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Linear Shockwave Therapy for Erectile Dysfunction

Clinical Review of Low Intensity Shockwave Therapy

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Efficacy and Safety of Linear Focused Shockwaves for Erectile Dysfunction (RENOVA) – A Second Generation Technology

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Abstract: **Low Intensity Shockwaves (LISW) are known to produce revascularization and have been in evaluation and in use to treat erectile dysfunction (ED). The present study is aimed to assess the safety and efficacy of a dedicated shockwave device (Renova) on Vasculogenic ED patients. Fifty seven patients with mild to severe ED were treated by Renova and their erectile function was evaluated by the IIEF-EF, SEP and GAQ questionnaires, at baseline and at 1 and 3 months post treatment. The average IIEF-EF increased significantly from 14.7 at baseline to 21.6 at 1 month and 3 months post treatment. Out of 57 patients, 47 (82%) had a successful treatment. No adverse events were reported during the treatment and the follow-up duration. In conclusion, it appears that the performance of Renova has made it the new advanced treatment for erectile dysfunction.**

Key words: Erectile dysfunction, extracorporeal shockwaves, low intensity shockwaves.

Introduction

Vasculogenic erectile dysfunction (ED) is defined as inability to get or keep an erection firm enough for sexual intercourse due to diseases such as diabetes mellitus and atherosclerotic vascular occlusive disease. Current methods for treating Vasculogenic ED aim at reducing symptoms instead of reversing the source of the disorder, which is in majority of patients is arterial or inflow disorders (Ref. 1). It has been demonstrated that shockwaves can enhance intrinsic angiogenesis and is used to treat ischemic heart disease (Ref. 2). Low-intensity shockwaves (LISW) have been evaluated for treating ED using a modified orthopedic device. The encouraging results that were seen in these studies were the first to show the effect of LISW on ED symptoms (Ref. 3-4). Recently published study conducted on rats with diabetes mellitus (DM) associated ED discovered that low-energy shockwave therapy (LESWT) significantly restored erectile function to levels almost similar to normal controls. The therapeutic efficacy of LESWT is possibly mediated by increased recruitment of mesenchymal stem cells (MSCs) that promote the regeneration of DM-damaged erectile tissues (Ref. 5). The present study was aimed to assess the efficacy and safety of a new dedicated shockwave device, Renova, which was designed to achieve

substantially superior organ coverage compared to the existing devices.

Patients and methods

Study Protocol

This study was a multicenter open-label prospective pilot study, conducted at 4 sites.

This study consisted of a screening phase, treatment phase and a 3 months follow-up phase. At screening phase, patients had an extensive medical and sexological history evaluation and physical examination. Inclusion criteria were heterosexual men in stable heterosexual relationship for at least 3 months, aged 20-80, with vascular ED (according to physician judgment) for at least 6 months, International Index of Erectile Function- Erectile Function Domain (IIEF-EF) score of 6 to 25 (Ref. 7). Recruited patients were both responders and non-responders to phosphodiesterase type 5 inhibitors (PDE5-I). The Exclusion criteria were hormonal, neurological or psychological pathology, past radical prostatectomy, any unstable medical or psychiatric condition, spinal cord injury, penile anatomical abnormalities, clinically significant chronic hematological disease, usage of anti-androgens, recovering from cancer in the past 5 years or radiotherapy in pelvic region.

At baseline and follow-up visits IIEF-EF and Sexual Encounter Profile (SEP) - questions 2 and 3 questionnaires were used (Ref. 7-8). Global Assessment Questions (GAQ, Ref. 9) were used at follow-up as well. The IIEF-EF questionnaire is widely accepted as the best method to verify ED progress. It includes 6 questions regarding erectile function, and its score range is 1-30 points. The SEP questionnaire includes 2 questions: 1) Over the past 4 weeks, were you able to insert your penis into your partner's vagina? 2) Over the past 4 weeks, did your erection last long enough for you to have successful intercourse? There are 2 possible answers: Yes or No. The GAQ questionnaire includes 2 questions: 1) Over the past 4 weeks, has the treatment you have been taking improved your erectile function? 2) If yes, has the treatment improved your ability to engage in sexual activity over the past 4 weeks? Similar to the SEP questionnaire, there are 2 possible answers: Yes or No.

At all study endpoints, patients were evaluated while under the same conditions in terms of pharmacotherapy as they were at baseline evaluation.

Patients committed to avoid using any ED treatment other than PDE5-I oral medication throughout the study duration.

The treatment consisted of four weekly treatment sessions. During each session 3600 shocks of 0.09mJ/mm^2 were applied. Shocks were applied at the penis shaft at right corpus cavernosum and left corpus cavernosum, and at the crura at right crus and left crus, 900 shocks at each area. The treatment areas were the same for each session, so that at the end of the full treatment (4 sessions) each area has received 3600 shocks of 0.09mJ/mm^2.

Follow-ups were conducted at 1 and 3 months post treatment and were consisted of adverse events report, IIEF-EF, SEP and Global Assessment Questions (GAQ). The primary success criterion, regarding to efficacy, was defined as an increase of IIEF-EF score from baseline to the second follow up (3 months post treatment) according to the severity of the symptoms by the minimal clinically important differences in the erectile function domain of the International Index of Erectile Function scale (Ref. 6) as described in table 1.

IIEF-EF Baseline Score	Success Factor
6-10	improvement of 7 points or more
11-16	improvement of 5 points or more
17-25	improvement of 2 points or more

Table 1 - The success criteria of this study according to Rosen et al (Ref. 6)

Treatment device

As being the first dedicated shockwave system for ED, Renova (Direx Group Ltd) differs from other shockwave devices in several aspects. Instead of generating shockwaves that converge on a single focal point and requires moving the shockwave source to multiple positions along the penis, Renova is based on Linear Shockwave Therapy (LSWT) which enables generation of a 70mm long and 40mm depth treatment area along the target organ. In addition, Renova enables efficient positioning when attempting to apply to the crura. Renova's electromagnetic generator delivers shockwaves with a maximum energy density of 0.09mJ/mm^2, meaning, they deliver 10% of the pressure used for disintegrating kidney stones. Shocks are delivered at a maximum rate of 300 PPM (5 Hz), therefore, a treatment session of 3600 shocks lasts approximately 15 minutes.

Statistical analysis

Patients' demographic variables were summarized by descriptive statistics. The average score of each questionnaire and its standard deviation was calculated at baseline, 1 and 3 months follow-up. Student's t test were used at significance level of <0.05.

Results

57 middle aged men (mean: 56.9 ± 9.9 yr, range: 33-84 yr) with Vasculogenic ED were recruited for this study. 43.9% (25 patients) have suffered from cardiovascular disease. 86.0% (44 patients) were PDE5-I responders. Table 2 summarizes the patients' demographic characteristics in division to baseline ED severity. Patients' baseline IIEF-EF score ranged between 6 and 25.

There was no decrease of scores between 1 and 3 months follow-up. Tables 3 and 4 summarize the effect of low-intensity extracorporeal shockwave therapy on the IIEF-EF, SEP and GAQ scores and show the change in these scores from baseline to 3 months post treatment.

No adverse events were reported during and following treatment.

Baseline ED Severity	Age	Cardiovascular disease	Response to PDE5-I
Severe	64.2±5.2	53.8%	69.2%
Moderate	57.8±10.7	68.2%	86.4%
Mild to Moderate	52.2±7.8	17.6%	94.1%
Mild	48.6±10.3	0.0%	100.0%
Total	**56.9±9.9**	**43.9%**	**86.0%**

Table 2 - Patients' demographic characteristics

Baseline ED Severity	Number of Patients	Baseline IIEF-EF		IIEF-EF Improvement Points		% Success
		Range	Average	Success Criterion	Results	
Severe	13	6-10	8.5±1.2	7	8.2±5.9	61.5%
Moderate	22	11-16	13.3±1.8	5	7.7±4.5	77.3%
Mild to Moderate	17	17-21	18.6±1.5	2	5.7±2.1	100.0%
Mild	5	22-25	23.6±1.3	2	3.8±0.8	100.0%
Total	**57**	**6-25**	**14.7±4.9**	**6.9±4.2**		**82.5%**

Table 3 – The results of the International Index of Erectile Function- Erectile Function domain (IIEF-EF), prior to and 3 months following low-intensity extracorporeal shockwave therapy. Success is determined according to table 1.

Baseline ED Severity	SEP 2		SEP 3		GAQ 1	GAQ 2
	Baseline	Follow-up	Baseline	Follow-up	follow-up	Follow-up
Severe	23.1%	76.9%	7.7%	53.8%	76.9%	61.5%
Moderate	45.5%	95.5%	9.1%	50.0%	72.7%	54.5%
Mild to Moderate	94.1%	100.0%	47.1%	100.0%	100.0%	100.0%
Mild	100.0%	100.0%	80.0%	100.0%	100.0%	100.0%
Total	**59.6%**	**93.0%**	**26.3%**	**70.2%**	**84.2%**	**73.7%**

Table 4 – The percentages of "Yes" answers to the Sexual Encounter Profile (SEP) questions and the Global Assessment Questions (GAQ) prior to and at 3 months following low-intensity extracorporeal shockwave therapy. The last questionnaire deals with the treatment; therefore it was used only after treatment.

Discussion

As seen in table 3, Renova treatment has succeeded in 47 out of 57 cases, meaning 82.5% success. Among the successful patients, the average IIEF-EF score increase was 8 points. When reviewing the baseline IIEF-EF scores of failed patients, it appears that their average score is 11.4, which is lower than the general baseline IIEF-EF score in more than 20%. Success rate for more severe cases is lower than the general success rate. When considering the numerical change in IIEF-EF, only 5 patients (8%) have not experienced any change in their erectile function.

When reviewing the change in SEP scores, a significant increase between baseline and follow-up is noticeable. These questions can indicate directly on the patients erectile function condition, since they are referring directly to the patient's ability to perform successful intercourse.

When reviewing the individual answers for the GAQ questions, it appears that 73% of the patients (42 patients) have answered both of these questions with "Yes". Since these questions are intended to evaluate the treatment, these results indicate on a successful treatment.

Conclusions

The results of this study indicate success of this second generation technology for treating ED with linear low-intensity shockwaves. This study shows that Initial follow up data from almost 60 patients demonstrate a clear therapeutic success in over 80% of patients. Pain is tolerated by 100% of

the treated patients and no side effects have been recorded, demonstrating the suitability of this treatment to millions of men who find themselves limited by the currently available solutions. Possible changes in the protocol should be investigated, such as adjusting the number of treatment session to the baseline ED severity.

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Does Low Intensity Extracorporeal Shock Wave Therapy Have a Physiological Effect on Erectile Function? Short-Term Results of a Randomized, Double-Blind, Sham Controlled Study

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Purpose: We investigated the clinical and physiological effect of low intensity extracorporeal shock wave therapy on men with organic erectile dysfunction who are phosphodiesterase type 5 inhibitor responders.

Materials and Methods: After a 1-month phosphodiesterase type 5 inhibitor washout period, 67 men were randomized in a 2:1 ratio to receive 12 sessions of low intensity extracorporeal shock wave therapy or sham therapy. Erectile function and penile hemodynamics were assessed before the first treatment (visit 1) and 1 month after the final treatment (followup 1) using validated sexual function questionnaires and venoocclusive strain gauge plethysmography.

Results: Clinically we found a significantly greater increase in the International Index of Erectile Function-Erectile Function domain score from visit 1 to followup 1 in the treated group than in the sham treated group (mean ± SEM 6.7 ± 0.9 vs 3.0 ± 1.4, $p = 0.0322$). There were 19 men in the treated group who were initially unable to achieve erections hard enough for penetration (Erection Hardness Score 2 or less) who were able to achieve erections sufficiently firm for penetration (Erection Hardness Score 3 or greater) after low intensity extracorporeal shock wave therapy, compared to none in the sham group. Physiologically penile hemodynamics significantly improved in the treated group but not in the sham group (maximal post-ischemic penile blood flow 8.2 vs 0.1 ml per minute per dl, $p < 0.0001$). None of the men experienced discomfort or reported any adverse effects from the treatment.

Conclusions: This is the first randomized, double-blind, sham controlled study to our knowledge that shows that low intensity extracorporeal shock wave therapy has a positive short-term clinical and physiological effect on the erectile function of men who respond to oral phosphodiesterase type 5 inhibitor therapy. The feasibility and tolerability of this treatment, coupled with its potential rehabilitative characteristics, make it an attractive new therapeutic option for men with erectile dysfunction.

Key Words: erectile dysfunction, high-energy shock waves, penis, hemodynamics

Abbreviations and Acronyms

ED = erectile dysfunction

EHS = Erection Hardness Score

FMD = flow mediated dilatation

FU1 = followup 1

FU2 = followup 2

IIEF = International Index of Erectile Function

IIEF-EF = International Index of Erectile Function-Erectile Function domain score

LI-ESWT = low intensity extracorporeal shock wave therapy

PDE5i = phosphodiesterase type 5 inhibitors

V1 = visit 1

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Study received institutional ethics review board approval.

