation frequencies (0, 2.5, 5, 7.5, 10, 12.5 and 15Hz) in anaesthetized control Wistar rats (n=13) and control GK rats (n=9). Two additional groups of GK rats were treated either with acute *iv* injection of sildenafil at 0.3 mg/kg (n=12) or Li-ESWT (n=11). Erectile responses were expressed as ΔICP/MAP **(**A) AUC_{45}/MAP (B) and AUC_{tot}/MAP (C) Data represent mean values ± SEM. ns, non-significant; * p<0.05; **p<0.01; ****p<0.0001, Two way ANOVA.

Figure 4. **Effect of the combination of acute sildenafil and Li-ESWT in type II diabetic GK rats with ED.** Erectile responses elicited by ES CN **(**6 V, 1 ms for 45 s) at increasing stimulation frequencies (0, 2.5, 5, 7.5, 10, 12.5 and 15 Hz) in anaesthetized control GK (n=9) or GK rats that received either acute *iv* injection of sildenafil at 0.3 mg/kg (n=12), Li-ESWT

(n=11) or a combination of acute *iv* injection of sildenafil and Li-ESWT (n=11). Erectile responses were expressed as ΔICP/MAP) **(**A) AUC_{45}/MAP) (B) and AUC_{tot}/MAP (C). Results represent mean values ± SEM. ns, non-significant; * p<0.05; **p<0.01; ***p<0.001; ****p<0.0001, Two way ANOVA.

Figure 5. **Effect of Li-ESWT on endothelium-dependent, -independent and nitrergic relaxations of cavernosal strips from type II diabetic GK rats with ED.** A) Endothelium-dependent relaxant responses to ACh (10^{-9} to 10^{-4} M) were studied on PHE-precontracted cavernosal strips from control Wistar rats (n=11), control GK rats (n=11) or GK rats treated with Li-ESWT (n=9). B) Endothelium-independent relaxant responses to SNP (10^{-9} to 10^{-5} M) were studied on PHE-precontracted cavernosal strips from control Wistar rats (n=13), control GK rats (n=11) or GK rats treated with Li-ESWT (n=11). C) Nitrergic relaxant responses induced by EFS (1, 2, 4, 8, 16 and 32 Hz) were studied on PHE-precontracted cavernosalstrips from control Wistar rats (n=13), control GK rats (n=11) or GK rats treated with Li-ESWT (n=11). Data represents mean values ± SEM. ns, non-significant; * p<0.05; **p<0.01; ****p<0.0001, Two way ANOVA.

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Table 1. Experimental groups considered

Groups	**Strain**	**Li-ESWT treatment**	**N**
Control	Wistar	No	13
GK	Goto-Kakizaki	No	12
GK-ESWT		Yes	12
GK-Acute sil		No	12
GK-ESWT+Acute sil		Yes	12

Figure 1.

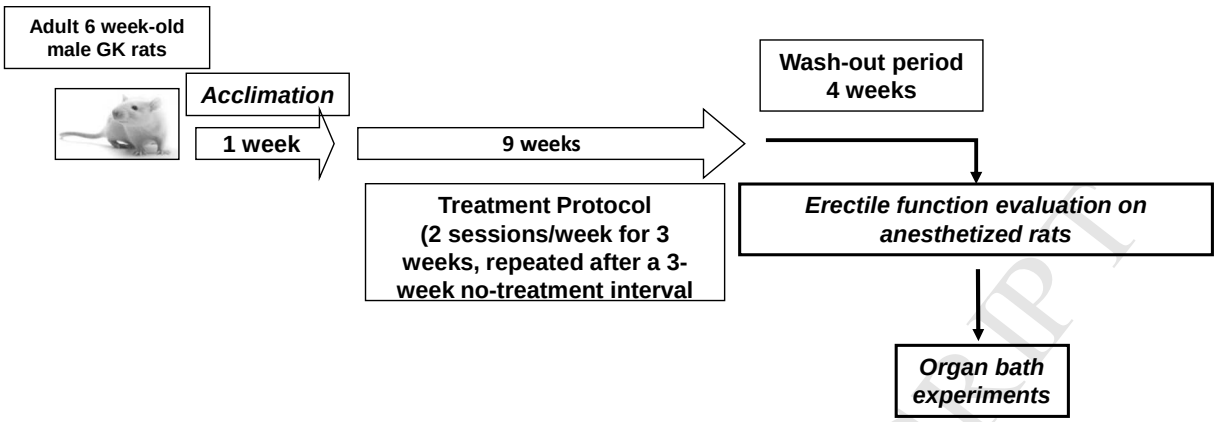


Figure 2.

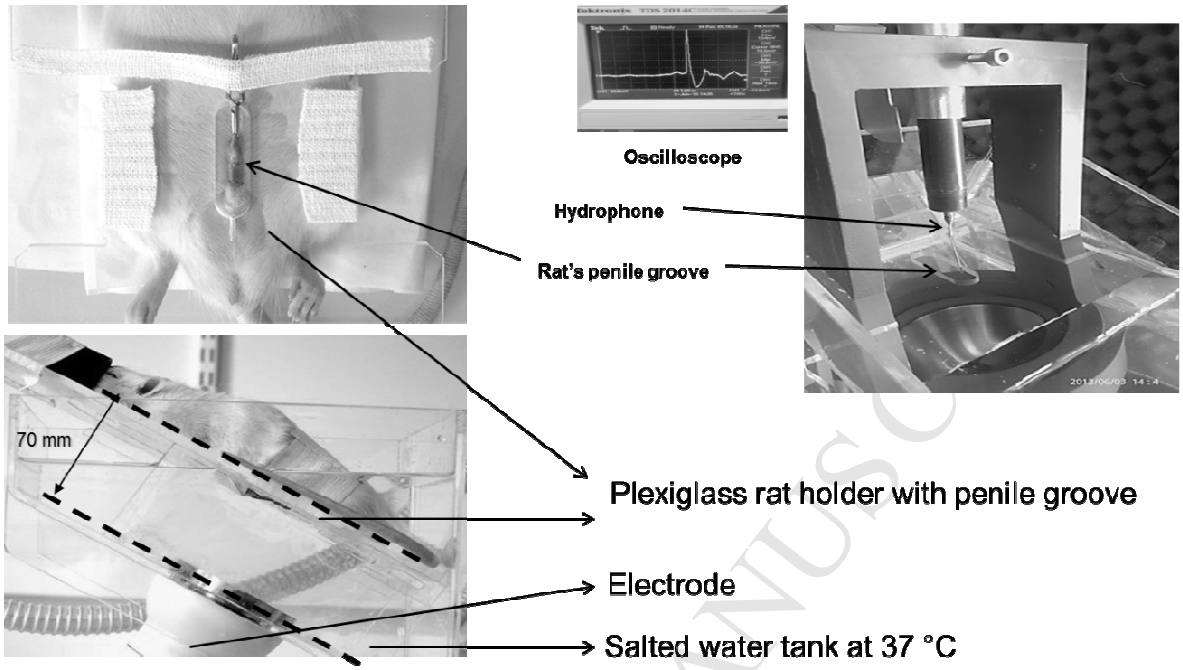


Figure 3.