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† Financial interest and/or other relationship with Medispec, Ltd.

NUMEROUS therapeutic strategies exist for improving erectile function. While these therapies have been proven to be safe and effective, they are limited for use before the sexual act and do not modify the physiological mechanism of penile erection.[1] Gene and stem cell therapies are current examples of treatment strategies whose therapeutic goals are to restore erectile function as part of the present trend to shift the field of ED treat-

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ments away from on demand palliative treatments.[2,3]

Adopting this new treatment strategy we began exploring the use of LI-ESWT to achieve this goal.[4,5] Using LI-ESWT as a treatment modality is not new. In 1990 Young and Dyson discovered that therapeutic ultrasound encourages angiogenesis by enhancing the expression of vascular endothelial growth factor.[6–8] This finding led clinicians to begin using shock wave therapy in the treatment of coronary artery disease,[9] bone fractures,[10] calcifying tendonitis[11] and diabetic foot ulcers.[12]

The results of our pioneer pilot study demonstrated that LI-ESWT improved erectile function and penile hemodynamics in men with ED who respond to pharmacotherapy.[4] We also reported that LI-ESWT effectively converted PDE5i nonresponders to responders.[5] While these results were encouraging, our studies were limited by the small sample size and lack of an appropriate control group. To validate our previously published results and to demonstrate whether LI-ESWT has a true physiological effect on the erectile mechanism, we conducted a larger, randomized, double-blind, sham controlled study in men with ED and cardiovascular risk factors who responded to PDE5i.

MATERIALS AND METHODS

The study protocol was reviewed and approved by our institution's Ethics Review Board. All participants gave written informed consent before entering the study.

Screening, Inclusion and Exclusion Criteria

We recruited men with a history of ED for at least 6 months who were already responding to PDE5i from our outpatient ED clinic between July 2009 and October 2010. A total of 77 men underwent an initial screening, including a complete medical history and physical examination (fig. 1). For study inclusion each man had to have an IIEF-EF of 19 or greater while on PDE5i and had to be in a stable heterosexual relationship for more than 3 months. Each man also had to agree to discontinue PDE5i during the entire study period. Men were excluded from analysis if they had undergone radical prostatectomy, received pelvic radiotherapy or hormonal therapy, were receiving ongoing treatment for a psychiatric condition, or had any anatomical, neurological or hormonal abnormalities. Ultimately 10 men met the exclusion criteria.

Study Protocol

The 67 participants who met the inclusion criteria underwent a 4-week PDE5i washout period. At V1 the men were assigned into 2 groups of those who received LI-ESWT (treated group) and those who were given sham therapy (sham group) in a 2:1 ratio using a computer generated table of random numbers. At the same visit each man completed a full IIEF and EHS questionnaire while not on PDE5i. The penile hemodynamics of each man was also evaluated at V1 using our previously described FMD technique in which penile blood flow is measured at rest and after a 5-minute ischemic period using venoocclusive strain gauge plethysmography.[13,14] Each subject then began the 9-week treatment period, which was comprised of 2 treatment sessions per week for 3 weeks that were repeated after a 3-week no treatment interval. A month after the final treatment session (FU1) erectile function and penile hemodynamics were reassessed while the men were still not taking PDE5i (fig. 2).

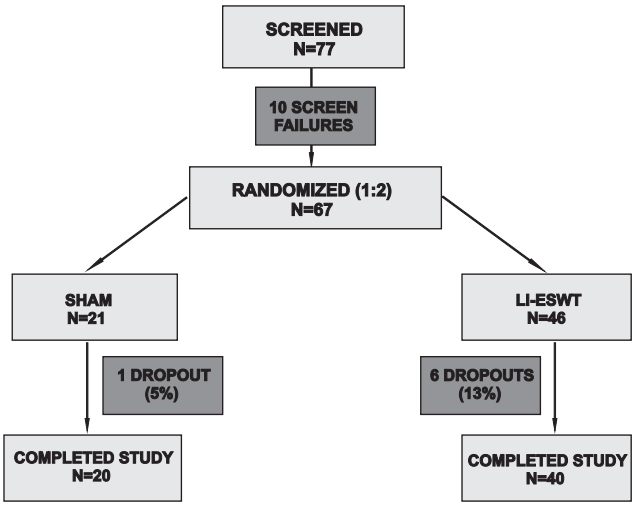

Figure 1. Patient screening and randomization flowchart

Specifics of LI-ESWT

We applied a standard commercial gel normally used for sonography to the penis. The shock waves were delivered to the distal, mid and proximal penile shaft, and the left and right crura using a specialized focused shock wave probe (Omnispec ED1000, Medispec Ltd., Yehud, Israel) as described in our previous studies (fig. 3).[4,5] Since the depth of the shock waves reached both corpora, treatment was delivered on 1 side of the penile shaft only. The 300 shocks at an energy density of 0.09 mJ/mm^2 and a frequency of 120 shocks per minute were delivered at each of the 5 treatment points. Each treatment session was 15 minutes. Due to the low energy density, no local or systemic analgesia was needed.

Followup

To improve the recruitment and compliance rates, all men were eligible to receive an additional treatment course if they were unsatisfied with the initial outcome and had an IIEF-EF of less than 25 at FU1 without PDE5i, regardless of the group to which they were originally assigned. The IIEF of the men who did not undergo additional treatment was reevaluated after 3 months (FU2).

Randomization and Sham Treatment

At randomization each man received a numeric identifier code that was paired to a treatment or sham probe supplied by the manufacturer. The sham probe looked identical to and made the same noise as the treatment probe, but contained a metal plate that prevented the shock wave energy from being applied to the penis. Since the noise and vibration of the probes used in both groups were

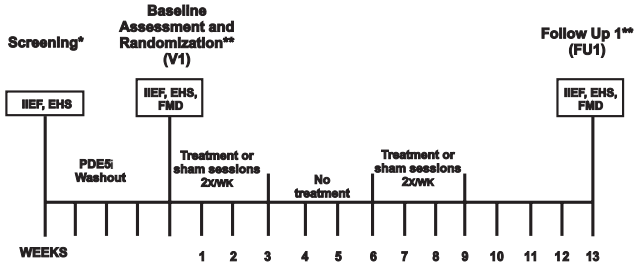

Figure 2. Study flowchart. Single asterisk indicates with PDE5i. Double asterisk indicates without PDE5i.

similar, and the treatment was painless, the operator and subject were blind to the treatment type.

Main Outcome Measures

We used the IIEF-EF to evaluate erectile function. Treatment success was defined as a 5-point or greater improvement in the IIEF-EF between V1 and FU1 because this value indicates an improvement of erectile function by at least 1 severity category. The secondary outcome measures were defined as significant increases in the IIEF subcategories, an increase in EHS from 2 or less at V1 to 3 or more at FU1, and an improvement in penile blood flow.

Statistical Analysis

The data were analyzed using statistical software (JMP®, SAS), and the data are expressed as median and range or mean ± SEM. The values of the study parameters from the 2 study groups were compared by Student's t test with pooled variances or the Wilcoxon signed rank test as appropriate. The linear relationship between changes in the IIEF-EF and changes in penile blood flow at FU1 was assessed by Spearman's rank order correlation. A chi-square contingency analysis was used to examine the relationship between the IIEF-EF and penile hemodynamics, with statistical significance set at 5%.

RESULTS

The baseline characteristics of the 2 study groups were similar (table 1). Six (13%) men in the treated group and 1 (5%) man in the sham group did not complete the study protocol (fig. 1). Of these men 3 took PDE5i, 2 could not meet the necessary time commitments, 1 separated from his wife and 1 had a prolonged hospitalization.

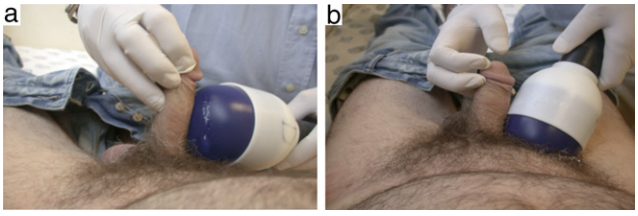

Figure 3. Application of shock wave probe to penile shaft (*a*) and crura (*b*).

Table 1. *Baseline characteristics of the study population at randomization while off PDE5i therapy*

	Sham	Treatment
No. men	20	40
Median age (range)	57 (35–77)	58 (27–72)
Median mos ED (range)	60 (6–240)	42 (6–240)
Concomitant condition (% of men):		
Cardiovascular risk factors*	60	75
Coronary artery disease	10	20
Diabetes mellitus	30	30
Mean ± SEM IIEF-EF domain scores	11.5 ± 0.86	12.6 ± 0.75
Median IIEF-EF domain scores (range)	12.5 (6–17)	13.5 (6–19)
Disease stratification (% of men):†		
Severe dysfunction (IIEF-EF 0–6)	20	12.5
Moderate dysfunction (IIEF-EF 7–12)	30	32.5
Mild to moderate dysfunction (IIEF-EF 13–18)	50	42.5
Mild dysfunction (IIEF-EF 19–24)	0	12.5

All values not significant ($p > 0.05$).

* Including at least 1 of cigarette smoking, hypercholesterolemia, hypertension or obesity.

† Statistical assessment of possible treatment group differences in disease severity distributions of patients could not be performed due to the small numbers in some subgroups.

Efficacy

At FU1 the mean IIEF-EF in the treated group increased by 6.7 points while the score in the sham group increased by 3.0 points ($p = 0.0322$, fig. 4). There were 26 (65%) men in the treated group and 4 (20%) in the sham group who had a 5-point or greater increase in IIEF-EF ($p = 0.0001$). The treated men had significantly improved mean scores in the IIEF subcategories of Sexual Desire ($p = 0.0348$) and Overall Satisfaction ($p = 0.0054$, fig. 4). Of 28 men in the treated group who had an EHS of 2 or less at V1, 19 reported an increase in EHS to 3 or greater at FU1 vs no men in the sham group (fig. 5).

Penile hemodynamics were assessed in 59 of the 60 men who presented at FU1 (1 man in the treated group refused this assessment after treatment). Penile hemodynamics improved significantly in the treated group (table 2, $p < 0.0001$). Furthermore, we noted a strong positive correlation between changes in the IIEF-EF and changes in the resting and maximal post-ischemic penile blood flow at FU1 ($p < 0.0001$). The IIEF-EF and the post-ischemic maximal blood flow improved ($p < 0.001$) in 22 (56%) men in the treated group and 1 (5%) man in the sham group.

Adverse Events

Unlike painful higher intensity shock wave energy used to treat nephrolithiasis and Peyronie disease (0.2 to 1.1 mJ/mm^2), the low intensity shock wave energy (0.09 mJ/mm^2) used in this study was not associated with any pain or side effects such as ecchymoses or hematuria.

Post-Study Followup

A total of 23 men including 16 (80%) from the sham group opted to receive a second series of treatments