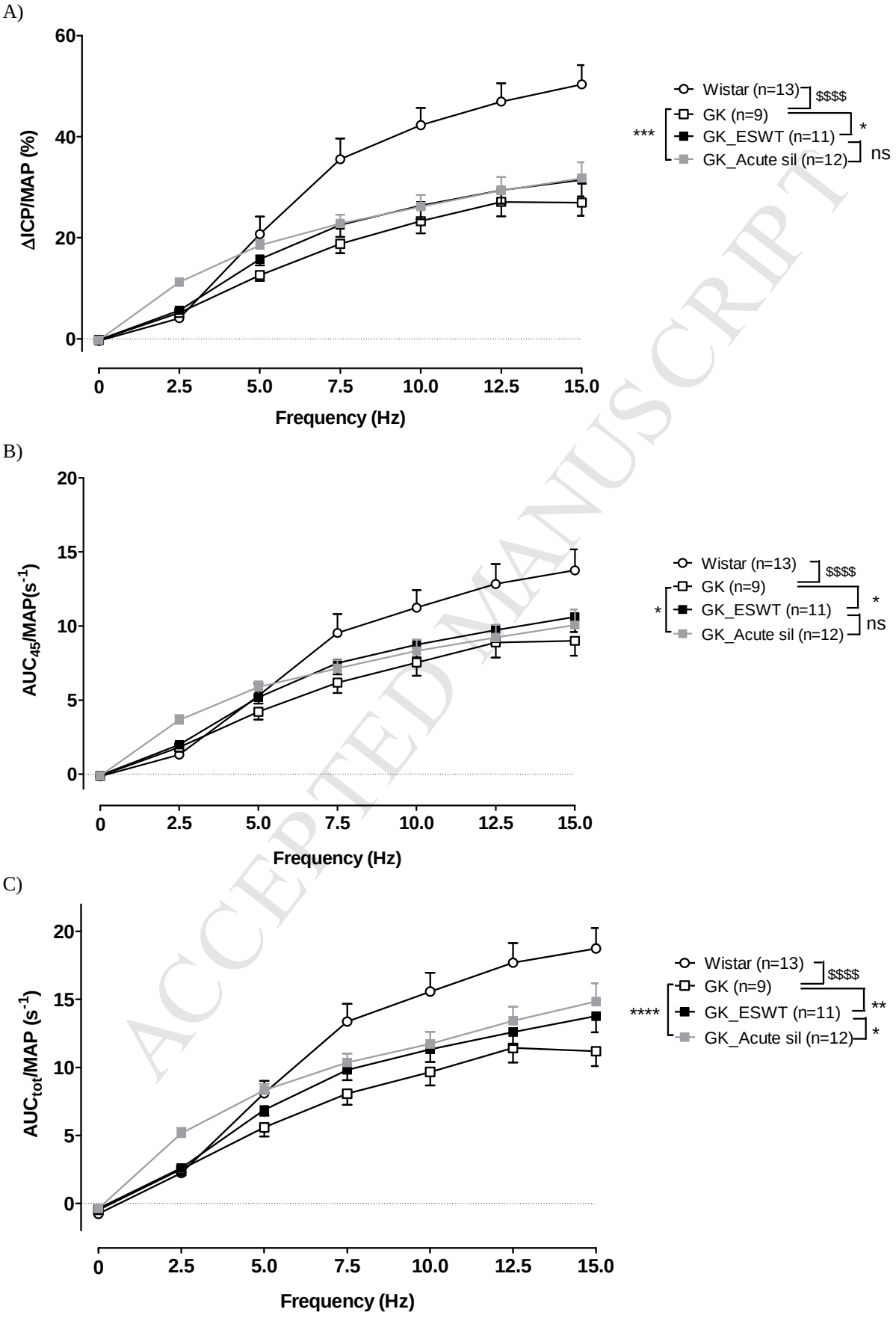
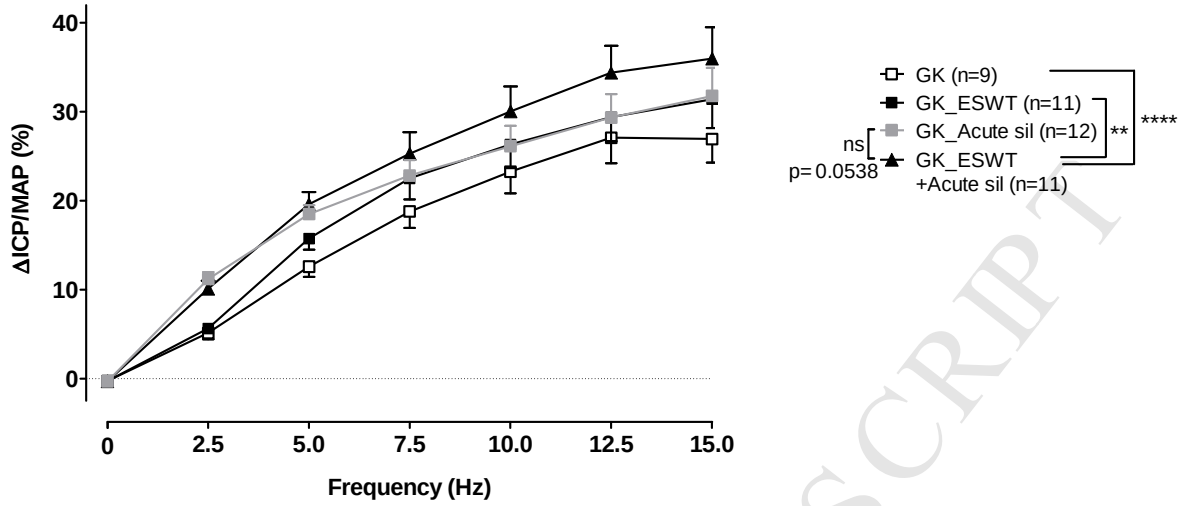


Figure 4.

A)



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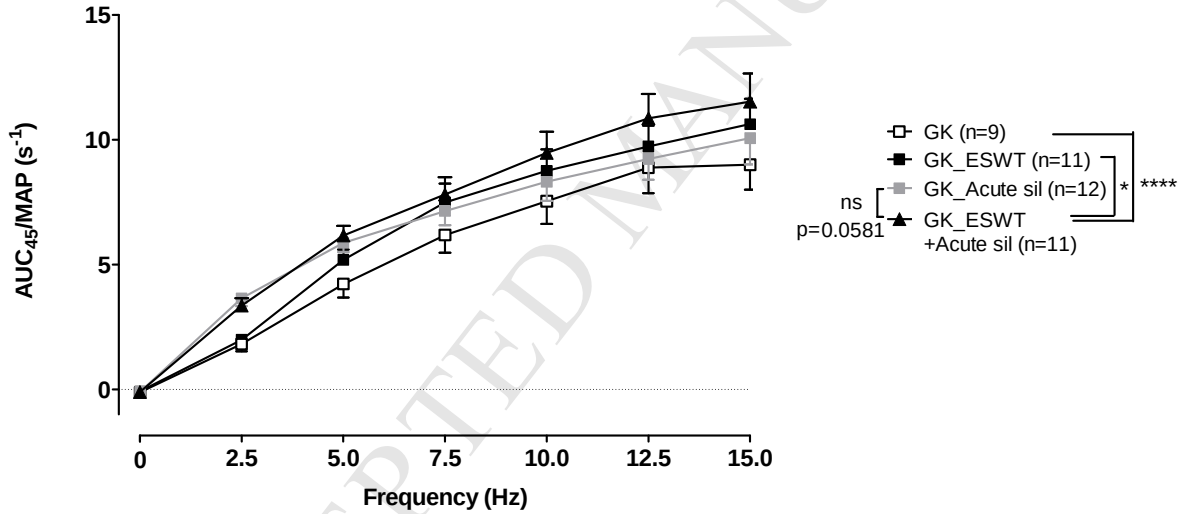


C)