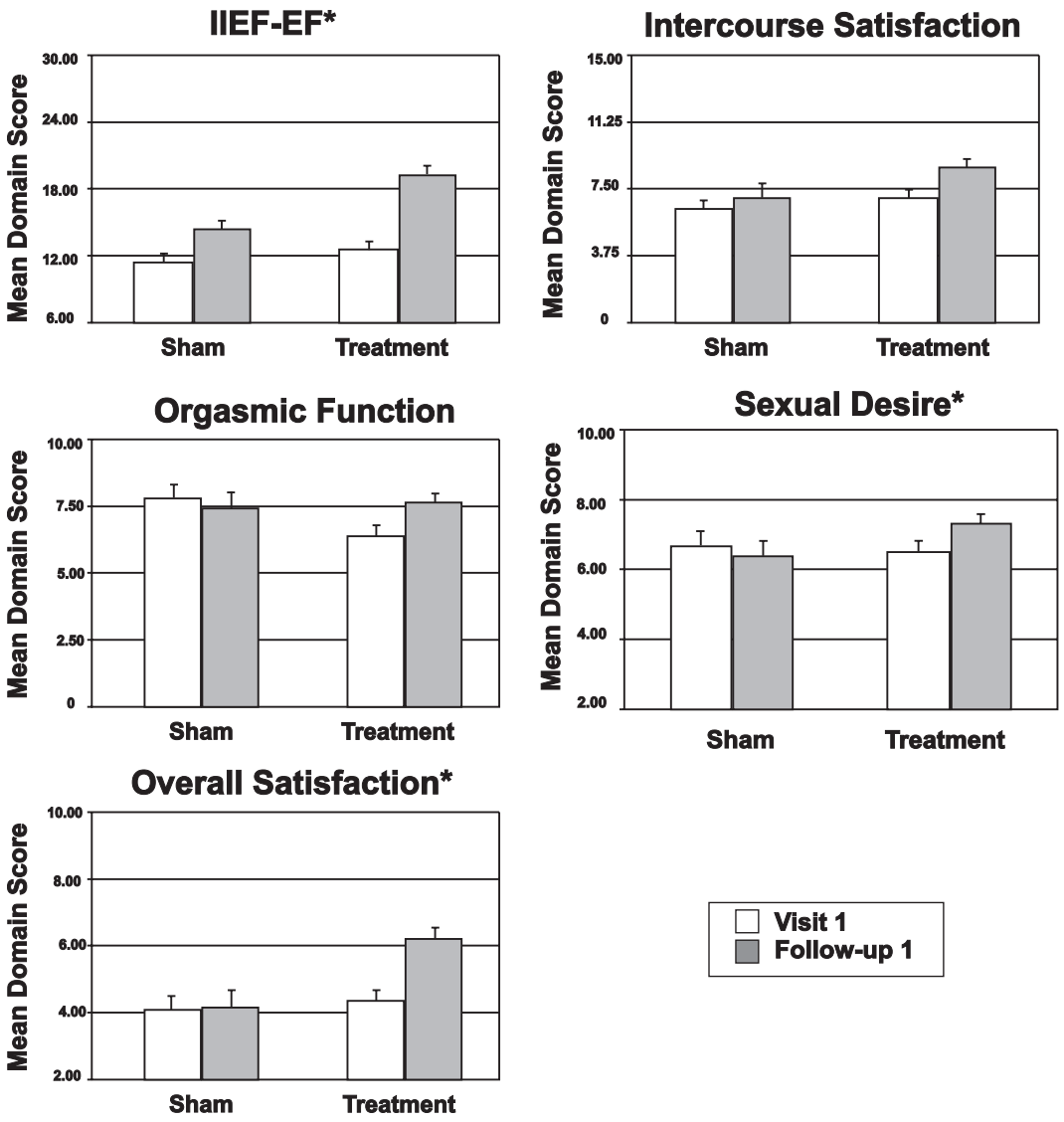

Figure 4. IIEF domain scores (mean ± SEM) for men treated with LI-ESWT or sham therapy at V1 or FU1. Asterisk indicates $p < 0.05$ and represents significance of difference between 2 groups.

without knowing their original group (fig. 6). Mean IIEF-EF of men continuing on to a second round of treatments was 12.2 at FU1, while the remaining 36 men who had followup at 3 months had an additional increase in mean IIEF-EF from 20.7 at FU1 to 22.1 at FU2.

DISCUSSION

Due to the skepticism surrounding this novel treatment, insufficient scientific background and disappointing results of penile shock wave therapy in Peyronie disease, it was crucial to further establish the validity of LI-ESWT by conducting a randomized, double-blind, sham controlled study. We chose to use measurement tools that are validated and widely accepted such as the IIEF and EHS. While validated in men receiving on demand PDE5i, these questionnaires have a high degree of sensitivity and specificity for detecting treatment related changes in the erectile mechanism.[15–17] Since LI-ESWT is a nonpharmacological intervention whose effect is not defined per sexual encounter but during a prolonged period, questionnaires such as the sexual encounter profile were not used.

We postulated that the underlying mechanism of LI-ESWT action is to improve penile hemodynamics. To confirm this hypothesis, objective and quantifiable measures of penile hemodynamics are required. Our experience with nocturnal penile tumescence testing in our first pilot study led us to conclude that nocturnal penile tumescence is not suitable to be used as an investigative tool due to difficulties in interpreting the results in terms of meaningful pa-

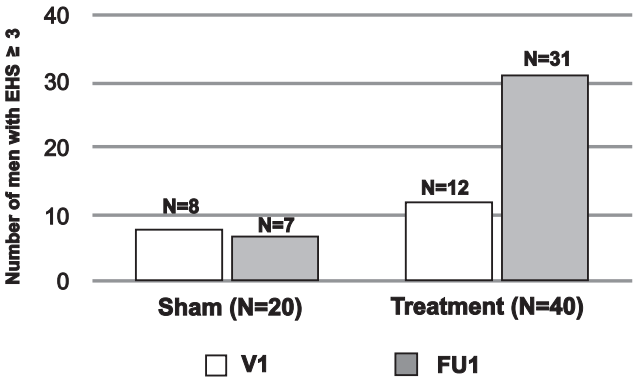

Figure 5. Number of men with EHS 3 or greater at V1 and FU1. For EHS clinical interpretation, grade definitions characterizing penis are grade 1—larger but not hard, grade 2—hard but not hard enough for penetration, grade 3—hard enough for penetration but not completely hard, grade 4—completely hard and fully rigid.

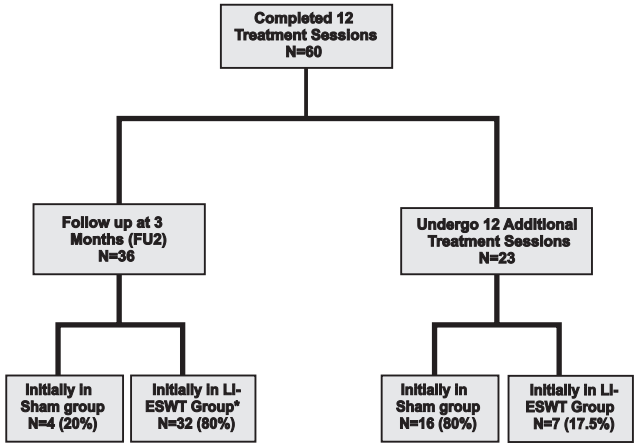

Figure 6. Patient followup after 12 treatment sessions. Asterisk indicates 1 patient (2.5%) was lost to FU2.

rameter changes and changes in penile hemodynamics. We did not use duplex ultrasonography because it mainly measures cavernous artery flow, is operator dependent, and is reliant on the timely response of injected vasoactive agents and patient disposition. Although it is an excellent test to evaluate penile vascular status, duplex ultrasonography may be problematic for the comparison of changes in penile hemodynamics before and after intervention. We used venoocclusive plethysmography to measure penile hemodynamics because it can objectively assess penile perfusion in the flaccid state in a simple and reproducible fashion, it is not operator dependent and it has previously been proven to reflect changes in erectile function after intervention.[13,14] Furthermore, while our group was the first to describe the FMD technique in the penis, it is not principally different from the widely used FMD technique to assess endothelial function in the brachial artery.

The IIEF-EF of the treated men significantly improved at FU1. The increase was not as great as the increases in the IIEF-EF that were reported in studies that introduced the therapeutic effects of PDE5i.[18–20] Admittedly, comparing the efficacies of an on demand treatment to a nonpharmacological rehabilitative intervention that is unrelated to the sexual act is inherently problematic. Unlike the ED naive cases in the first sildenafil studies that had not previously experienced treatment success, those in our study had a different definition of therapeutic success because they already had a positive experience with PDE5i. Furthermore, many of the original PDE5i studies included a mixed ED population, as opposed to our group of men with similar ED risk factors. Our exclusion criteria may also account for the 25% sham effect seen in our study compared to a placebo effect as high as 46% reported in the original PDE5i studies.[21] The results of later studies that excluded patients with psychogenic ED, and examined the effect of PDE5i on men with organic ED and cardiovascular risk factors, are comparable to the results of our study.[22,23] Nevertheless, it is possible that our empirical LI-ESWT protocol is less effective than PDE5i therapy.

Table 2. *Changes in penile blood flow at FU1*

	Resting Blood Flow (ml/min/dl)	Max Blood Flow (ml/min/dl)
Sham:		
Median	0.2	−0.1
Min	−6.7	−9.2
Max	7.6	18.5
Treatment:		
Median	4.6	8.2
Min	−15.5	−17.0
Max	80.2	124.8

All values $p < 0.0001$.

An unexpected finding was the significant improvement in the IIEF Sexual Desire domain scores of the treated men, a finding that has been reported in at least 1 of the previous studies that evaluated pharmacotherapy.[19] While our finding was statistically significant, the clinical importance of a 1-point increase in this score remains unclear.

We did not find statistically significant improvement in the IIEF Sexual Satisfaction domain score. We attribute this lack of improvement to our subjects' previous positive experience with PDE5i. Nevertheless, the IIEF Overall Satisfaction domain score did increase significantly after treatment, indicating a beneficial effect of LI-ESWT.

The EHS data also revealed that more men in the treated group than in the sham group were able to achieve erections sufficiently hard for penetration.

Ease of definition and applicability make the EHS a valuable tool for simple clinical assessment. However, it is statistically ill suited for pre-post and 2-group study designs such as ours.

Physiological evidence that LI-ESWT improves penile hemodynamics comes from the finding that the 2 measures of penile blood flow improved significantly in the treated group and were positively correlated with the increases in IIEF-EF. Moreover, in seeking a success criteria based on clinical and physiological outcomes, we found that of the patients who had a 5-point or greater improvement in the IIEF-EF and improved penile hemodynamics all but 1 came from the treated group. Further supporting our contention that LI-ESWT improves penile hemodynamics is our finding that most of the treated men reported improvement in erectile function between treatment sessions 6 and 8, which is probably the time needed for LI-ESWT to induce the physiological changes.

While the purpose of this study was to evaluate the physiological effects of LI-ESWT on the penis, our finding that the IIEF-EF remained increased 3 months after the final treatment suggests that the positive physiological effect is preserved. This finding is similar to that of our previous study demonstrating that the subjects' IIEF-EF remained high at the 3 and 6-month followup.[4]

The treatment protocol that we used in all our studies to date was based on that described in the cardiology literature.[24,25] This empirical protocol had not been previously tested in animal or human penile tissue and, therefore, will likely change as more protocols are examined.

Although our final study population was comprised of only 60 men, this number of participants was sufficient to achieve our main goal of determining whether our treatment protocol could yield a genuine physiological effect on cavernous tissue.

To date, no deleterious side effects have been reported in the long-term followup of patients undergoing high intensity penile shock wave therapy for the treatment of Peyronie disease,[26,27] despite findings that such shock waves may lead to the collagenization of corporal smooth muscle in the rat.[28] While our subjects did not report any adverse effects to the treatment, the long-term risk of LI-ESWT on penile tissue has yet to be fully elucidated.

CONCLUSIONS

This is the first randomized, double-blind, sham controlled study in which LI-ESWT has been shown to have a beneficial effect on erectile function in men with ED and cardiovascular risk factors. While we do not know the precise mechanism of action of LI-ESWT, our objective measures lead us to presume that this therapy works by improving penile hemodynamics. We also found that this treatment is feasible and tolerable, and is unique in that it has rehabilitative characteristics. Additional studies with long-term followup are now needed to fully evaluate the efficacy of this new therapy and confirm our findings. These studies must be backed by basic science research whose aims are to fully understand the mechanism of action of this energy. With this additional knowledge, our hope is that LI-ESWT will make its way into the armamentarium of treatment options currently being used in the long-term clinical management of ED.

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Low-Intensity Extracorporeal Shock Wave Therapy in Vascular Disease and Erectile Dysfunction: Theory and Outcomes

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ABSTRACT

Introduction. Low-intensity extracorporeal shock wave therapy (LI-ESWT) to the penis has recently emerged as a new and promising modality in the treatment of erectile dysfunction (ED).
Aim. To review the published literature on the mechanism of action of LI-ESWT; and to report our clinical data on its efficacy in men with vasculogenic ED.
Methods. A Medline search using the relevant keywords on this topic has been done.
Results. From the results of numerous preclinical and animal studies that have been done to date, sufficient evidence shows that the underlying mechanism of action of LI-ESWT is probably neovascularization. Therefore, local application of LI-ESWT to the corpora cavernosa may potentially act in the same mechanism and increase corporal blood flow. We found that the application of LI-ESWT to patients who responded to oral therapy (PDE5i) eliminated their dependence on PDE5i and they were able to successfully achieve erections and vaginal penetration (60-75%). Furthermore, PDE5i non-responders became responders and capable of vaginal penetration (72%). Additionally, LI-ESWT resulted in long-term improvement of the erectile mechanism.
Conclusions. LI-ESWT has the potential to improve and permanently restore erectile function by reinstating the penile blood flow. Although these results on LI-ESWT are promising, further multi- centered studies with longer follow-up are needed to confirm these findings. **Gruenwald I, Kitrey ND, Appel B, and Vardi Y. Stem low-intensity extracorporeal shock wave therapy in vascular disease and erectile dysfunction: Theory and outcomes. Sex Med Rev 2013;1:83–90.**

Key Words. Low-Intensity Extracorporeal Shock Waves; Erectile Dysfunction; Therapy

Introduction

Throughout the ages, masculinity and sexual function have always been strongly linked. Erectile dysfunction (ED) is considered a sign of weakness and vulnerability, and men with ED see themselves as impotent in the wide sense of the word. Hence, the impact of ED on self-esteem and self-confidence is enormous and adversely affects quality of life [1]. From a medical standpoint, improving erectile function has always been fundamental to treating these stigmata. Fortunately, the quest for improving erectile function has been quite successful. The discovery in the mid-1980s that nitric oxide (NO) production by penile nerve terminals and vascular endothelium is essential for normal erection improved our understanding of the pathophysiological processes that underlie ED [2]. This discovery also provided an explanation for the link between penile endothelial dysfunction and poor penile blood flow that occurs in atherosclerosis, diabetic vasculopathy, and diabetic neuropathy. This discovery also led to the improvement and development of therapies that specifically targeted penile endothelial cells, such as intracorporal injection of vasodilators and phosphodiesterase-5 inhibitors (PDE5is) [3]. Even so, none of these treatments addressed the problem of impaired blood supply to the corpora, and none of these therapies are curative because they do not restore corporal blood flow and/or endothelial function. Hence, the challenge in ED

management is to develop a therapeutic modality that will reinstate corporal blood flow and improve ED.

Historically, the first therapy that attempted to cure arteriogenic ED by increasing blood flow to the corpora was the surgical revascularization, a technique that was pioneered by Michal et al. in 1973 [4]. Unfortunately, surgical treatment for ED was not further developed because the results were relatively poor (effective in only about 50% of all cases), it was restricted to young men with traumatic arterial occlusion [5], and the data on long-term outcome were limited. Interestingly, a minimally invasive approach to restore corporal blood flow and cure ED has been recently developed, namely the insertion of endovascular stents in the internal pudendal artery in a group of PDE5i nonresponders of whom 50–60% have pudendal artery stenosis. Because these studies are still preliminary, more research is needed to evaluate the efficacy of this procedure and its long-term outcome [6].

Today, new experimental long-lasting treatments of ED [4] are being explored, such as regenerative medicine where (i) the damaged tissue is replaced after laboratory culturing of normal tissue or an autologous stem cell transplant, or (ii) the body's own repair mechanisms are stimulated to heal the damaged tissues. Gene therapy in which plasmids as used to deliver genetic material, such as neuromodulatory factors and brain-derived growth factors (neurotrophins), in order to alter gene expression in the penile smooth muscle, endothelial, or nerve cells is being explored as another potential therapy for ED. Detailed descriptions of these new treatments are beyond the scope of this review and can be found elsewhere [6,7].

Why Did We Choose Low-Intensity Extracorporeal Shock Wave Therapy for ED?

In modern medicine, the use of different types of energy for diagnostics and therapy is widespread. In particular, sound or shock waves (SWs) of various intensities have been used therapeutically for the last two decades in medicine. High-intensity SW therapy has revolutionized the treatment of urinary calculi, and medium-intensity SW therapy is now used for treating joint pain, tendonitis, and bursitis. Low-intensity extracorporeal SW therapy (LI-ESWT) interested us because data from both in vitro and in vivo studies have consistently shown that this energy can stimulate angiogenesis. The idea of applying LI-ESWT to the penis came from animal studies in which shockwave energy was applied to the myocardium of pigs, where it has been reported that LI-ESWT improved ischemia-induced myocardial dysfunction [8]. Extrapolating these findings to ED, we postulated that LI-ESWT of the penis would improve penile blood flow and endothelial function by stimulating angiogenesis in the corpora.

What Do We Know on the Biological Effects of Low-Intensity SWs?

SWs have two important features: they carry energy, and they are able to propagate through a medium. SWs are a sequence of single sonic pulses and are characterized by a fast pressure rise (<10 nanoseconds), a high-pressure peak (100 MPa), and a short lifecycle (10 microseconds) [9]. When SWs are noninvasively focused on an organ or tissue, their energy creates a high-pressure load that only affects the targeted area.

Although the underlying mechanism of their biological action is not completely understood, it is theorized that the tissue is first compressed due to the positive pressure from the energy that is carried by the SW and then expands due to the tensile properties of the tissue [10]. Nishida described this phenomenon as a cavitation because it resembled a micrometer-sized violent collapse of bubbles. Because the physical forces that are generated by cavitation are highly localized, it is thought that SW induces a localized stress on cell membranes in the same way that shear stress affects endothelial cell membranes [11]. This shear stress then triggers a chain of events that cause the release of angiogenic factors, such as increased local NO production through the increased activity of endothelial NO synthase (eNOS) and neuronal NOS (nNOS), platelet-derived growth factor, and vascular endothelial growth factor (VEGF) [12]. In addition to this effect, SWs have been reported to cause membrane hyperpolarization, activation of the Ras signaling pathway [13], non-enzymatic synthesis of NO [14], and induction of stress fibers and intercellular gaps [15].

LI-ESWT: In Vitro and Animal Studies

Wang and his colleagues studied the biological effects of low-intensity SWs (LISWs) extensively [16] and discovered that LISW stimulates endothelial cell proliferation with the expression

of eNOS, VEGF, and proliferating cell nuclear antigen. The angiogenic markers increased after 1 week and continued to rise for 8 weeks, while the processes of neovascularization and cell proliferation started 4 weeks and persisted for more than 12. The same group also reported that LI-ESWT stimulated neovascularization of the tendon-bone junction in dogs [17] and rabbits [16]. LISW can elevate VEGF and VEGF-messenger RNA levels in human umbilical vein endothelial cells as well [8], and it improves angiogenesis, blood flow, and wound repair in nude mice with experimental burns [18].

Stem cells and progenitor cells have the ability to divide and differentiate into specialized cell types. Their pivotal role in the neovascularization of ischemic tissues was widely studied in recent years. LISW affect stem cell recruitment in tissue repair. Chen et al. [19] studied the changes in cell morphology and histology in healing bones of rats following LI-ESWT. They showed that LI-ESWT increased the number of mesenchymal stem cells (MSCs) in the defect, which later differentiated into osteoblasts and chondrocytes. LI-ESWT significantly increased the expressions of growth factors (transforming growth factor β1 and VEGF-A), which have a chemotactic and mitogenic role in the repair process. Aicher et al. [20] investigated the effect of LI-ESWT on the attraction of human autologous circulating progenitor cells (CPCs) in rats with induced chronic ischemia. LISW-treated ischemic muscles attracted significantly more labeled CPCs than the untreated muscles, and the treatment significantly increased the blood flow in the ischemic muscles.

Nishida and colleagues [8] investigated the effects of LI-ESWT in pigs with experimentally induced chronic myocardial ischemia and found that LI-ESWT significantly upregulated VEGF expression in ischemic myocardium. It improved regional myocardial blood flow and left ventricular (LV) ejection fraction. This beneficial effect of LI-ESWT on LV remodeling has also been demonstrated in studies that involved pigs with experimentally induced myocardial infarction [21] and myocardial ischemia-reperfusion injury [22].

The effect of the LI-ESWT on the erectile tissue has only recently been studied. Qiu et al. [23] investigated the effects of LI-ESWT on erectile function in diabetes mellitus rats using a protocol that is similar to the one used to treat men with ED. According to the changes in the intracavernous pressure following electrostimulation of the cavernous nerve to assess erectile function, they found that erectile function was significantly decreased in all diabetic rats and that this effect was less in the LI-ESWT group. Histologically, they found much less nNOS-containing nerves in the dorsal nerves of the penis, around the dorsal arteries, and in the corpora cavernosa. nNOS-containing nerves, endothelial and smooth muscle cells, and MSCs were more abundant in the LI-ESWT group. Such findings support the notion that the mechanism of the therapeutic action of LI-ESWT is the recruitment of MSC, which was postulated by Chen et al. [19].

The Effect of LI-ESWT in Humans

Two well-designed studies have informed on positive effects of LI-ESWT in human patients with severe ischemic heart disease. In the first study, Kikuchi et al. [24] reported that LI-ESWT improved the LV ejection fraction and stroke volume in eight patients, as well as significantly ameliorating the severity of the chest pain after a 6-minute walk. Yang et al. [25] have also reported that LI-ESWT ameliorated the severity of angina pectoris and ischemic heart failure assessments in 25 patients with coronary heart disease. Comparable results have also been reported by Vasyuk et al. [26], Wang et al. [27] and Zimpfer et al. [28] in patients with severe coronary artery disease and refractory angina to whom LI-ESWT was applied. Other reports on the capability of LI-ESWT to heal and repair ischemic wounds were published. Larking et al. [29] examined the effect of LI-ESWT on 16 static chronic ulcers in a placebo-controlled study on a group of patients with complex neurological disabilities. They showed a clear improvement in wound healing and a significant difference compared with placebo after 3 weeks of treatment. A review published on the healing effect of LI-ESWT on chronic ischemic wounds [30] reports very positive results on a wide range of ischemic wounds and ulcers with success rates ranging from the lowest success rate of 36% in venous stasis wounds to 66.7% in decubital ulcers to 100% in burn wounds. In the same publication, they report on success rates of LI-ESWT from nine studies published in peer-reviewed journals on a total of more than 550 patients with chronic soft tissue wounds ranging from 25% [31] to 100% [32].

How Is LI-ESWT Used to Treat ED?

The protocol of LI-ESWT that we selected was based on the accumulated clinical experiences in

which LI-ESWT was used in patients with cardiovascular disease. The protocol required modifications because one of the challenges was to apply LISWs to the whole area of the corpora cavernosa including the crus. Another challenge was to adapt the SW delivery probe to the penis' anatomy because the corpora cavernosa lies immediately under the penile skin. Our final protocol consisted of two treatment sessions per week for three successive weeks, followed by a 3-week no-treatment period, and a repeated twice-weekly treatment session of 3 weeks. After stretching the penis, LISWs (300 SWs/treatment point and 1,500 SWs/session) were applied to three points along the penile shaft and two over the crus at an intensity of 0.09 mJ/mm^2 and a frequency of 120 shocks/minute for about 3 minutes at each point. A water-based gel was spread on and around the penis just before starting the treatment.

The SW Device

For all treatment sessions, LISWs were generated and delivered by an extracorporeal shockwave generator (Omnispec ED1000, Medispec Ltd., Germantown, MD, USA), which is a compact electrohydraulic SW generator with a focused SW source. This device's spark voltage is 10–24 kV and is able to provide SWs with a focal penetration depth of 180 mm and a focal width of 24 mm.

Which Patients Were Eligible for LI-ESWT?

In our studies, only patients who had ED for more than 6 months and were sexually active in a stable heterosexual relationship for more than 3 months were considered for inclusion. Their International Index of Erectile Function questionnaire—Erectile Function (IIEF-EF) domain score had to be below 20, and at least 50% of their sexual intercourse attempts were unsuccessful. Additional inclusion criteria depended on the specific aims of the study (PDE5i responders, PDE5i non-responders, and patients who have had a radical prostatectomy—see Table 1). General exclusion criteria would include ED patients with a neurological pathology, an unstable medical or psychiatric condition, pelvic surgery (other than prostatectomy), or patients who had a spinal cord injury. Patients with an anatomical abnormality of their penis, cancer, or with a cardiovascular condition that prevented normal sexual activity were also excluded.

Evaluation and Outcome Measures

At screening, all patients were thoroughly interviewed about their medical and sexual history and underwent a complete physical examination. The total IIEF questionnaire, the Erection Hardness Scale, the Quality of Erection, the Self-Esteem and Relationship questionnaires, and the Clinical Global Impression of Change rating scale were used to subjectively determine the sexual function of each patient. Objective evaluation parameters included measurement of penile blood flow and endothelial function using plethysmography in all studies, nocturnal penile tumescence (NPT) in some studies, and Doppler ultrasonography to ascertain the cause of ED. All indices of sexual and penile function were obtained before and at least 1 month after LI-ESWT, as well as at 3, 6, 12, 18, and 24 months after treatment completion.

LI-ESWT for ED: Clinical Studies and Results

The aim of our first study was to evaluate the feasibility, efficacy, and safety of LI-ESWT in 20 selected middle-aged men with mild-to-moderate vasculogenic ED [33] who were PDE5i responders. One month after LI-ESWT, erectile function improved in 15 men. The average increase in the IIEF-EF domain score was 7.4 (13.5–20.9, $P = 0.001$). Specifically, the IIEF-EF domain score

Table 1 Study trials

- The Effect of Low-Intensity Extracorporeal Shockwave Therapy for Erectile Dysfunction (ED)—Pilot [33]
- The Effect of Low-Intensity Extracorporeal Shock Wave Therapy on Men with ED Who Respond to PDE5 Inhibitors (PDE5i)—a Double-Blind Placebo-Controlled [35]
- The Effect of Low-Intensity Extracorporeal Shockwave Therapy on Men with ED Who Did Not Respond to PDE5i—Pilot [34]
- Low-Intensity Shock Wave Therapy on Men with ED Who Are Nonresponders to PDE5i—a Double-Blind Placebo-Controlled Study (ongoing—ClinicalTrials.gov Identifier NCT01262157)
- The Effect of Low-Intensity Extracorporeal Shock Wave Therapy on Men with ED Who Respond to PDE5i—a Double-Blind Placebo-Controlled Multicenter Study (ongoing—ClinicalTrials.gov Identifier NCT01274923)
- Low-Intensity Extracorporeal Shock Wave Therapy for the Treatment of ED—a 4-Arm Comparator Study (between different new treatment protocols) (ongoing—ClinicalTrials.gov Identifier NCT01442077)

in 15 men increased by more than 5 points and by more than 10 points in seven men. Five men did not respond to LI-ESWT. Ten men reported that they had erections that were sufficiently rigid for vaginal penetration without PDE5i support. In the 15 men who responded to LI-ESWT, all NPT parameters improved as recorded by significant increases in the duration of the erections and penile rigidity. Finally, penile blood flow and endothelial function in these 15 men had improved significantly at the 1-month follow-up examination.