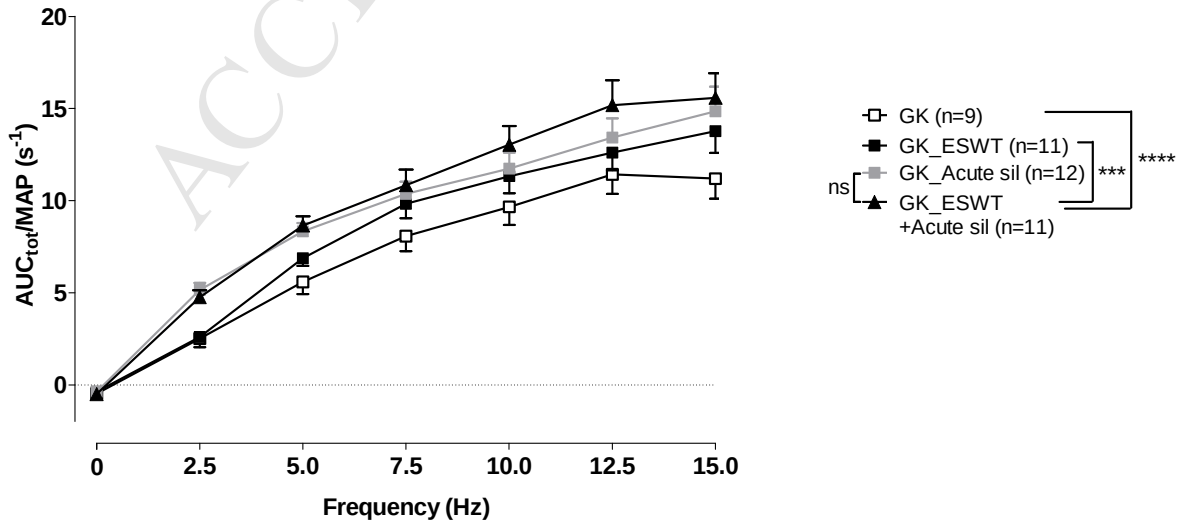
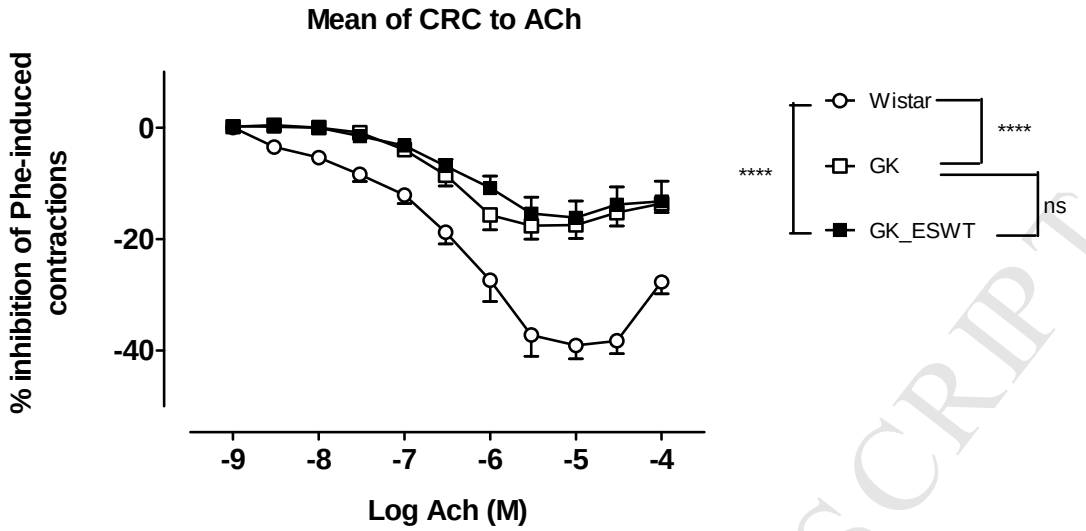


Figure 5.

A)