The aim of our second study was to investigate whether LI-ESWT could convert PDE5i nonresponders to PDE5i responders and enable them to achieve vaginal penetration with oral PDE5i therapy. To this end, we investigated the effect of LI-ESWT in 29 men with severe ED, who had multiple cardiovascular risk factors (23), cardiovascular disease (11), and diabetes mellitus (14), and were PDE5i nonresponders [34]. When these men started the study, the average IIEF-EF domain score was 8.8 ± 1 (with PDE5i). Three months after the completion of LI-ESWT, while using PDE5i, the IIEF-EF domain scores improved by at least 5 points in 76%, and the mean IIEF-EF domain score increased to 18.8 ± 1. At the end of the study, eight men achieved normal erections; their IIEF-EF domain score were greater than 25. Overall, 21 of the 29 men were converted to PDE5i responders. Penile blood flow and endothelial function significantly improved ($P = 0.0001$) in the men who responded to LI-ESWT. A significant correlation between the subjective assessment of sexual function using validated sexual function questionnaires and the objective measures of penile blood flow and endothelial function was found in the two studies. None of the men in the two studies reported treatment-associated pain or any adverse events during or after the treatment.

In view of the reassuring results from these first two studies, we then conducted a prospective, randomized, double-blind, sham-controlled study on 60 men with ED [35]. In this study, we found that mean IIEF-EF scores of the treated men were significantly higher than those of the sham-treated men. This increase was also accompanied by improvement in penile and cavernosal blood flows and penile endothelial function.

In order to establish the overall success rate of LI-ESWT, we then analyzed the data from all the study patients who participated in the different clinical trials and for whom we had follow-up data for at least 6 months (Table 1). This cohort comprised 184 patients of which 127 were PDE5i responders and 57 were PDE5i nonresponders with the following characteristics: their mean age was 58.5 years, their mean ED duration was 65.2 months, 51% had severe ED, 37% had moderate ED, and 12% had mild-to-moderate ED according to their IIEF-EF domain scores, 54.3% had cardiovascular risk factors, and 35.3% had diabetes mellitus. The mean IIEF-EF domain scores improved after LI-ESWT by 7 points with the greatest increase occurring in the men with severe ED (Figure 1). We also found that the increases in the IIEF-EF domain scores in the men who were PDE5i nonresponders were higher than those men who were PDE5i responders (Δ 7.52 vs. Δ 5.7 points, $P = 0.04$).

From this group of 184 men, we had data from the 1-year follow-up evaluation for 86 patients. When we compared 6-month and 12-month follow-up IIEF-EF domain scores of these 86 ED patients, we found that the scores had slightly

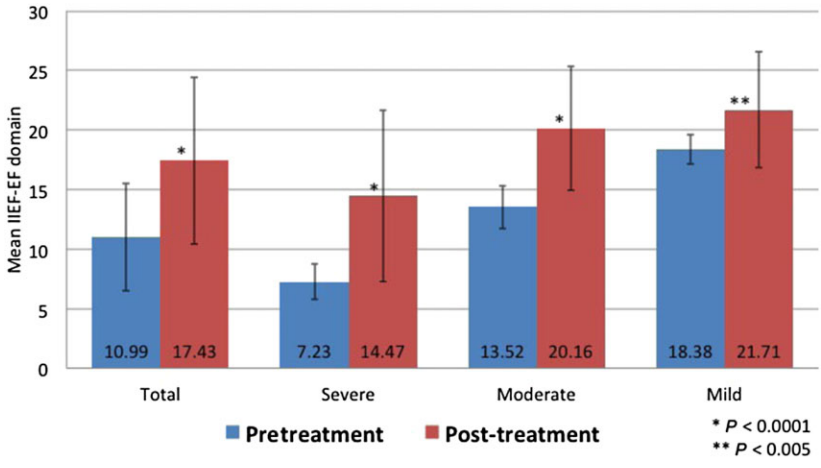

Figure 1 Improvement in International Index of Erectile Function—Erectile Function domain scores from baseline according to severity

increased (17.2 vs. 19.9, $P < 0.01$). We also compared the success of therapy of the treated patients to those who were given the placebo: success, which was defined by an at least 5-point change in the IIEF-EF domain score, occurred in 56% of the treated group, whereas success occurred in only 18% of the placebo-treated men ($P < 0.001$). Because LI-ESWT involves an intense interaction between the patient and the attending physician or nurse, a relatively high placebo effect could be expected. The success rate in 38 men with ED who received the sham-treatment protocol in our study was 21%. This result is less than the 20–35% success rate that is reported in men with ED and pharmacological treatment in other published placebo-controlled studies [36–38].

We are also investigating whether a second round of penile LI-ESWT in patients with ED whose first round of penile LI-ESWT was unsuccessful would be therapeutically beneficial. In this ongoing study, we are witnessing clinical improvements in some of these patients, but analyzed data on both objective and subjective parameters are not yet finalized at this time.

Discussion

After more than a decade of wide experience with oral therapies for ED, it has become evident that PDE5is improve erections but do not treat the underlying mechanism. Undoubtedly, the dependence on drugs for sexual function has its limitations, and a search for a better treatment is the next step in ED management. Although investigations in this direction, such as stem cell therapy or gene therapy are ongoing, no significant progress has yet been made and an ED cure is still being sought. When any new and unconventional therapeutic modality is introduced, it is prone to skepticism and criticism, especially when its mechanism of action is not fully understood and scientifically recognized. This doubt is particularly true when all the published clinical results originate from only one center and the total number of evaluated patients is relatively low. Doubts about any new and controversial therapy can be overcome by conducting larger scale multicenter studies in order to reach conclusive data. Some abstracts have already been published [39–41], and hopefully, this is just the beginning of more studies to come. Although efficacy and safety of LI-ESWT were established in our studies, our protocol was nonstandardized and empirical. Accordingly, information about the optimal treatment protocol, the best anatomical locations to apply the probes, and the amount of energy that needs to be applied at each treatment session still needs to be obtained. We also still do not know which clinical parameters could be used to predict which ED patients would most benefit from LI-ESWT. Finally, there is insufficient data on the mechanism of action of LI-ESWT at the cellular level, a crucial area of further investigation.

In our studies, we have done our best to use strict scientific methodology to collect accurate subjective data from our study population and objective measures in order to validate the efficacy and safety of LI-ESWT in ED. We are also fully aware of the limitations of each of our studies, be it a pilot study or a one-arm comparative study with a small cohort. On the other hand, we have also presented data from well-designed, double-blind, sham-controlled, randomized studies and from a multicenter trial, and the results of these studies are consistent and promising.

Using objective measures, we have shown that LI-ESWT significantly improves penile blood flow and endothelial function, and found that these improvements are positively correlated with the subjective measures of erectile function. These findings, as well as the affirmative reports of improved erection on follow-up visits after LI-ESWT, convey very encouraging indications of treatment success.

Some of the data that we recently collected from patients with 1-year follow-up data after LI-ESWT are reassuring. From their reports, the beneficial effects of LI-ESWT have not diminished or waned. Moreover, some even reported a continual improvement in their erections with time. We would like to emphasize that all our collected data were consistently positive even though they were collected from different studies, with different cohorts, a wide range of ED severity, and a variety of cardiovascular diseases and risk factors. This reproducibility of therapeutic benefit of LI-ESWT in ED adds another layer of evidence that the physiological effect of this unique treatment modality is genuine.

Although our results on the use of LI-ESWT are encouraging, more data from extensive research at both the basic and clinical levels and from other independent studies need to be accumulated before we include this treatment modality in the armamentarium of therapies for ED.

Summary

LI-ESWT is a new therapeutic option for rehabilitating the erectile mechanism and restoring erectile function in men with vasculogenic ED. The main characteristic of this revolutionary treatment is its potential to restore erectile function in these patients without any side effects and the need for a PDE5i. Because of its unique mechanism of action, LI-ESWT could be used to amplify the partial response to current treatments. Finally, LI-ESWT could also be used to prevent the progression of ED. With the acquisition of new knowledge on LI-ESWT, we anticipate that that this novel therapy will be widely used in the future in the clinical management of ED.

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Effects of Low-Energy Shockwave Therapy on the Erectile Function and Tissue of a Diabetic Rat Model

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ABSTRACT

Introduction. Low-energy shockwave therapy (LESWT) has been shown to improve erectile function in patients suffering from diabetes mellitus (DM)-associated erectile dysfunction (ED). However, the underlying mechanism remains unknown.

Aim. The aim of this study is to investigate whether LESWT can ameliorate DM-associated ED in a rat model and examine the associated changes in the erectile tissues.

Methods. Newborn male rats were intraperitoneally injected with 5-ethynyl-2-deoxyuridine (EdU; 50 mg/kg) for the purpose of tracking endogenous mesenchymal stem cells (MSCs). Eight weeks later, eight of these rats were randomly chosen to serve as normal control (N group). The remaining rats were injected intraperitoneally with 60 mg/kg of streptozotocin (STZ) to induce DM. Eight of these rats were randomly chosen to serve as DM control (DM group), whereas another eight rats were subject to shockwave (SW) treatment (DM+SW group). Each rat in the DM+SW group received 300 shocks at energy level of 0.1 mJ/mm^2 and frequency of 120/minute. This procedure was repeated three times a week for 2 weeks. Another 2 weeks later, all 24 rats were evaluated for erectile function by intracavernous pressure (ICP) measurement. Afterward, their penile tissues were examined by histology.

Main Outcome Measures. Erectile function was measured by ICP. Neuronal nitric oxide synthase (nNOS)-positive nerves and the endothelium were examined by immunofluorescence staining. Smooth muscle and MSCs were examined by phalloidin and EdU staining, respectively.

Results. STZ treatment caused a significant decrease in erectile function and in the number of nNOS-positive nerves and in endothelial and smooth muscle contents. These DM-associated deficits were all partially but significantly reversed by LESWT. MSCs (EdU-positive cells) were significantly more numerous in DM+SW than in DM rats.

Conclusion. LESWT can partially ameliorate DM-associated ED by promoting regeneration of nNOS-positive nerves, endothelium, and smooth muscle in the penis. These beneficial effects appear to be mediated by recruitment of endogenous MSCs. **Qiu X, Lin G, Xin Z, Ferretti L, Zhang H, Lue TF, and Lin C-S. Effects of low-energy shockwave therapy on the erectile function and tissue of a diabetic rat model. J Sex Med 2013;10:738–746.**

Key Words. Low-Energy Shockwave; Diabetes Mellitus; Erectile Dysfunction

Introduction

Erectile dysfunction (ED) is a prevailing health problem that seriously impacts the quality of life of men and their spouses or partners [1]. Although the majority of ED patients can be satisfactorily treated with phosphodiesterase type 5 inhibitors (PDE5), a substantial population (30–40%) cannot [2]. This includes patients who are intolerant to PDE5 inhibitors' side effects, taking nitrate medication for angina, or having certain types of ED refractory to PDE5 inhibitors. In particular, diabetes mellitus (DM) and surgery-induced cavernous nerve injuries (mainly due to radical prostatectomy) are currently the most

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common causes of refractory ED [2]. To treat these types of ED, one of the proposed strategies is low-energy shockwave therapy (LESWT), as seen in recently published studies [3–5] and ongoing clinical trials (NCT01317693, NCT01274923, NCT01442077, and NCT01317680 at http://clinicaltrials.gov). In one study involving 29 severe ED patients, LESWT was found to substantially increase erectile function scores with concomitant improvement of penile hemodynamics [3]. Noteworthy is that the majority of these patients (21 out of 29) were diabetic, and thus their positive response to LESWT raises the question whether LESWT is specifically effective for treating DM-associated ED. Although the answer awaits further clinical studies, it should be pointed out that, despite successful demonstration in clinical trials, LESWT as an ED treatment modality has not been investigated at the basic science level and has no known mechanistic basis.

LESWT has been investigated in animal models of heart failure [6], coronary arterial disease [7], ischemic myocardial dysfunction [8], ischemic tissue necrosis [9], chronic hind limb ischemia [10], and bone defects [11]. The outcomes invariably point to induction of angiogenesis as one of the underlying mechanisms for LESWT's therapeutic effects. In addition, one of these studies also identified recruitment of mesenchymal stem cells (MSCs) as a possible mechanism [11]. In ED field, angiogenesis is known to play important roles for the therapeutic effects of growth factors and gene therapies [12–14], and both exogenously applied and endogenously recruited MSCs have been shown to enhance recovery of erectile function in ED animal models [15,16]. Thus, the observed therapeutic effects of LESWT in ED patients are likely mediated by angiogenesis and MSC recruitment. In the present study, we tested this hypothesis; specifically, we investigated the effects of LESWT on erectile function and related tissue structures in a streptozotocin (STZ)-induced DM rat model. We also examined whether LESWT enhanced MSC recruitment by using the label-retaining cell (LRC) strategy [17] with 5-ethynyl-2-deoxyuridine (EdU) being the thymidine analog for such labeling [18].

Materials and Methods

Animals

All animal experiments in this study were approved by the Institutional Animal Care and Use Committee at our institution. Pregnant Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA, USA) for the investigation of childbirth-related urinary incontinence in separate projects. Their newborn male rats were used for this study. For the purpose of tracking endogenous MSCs, each pup received intraperitoneal injection of EdU (50 mg/kg, Invitrogen, Carlsbad, CA, USA) immediately after birth, as described previously [18–20]. At 8 weeks of age, eight of these rats were randomly selected to serve as normal control (N). The remaining rats were each injected intraperitoneally with 60 mg/kg of STZ (Sigma-Aldrich, St. Louis, MO, USA), and their blood glucose level was monitored weekly by checking tail vein blood with Accutrend strip (Roche Diagnostics, Indianapolis, IN, USA). Rats with fasting blood glucose of $\geq$200 mg/dL were designated as diabetic and selected for further tests. A total of 16 such rats were equally randomized into a diabetic group (DM) and a diabetic plus LESWT group (DM+SW).

Shockwave Treatment

Four weeks post-STZ injection, rats in the DM+SW group were treated with shockwaves as depicted in Figure 1 and explained in the following. Under anesthesia, each rat was placed in a supine position, its lower abdomen shaved, and its penis drawn out of the prepuce and held in place with a loop made of suture line and syringe. After application of ultrasound gel (Aquasonic, Parker Laboratories, Inc, Fairfield, NJ, USA) on the penis, a shockwave applicator (DermaGold, MTS

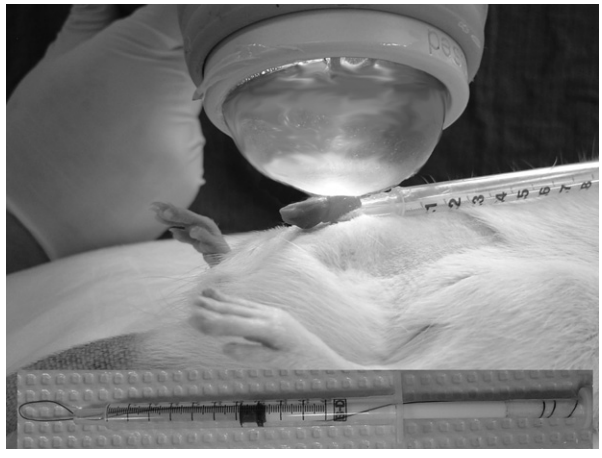

Figure 1 Shockwave application to the rat penis. Under anesthesia, the penis was drawn out of the prepuce, held in place with a loop made of suture line and syringe (shown in inset), applied with ultrasound gel, and treated with shockwave.

Europe GmbH, Konstanz, Germany) was placed in contact with the penis, and a total of 300 shocks were delivered at energy level of 0.1 mJ/mm^2 and frequency of 120/minute. This procedure was repeated three times a week for 2 weeks, and the entire treatment course is comparable with clinical shockwave treatment for ED patients [3–5]. Due to the fact that DermaGold is clinically approved to treat superficial wounds, its delivered shockwave is expected to penetrate a few centimeters (probably the thickness of a rat penis) in the contacted area.

Erectile Function Evaluation

Two weeks after the final shockwave treatment for rats in the DM+SW group, all 24 rats (in N, DM, and DM+SW groups) were tested for erectile function by measuring intracavernous pressure (ICP) in response to electrostimulation of cavernous nerves. Briefly, under ketamine (100 mg/kg) and midazolam (5 mg/kg) anesthesia, the cavernous nerves were exposed via midline laparotomy. The corpus cavernosum was cannulated with a heparinized (200 U/mL) 25G needle connected to a pressure transducer (Utah Medical Products, Midvale, UT, USA). The stimulus parameters were 20 Hz, pulse width of 0.2 ms, and duration of 50 seconds with three different current settings: 0.5 mA, 1.0 mA, and 1.5 mA. The maximum increase of ICP of three stimuli per side was selected for statistical analysis in each animal. ICP was normalized to mean arterial pressure (MAP), which was recorded using a 25G needle inserted into the aortic bifurcation.

Histology

At the conclusion of erectile function evaluation, the rats were sacrificed, and their penises were harvested for histology. The penis (mid-shaft portion) was fixed with 2% formaldehyde and 0.002% picric acid in 0.1 M phosphate-buffered saline (PBS) for 4 hours, followed by immersion in 30% sucrose in PBS overnight at 4°C. The fixed tissue was then embedded in optimal cutting temperature compound (Sakura Finetek, Torrance, CA, USA), cut into 5-μm-thick sections, mounted on glass slides (3 sections per slide), and subjected to immunofluorescent (IF) and EdU staining. For IF staining, the slides were placed in 0.3% H_2O_2/methanol for 10 minutes, washed twice in PBS for 5 minutes, and incubated with 3% horse serum in PBS/0.3% Triton X-100 for 30 minutes at room temperature. After draining this solution from the tissue section, the slides were incubated at room temperature with rabbit antineuronal nitric oxide synthase (nNOS) (1:100, SC-648, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or mouse antirat endothelial cell antigen (RECA; 1:500; MCA-970R, AbD Serotec, Raleigh, NC, USA) antibody overnight. Control tissue sections were similarly prepared except no primary antibody was added. After rinses with PBS, the sections were incubated with Alexa-488- or Alexa-594-conjugated secondary antibody (Invitrogen). Smooth muscle was stained by incubation with Alexa-488-conjugated phalloidin (Invitrogen) for 20 minutes at room temperature. Nuclei were stained with 4′,6-diamidino-2-phenylindole (Invitrogen). For tracking EdU-positive cells, tissue sections were incubated with Click-IT reaction cocktail (Invitrogen) for 30 minutes at room temperature.

Image Analysis and Quantification

The stained tissues were examined with Nikon Eclipse E600 fluorescence microscope (Nikon Instruments, Melville, NY, USA) and photographed with Retiga 1300 Q-imaging camera (Nikon Instruments Inc.) using the ACT-1 software (Nikon Instruments Inc.). For evaluation of cavernous smooth muscle and endothelial contents, two fields (both sides of the corpus cavernosum at 200× magnification) on each tissue section were photographed. For evaluation of arterial endothelial content, two arteries within the corpus cavernosum at 1000× magnification on each tissue section were photographed. For evaluation of dorsal nerve nNOS expression, two fields (the two largest dorsal nerve branches at 400× magnification) on each tissue section were photographed. For evaluation of nNOS expression around dorsal arteries, the two dorsal arteries on each tissue section at 400× were photographed. For evaluation of nNOS expression in the corpus cavernosum, two fields (both sides of the corpus cavernosum at 400× magnification) on each tissue section were photographed. In each of these photographic recordings, the images were generated in green, red, and blue channels, and these single-color images were then superimposed to generate the multicolor figures. For quantification, the single-color images were analyzed with ImagePlus 5.1 software (Media Cybernetics, Bethesda, MD, USA). To quantify cavernous endothelium, RECA-stained area (in red) was measured and expressed as pixel number. To quantify arterial endothelium, RECA-stained area (in red) was measured and expressed as a ratio (in percentage)

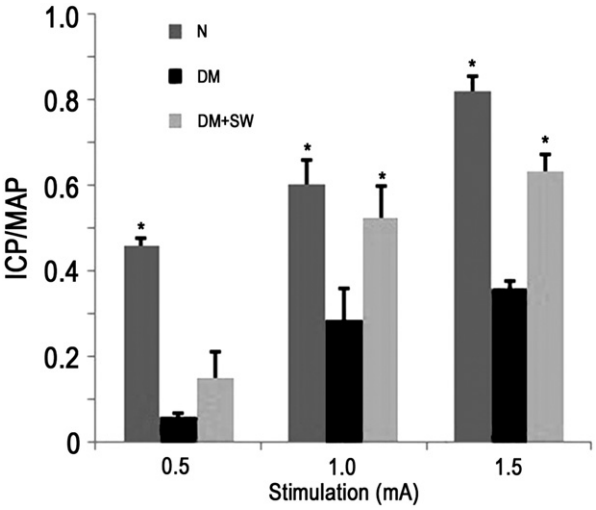

Figure 2 Evaluation of erectile function. Rats in N group (N = 8) were normal control. Rats in DM group (N = 8) were diabetic. Rats in DM+SW group (N = 8) were diabetic and treated with shockwaves. Their erectile function was evaluated as response in ICP to electrostimulation of cavernous nerves at three different amperages (0.5, 1.0, and 1.5). ICP was normalized with MAP. "*" denotes $P < 0.05$ when compared with the DM group. ICP = intracavernous pressure; MAP = mean arterial pressure.

to the phalloidin-stained area. To quantify nNOS expression, nNOS-stained dots (in red) were manually counted. To quantify cavernous smooth muscle, phalloidin-stained area (in green) was measured and expressed as a percentage of the entire corpus cavernosa.

Statistical Analysis

Data were analyzed using Prism 5 (GraphPad Software, San Diego, CA, USA) and expressed as mean ± standard error of mean (SEM). Multiple groups were compared using one-way analysis of variance followed by the Tukey–Kramer test for post hoc comparisons. Statistical significance was set at $P < 0.05$.

Results

LESWT Improves Erectile Function in Diabetic Rats

STZ treatment significantly impaired erectile function as seen in the sharp decline of the ICP/MAP value in DM rats vs. normal control (Figure 2). LESWT significantly restored erectile function to levels similar to normal control (at settings of 1.0 and 1.5 mA; Figure 2).

LESWT Promotes Nerve Regeneration

STZ treatment caused significant decreases of nNOS-containing nerves in the penis (Figure 3). LESWT partially but significantly restored these nNOS-positive nerves in the sinusoids, around the dorsal arteries, and within the dorsal nerves (Figure 3).

LESWT Restores Endothelial and Smooth Muscle Contents

STZ treatment caused a significant decrease of endothelial content in both the cavernous sinusoids and small arteries and which was partially but significantly reversed by LESWT (Figure 4). Likewise, STZ treatment caused a significant reduction of cavernous smooth muscle content and which was partially but significantly reversed by LESWT (Figure 5).

LESWT Enhances Recruitment of MSCs

MSCs, recognized by their ability to retain thymidine analog EdU, were significantly more numerous in the penis of rats in the DM+SW group than in the DM group (Figure 6).

Discussion

Despite tremendous advances in the management of ED in the past decade, DM-associated ED remains difficult to treat. To overcome this obstacle, one of the proposed therapeutic strategies is stem cell therapy, which has been actively pursued in several clinical and preclinical trials [16]. Another lesser known strategy is LESWT, which has been tested in clinical trials, but in sharp contrast to stem cell therapy, has not been investigated at the preclinical level. Thus, the present study was designed to provide, for the first time, a mechanistic basis for LESWT's therapeutic effects by using a well-established STZ-induced DM-ED rat model.