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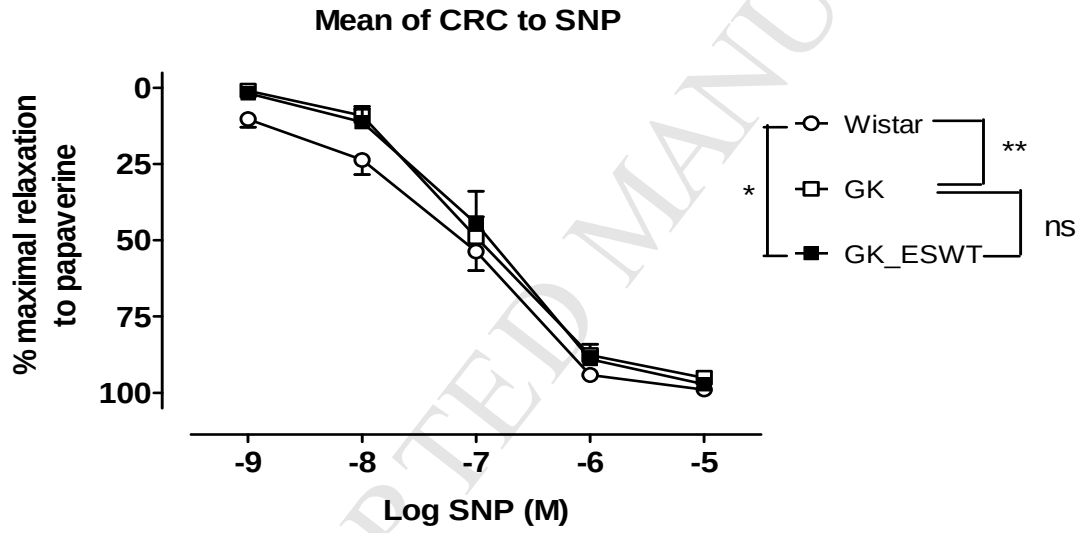

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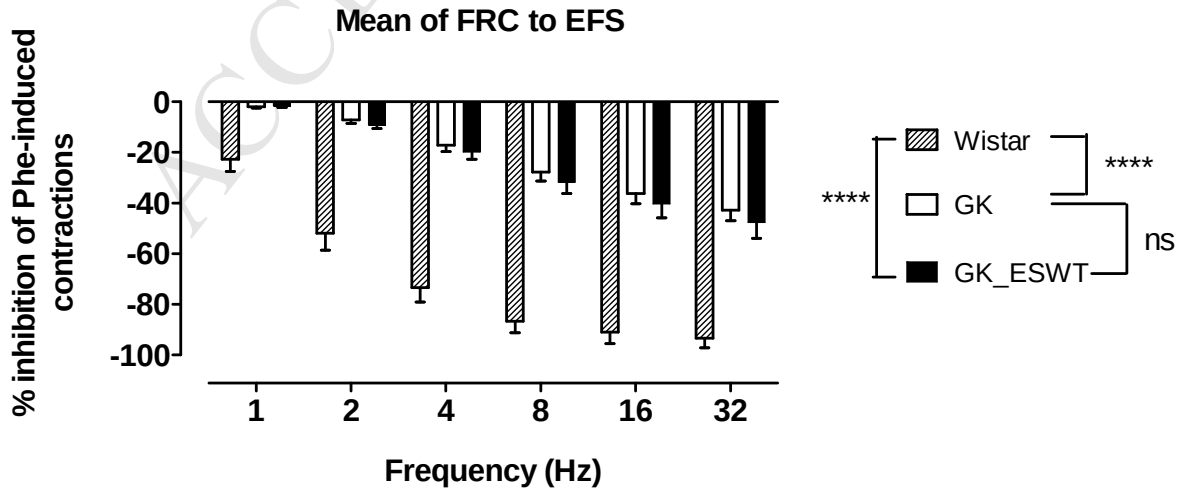

ABBREVIATIONS

ACh: acetylcholine

AUC_{45} : area under de curve measured during the first 45 s after the beginning of the electrical stimulation

AUC_{tot} : area under de curve measured during the entire erectile response

CC: corpus cavernosum

cGMP: cyclic guanosine monophosphate

CRC: concentration-response curve

ED: erectile dysfunction

EFS: electrical field stimulation

eNOS: endothelial nitric oxide synthase

ES CN: electrical stimulation of the cavernous nerve

FRC: frequency response curve

GK: Goto-Kakizaki

ICP: intracavernosal pressure

Li-ESWT: low intensity extracorporeal shockwave therapy

MAP: mean arterial pressure during the plateau phase

nNOS: neuronal nitric oxide synthase

NO: nitric oxide

PDE5-I: Phosphodiesterase type5 inhibitor

PHE: phenylephrine

T2DM : type II diabetes mellitus

Sil: sildenafil

SNP: sodium nitroprusside