STZ-induced diabetic rats have been consistently shown to have poor erectile function [21,22]. In the present study, we further confirmed this observation, and more importantly, we showed that LESWT significantly improved erectile function in STZ-induced diabetic rats. It has also been known that STZ treatment caused a significant loss of nNOS-positive nerves in rat penis [22,23], and a recent study also identified a significant reduction of nNOS-positive nerves in the penis of diabetic patients [24]. In the present study, we showed that, when compared with DM rats, shockwave-treated rats displayed significantly higher numbers of nNOS-positive nerves in different compartments of the erectile tissue, including the dorsal nerves, around the dorsal arteries,

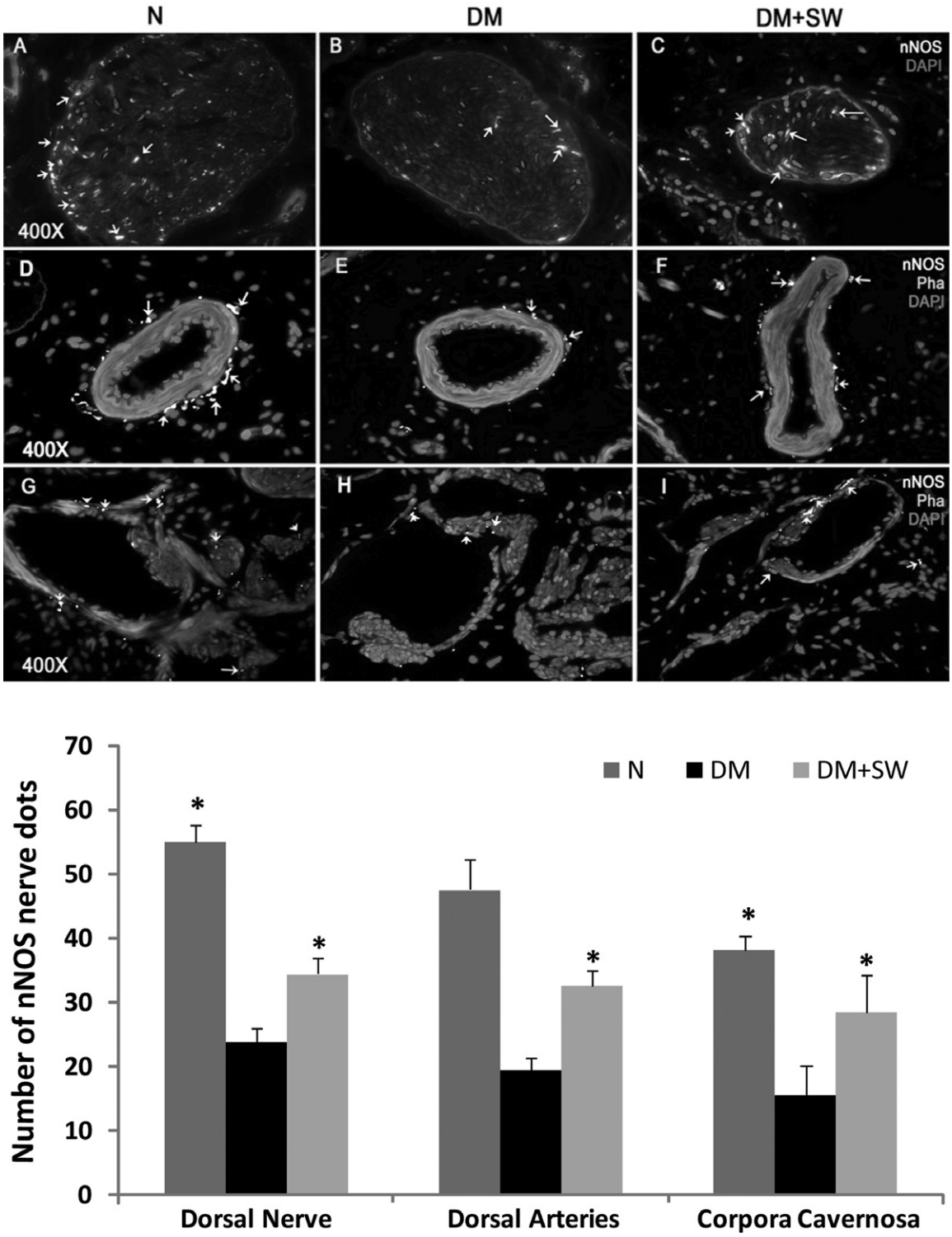

Figure 3 Evaluation of nNOS expression. Rats were grouped and treated as described in Figure 1. Their penile tissues were examined by IF staining for nNOS expression. The results are shown in the representative histological images with red, green, and blue stains indicating nNOS-positive nerves, smooth muscle, and cell nuclei, respectively. For clarity, the histological images are divided into the dorsal nerves (panels A–C), the dorsal arteries (panels D–F), and the sinusoids (panels G–I). White arrows point at representative nNOS-positive dots. Quantitative data of nNOS expression in these three tissue compartments are shown in the bar chart with the asterisk denoting $P < 0.05$ when compared with the DM group. IF = immunofluorescence; DAPI = 4′,6-diamidino-2-phenylindole; nNOS = neuronal nitric oxide synthase

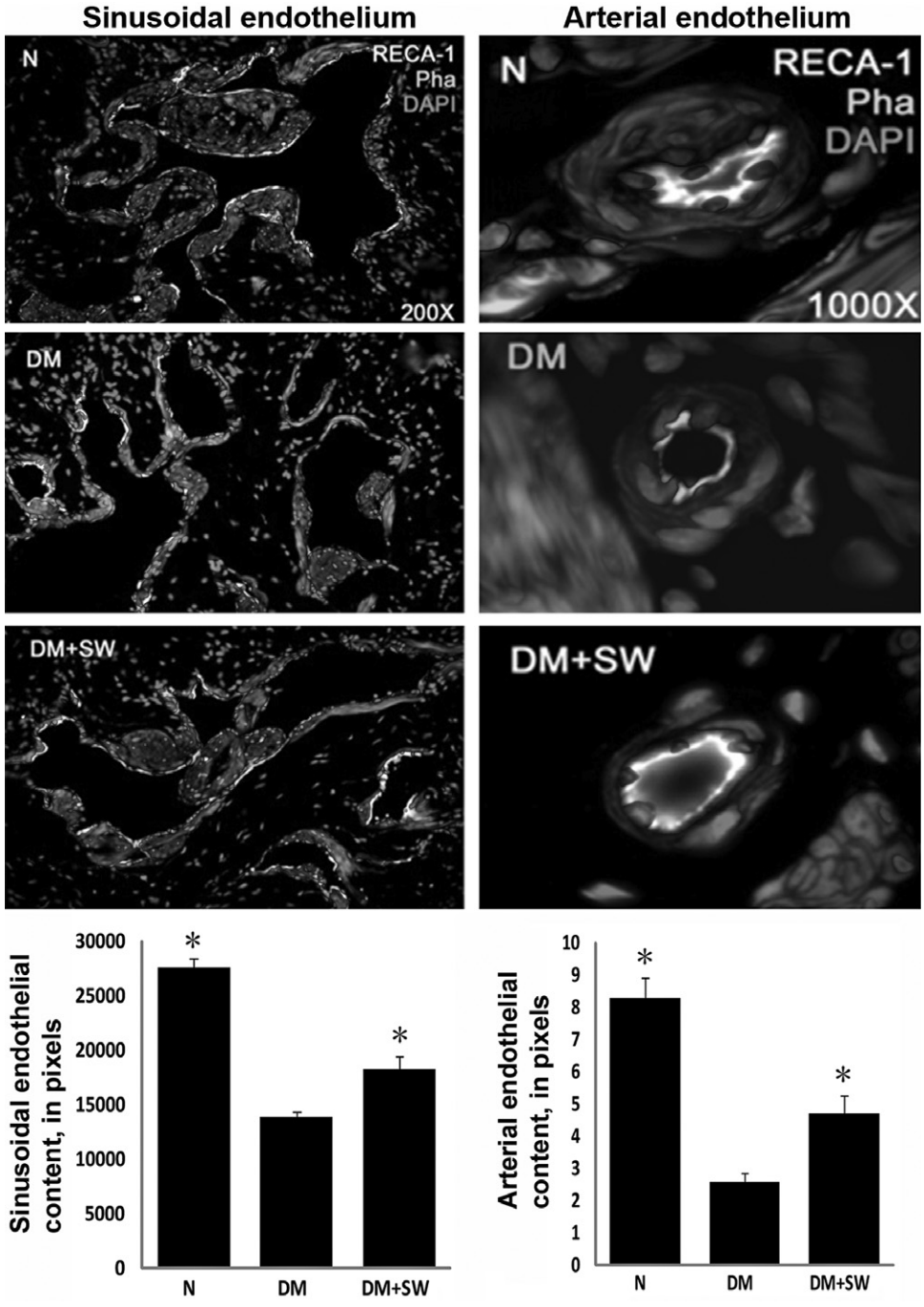

Figure 4 Evaluation of endothelial content. Rats were grouped and treated as described in Figure 1. Their penile tissues were examined by IF staining for RECA expression. The results are shown in the representative histological images with red, green, and blue stains indicating the endothelium, smooth muscle, and cell nuclei, respectively. Quantitative data of RECA expression in cavernous sinusoids and arteries are shown in the left and right bar charts, respectively, with the asterisk denoting $P < 0.05$ when compared with the DM group. IF = immunofluorescence; RECA = rat endothelial cell antigen; DAPI = 4′,6-diamidino-2-phenylindole

and in the corpora cavernosa. This preservation of nNOS-positive nerves thus appears to be an underlying mechanism for LESWT's therapeutic effects on diabetic patients.

Endothelial injury and dysfunction in cavernous tissue have been consistently identified in diabetic men with ED and in diabetic animal models [25]. Specifically, a reduced cavernous endothelial

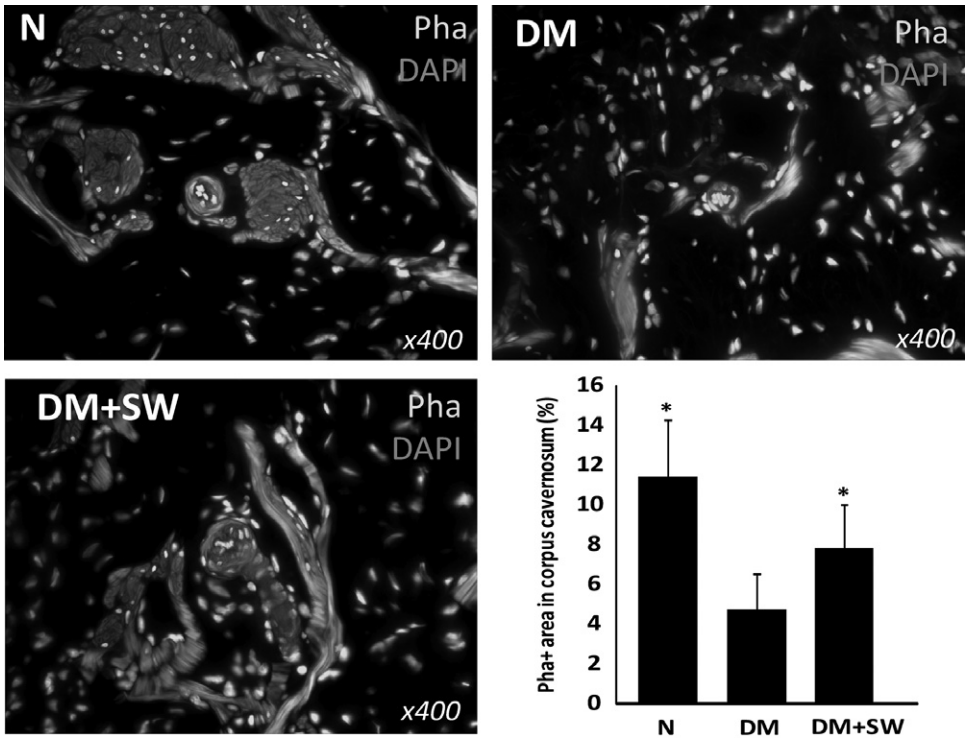

Figure 5 Evaluation of smooth muscle content. Rats were grouped and treated as described in Figure 1. Their penile tissues were examined by fluorescent phalloidin staining for smooth muscle. The results are shown in the representative histological images with green and blue stains indicating the smooth muscle and cell nuclei, respectively. Quantitative data of cavernous smooth muscle content are shown in the bar chart with the asterisk denoting $P < 0.05$ when compared with the DM group. DAPI = 4′,6-diamidino-2-phenylindole

content is one of the most consistent features of STZ-induced diabetic rats [22,26]. In the present study, we found that the endothelial contents in both the cavernous sinusoids and arteries were significantly reduced in STZ-treated rats. More importantly, we also found that LESWT was able to significantly restore the endothelial contents in both of these two tissue compartments. Thus, protection or regeneration of the endothelium represents another possible underlying mechanism for LESWT's therapeutic efficacy in diabetic patients. In addition, it has also been shown that diabetic men and animals have reduced cavernous smooth muscle content [22,26,27]. In the present study, we confirmed this finding in the STZ-treated rats, and more importantly, we showed that LESWT was able to significantly restore the smooth muscle content.

In all ED-related stem cell studies that have performed histological examination of the erectile tissue, restoration of nNOS-positive nerves, the endothelium, and the smooth muscle has also been consistently observed [16]. In addition, these studies also invariably pointed out that the beneficial tissue effects were likely mediated by stem cell's paracrine capacity [16]. On the other hand, in non-ED fields, the involvement of stem cells in the therapeutic effects of LESWT has been observed in two instances. In one study of a rat model of bone defects, LESWT was found to result in the recruitment of MSCs and increased expression of Transforming growth factor-beta (TGF-β) and vascular endothelial growth factor (VEGF) in the defect tissues [11]. In another study of a rat model of chronic hind limb ischemia, LESWT was also found to enhance recruitment of endothelial progenitor cells in the ischemic tissue [10]. Thus, it is conceivable that the tissue effects of LESWT as observed in the present study might have a stem cell component.

LRC is a frequently employed strategy for the identification of resident or migrated stem cells in postnatal tissues [18–20]. It commonly involves the injection of thymidine analog bromodeoxyuridine (BrdU) in neonatal animals, followed by the immunohistochemical localization of BrdU in the tissue of interest weeks or months later. Because detection of BrdU is technically difficult, we recently introduced another thymidine analog EdU as a replacement for BrdU [18–20]. In the

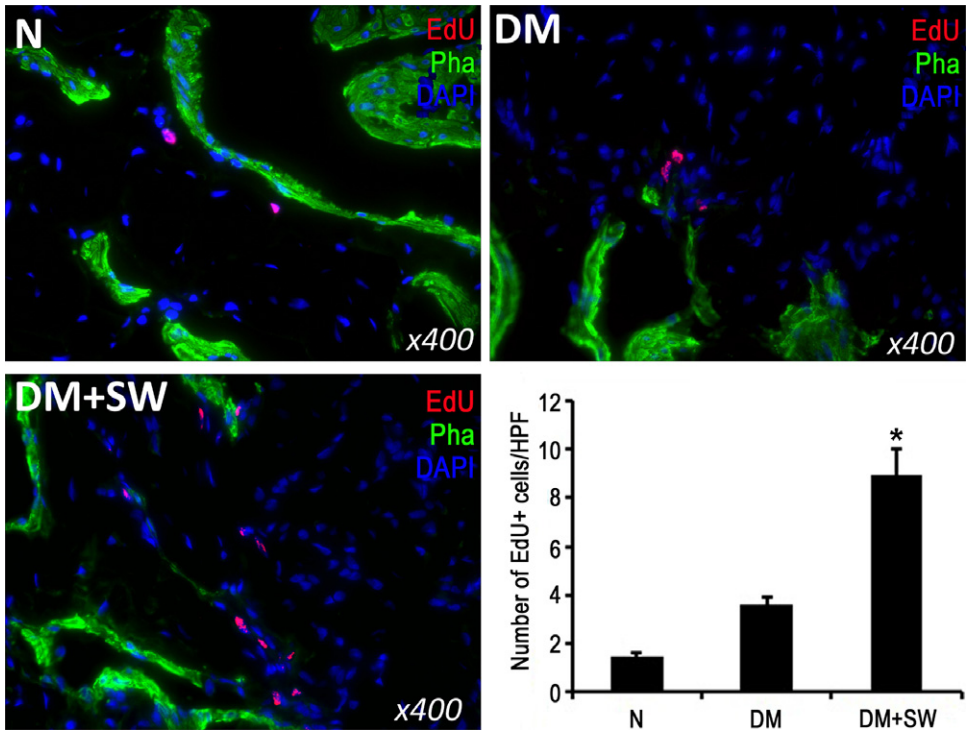

Figure 6 Evaluation of label-retaining cells. Rats were intraperitoneally injected with thymidine analog EdU immediately after birth. They were then grouped and treated as described in Figure 1. At 14 weeks post-EdU injection, their penile tissues were examined by fluorescent chemical staining for EdU-positive cells. The results are shown in the representative histological images with red, green, and blue stains indicating EdU-positive cells, smooth muscle, and cell nuclei, respectively. Quantitative data of EdU-positive cells are shown in the bar chart with the asterisk denoting $P < 0.05$ when compared with the DM group. HPF = high power field (400×); EdU = 5-ethynyl-2-deoxyuridine

present study, we repeated this strategy for the identification of migrated stem cells in the rat penis. The results show that diabetic rats with LESWT had a significantly higher number of EdU+ cells in the penis than diabetic rats without LESWT, suggesting an increased recruitment of MSCs.

In summary, the present study showed that STZ-induced DM is associated with ED and reduced erectile components (nerves, endothelium, and smooth muscle), and LESWT was able to partially but significantly restore these function and tissues. Furthermore, we also showed that these beneficial effects of LESWT were possibly mediated by increased recruitment of MSCs into the erectile tissue. However, it should be cautioned that the present study is still preliminary and requires further validation. Specifically, at 4 weeks after STZ treatment, the nitrergic degeneration has not yet taken place [23], but in clinical situations, most patients already have nitrergic degeneration and hence no response to PDE5. Thus, in future studies, extended time points should be explored in order to better simulate the clinical situation. In addition, the effect of LESWT on the vascular supply to the penis and major pelvic ganglion should be further investigated, and Western blot analysis should be employed to more accurately quantify the nNOS, endothelial, and smooth muscle contents. Finally, the identity and properties of the EdU+ cells (paracrine? differentiation?) also need to be investigated.

Conclusions

LESWT's therapeutic efficacy for DM-associated ED is possibly mediated by increased recruitment of MSCs that promote the regeneration of DM-damaged erectile tissues.

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Statement of Authorship

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Platinum Priority – Sexual Medicine

Editorial by Andrea Salonia on pp. 1017–1019 of this issue

Minimal Clinically Important Differences in the Erectile Function Domain of the International Index of Erectile Function Scale

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Abstract

Background: Despite widespread adoption of the six-item erectile function (EF) domain of the International Index of Erectile Function (IIEF) as a clinical trial end point, there are currently no objective data on what constitutes a minimal clinically important difference (MCID) in the EF domain.
Objective: Estimate the MCID for the IIEF EF domain.
Design, setting, and participants: Anchor-based MCIDs were estimated using data from 17 randomized, double-blind, placebo-controlled, parallel-group clinical trials of the phosphodiesterase type 5 inhibitor (PDE5-I) tadalafil for 3345 patients treated for 12 wk.
Measurements: The anchor for the MCID is the minimal improvement measure calculated using change from baseline to 12 wk on IIEF question 7: "Over the past 4 weeks, when you attempted sexual intercourse how often was it satisfactory for you?" MCIDs were developed using analysis of variance (ANOVA)– and receiver operating characteristic (ROC)–based methods in a subset of studies ($n = 11$) by comparing patients with and without minimal improvement ($n = 863$). MCIDs were validated in the remaining six studies ($n = 377$).
Results and limitations: The ROC-based MCID for the EF domain was 4, with estimated sensitivity and specificity of 0.74 and 0.73, respectively. MCIDs varied significantly ($p < 0.0001$) according to baseline ED severity (mild: 2; moderate: 5; severe: 7). MCIDs consistently distinguished between patients in the validation sample classified as no change or minimally improved overall and by geographic region, ED etiology, and age group. MCIDs did not differ by age group, geographic region, or ED etiology. Current analyses were based on 17 clinical trials of tadalafil. Results need to be replicated in studies using other PDE5-Is or in nonpharmacologic intervention studies.
Conclusions: The contextualization of treatment-related changes in terms of clinically relevant improvement is essential to understanding treatment efficacy, to interpreting results across studies, and to managing patients effectively. This analysis provides, for the first time, anchor-based estimates of MCIDs in the EF domain score of the IIEF.

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1. Introduction

Self-report measures, often referred to as *patient-reported outcomes* (PROs) [1], are widely used in sexual medicine research [2,3]. Among PRO measures used in assessing sexual function, none is more widely used currently than the International Index of Erectile Function (IIEF) [4]. The IIEF was recommended by the International Consultation on Sexual Medicine in 2004 and 2010 as the gold standard self-report questionnaire for measuring erectile function (EF) in clinical trials and observational studies and has been accepted and recommended by regulatory agencies

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worldwide for approval of erectile dysfunction (ED) therapies. A recent PubMed search indicated >1400 citations of the IIEF since its development in 1996. Recently, an abbreviated version of the IIEF [5] has been adopted as a screening tool for clinicians. Multiple validation studies and systematic reviews of the IIEF have been published supporting its use in both clinical and research settings [3,6,7].

The EF domain of the IIEF [4] is the primary measurement domain of the IIEF and is a commonly used primary end point in clinical trials of ED. The psychometric properties of the EF domain have been extensively reported [3–7], but surprisingly, there are no studies in the literature of the minimal amount of change needed in the EF domain to be clinically meaningful to patients. This is commonly referred to as the *minimal clinically important difference* (MCID), which has been defined as the smallest difference in a score in the domain of interest that patients perceive as beneficial and that would mandate, in the absence of side effects and excessive cost, a change in the patient's management [8]. Identifying a clinically meaningful change in the EF domain of the IIEF is critical to understanding efficacy, to interpreting study results, and to managing patients.

The primary objective of this analysis was to estimate the MCID for the EF domain of the IIEF using anchor-based methods (favored by regulatory agencies [1] and clinical investigators) in 17 randomized, double-blind, placebo-controlled, parallel-group clinical trials with virtually identical designs that assessed the efficacy of the phosphodiesterase type 5 inhibitor (PDE5-I), tadalafil for use in men with ED.

2. Methods

2.1. Study design

An integrated post hoc analysis was performed according to a prespecified statistical analysis plan on data collected from 17 randomized, double-blind, placebo-controlled, parallel-group clinical trials with identical designs that were conducted at 148 centers in North and South America, Europe, Asia, and Australia from 1999 to 2004. Details about the general study design, efficacy and safety measures, and statistical analyses have been published in previous integrated analyses of 5 [9] and 11 [10,11] tadalafil trials.

Briefly, following a screening visit, patients who made at least four attempts at sexual intercourse during a 4-wk treatment-free run-in period were randomly allocated to 12 wk of treatment with placebo (n = 1002) or on-demand tadalafil at fixed doses of 10 mg (n = 527) or 20 mg (n = 1816). Patients were seen at 4-wk intervals until they completed the study or discontinued early for any reason. All 17 studies included IIEF, Sexual Encounter Profile (SEP), and Global Assessment Question (GAQ) as efficacy measures.

Studies were approved by institutional review boards, and each patient gave written informed consent. Studies were conducted in accordance with the Declaration of Helsinki and guidelines for Good Clinical Practice.

2.2. Study population

Men aged >18 yr who had a minimum 3-mo history of mild to severe ED of organic, psychogenic, or mixed etiology (as determined by the investigator) with a steady female partner were eligible to participate in these studies. Patients were excluded if they failed to achieve erection following radical prostatectomy or pelvic surgery, had clinically significant penile deformities or penile implants, had clinically significant renal or hepatic insufficiency, or had a recent history of spinal cord trauma. Patients were also excluded from trials if they had an underlying cardiovascular disorder sufficiently severe or unstable to make sexual intercourse unadvisable (eg, unstable angina, recent myocardial infarction or stroke, recent myocardial revascularization, poorly controlled blood pressure). Men treated with nitrates, antiandrogens, or cancer chemotherapy also were excluded from study.

2.3. Outcomes

The IIEF is a self-administered questionnaire that assesses five domains of male sexual function, including EF, orgasmic function, sexual desire, intercourse satisfaction, and overall satisfaction. The IIEF was administered at baseline and following treatment. Values on the EF domain were set to missing if more than one of the individual items was missing; if the answer to one item was missing, then the average of the remaining items was imputed. Lower EF domain scores indicate more severe ED.

For the purposes of determining the MCID of the EF domain, the change from baseline to week 12 of the EF domain score was used in calculations and models. In the event that the patient discontinued the study prior to week 12 or the week 12 assessment is otherwise missing, the last observed postbaseline value was analyzed.

2.4. Anchor

The clinical anchor is the minimal improvement measure calculated using IIEF question 7 (Q7): "*Over the past 4 weeks*, when you attempted sexual intercourse how often was it satisfactory for *you*?" The following responses are possible: 0, "Did not attempt intercourse"; 1, "Almost never or never"; 2, "A few times (much less than half the time)"; 3, "Sometimes (about half the time)"; 4, "Most times (much more than half the time)"; and 5, "Almost always or always." This item was selected as relevant to the US National Institutes of Health (NIH) definition of ED involving ability to have satisfactory intercourse [12] and its prior use in the development and validation of severity cut points on the EF domain [13]. Minimal improvement in the anchor from baseline to week 12 was defined as a change from little or no satisfactory intercourse at baseline (either 1, "almost never," or 2, "a few times") to satisfactory intercourse sometimes (3, "sometimes"). No change at week 12 was defined as a rating of 1 or 2 at baseline, followed by a similar rating of 1 or 2 at week 12 (see Fig. 1). This measure is later referred to as the *Q7 measure of minimal improvement* (Q7MMI). Patients whose baseline response was 0, "Did not attempt intercourse," were not included in these analyses. Patients with a week 12 score >3 were excluded because these patients would have experienced more than a minimal improvement in intercourse satisfaction.

2.5. Development and validation data sets

Patients were selected for this analysis if, based on the IIEF Q7 responses, they had little or no satisfactory intercourse at baseline (either 1, "almost never," or 2, "a few times") and no more than satisfactory intercourse sometimes at week 12 (3, "sometimes"), regardless of treatment group assignment. A total of 1240 of the 3345 patients in the 17-study data set qualified according to these criteria. Only 93 (2.8%) of the patients in the 17-study data set had missing data for Q7 at either baseline or week 12.

The database of 17 studies was divided into two main groups: the development data set, in which the MCID would be derived, and the validation data set, in which the estimates of the MCID would be evaluated for external validity, convergent validity, and consistency. The development data set was based on 11 of 17 randomly selected studies.

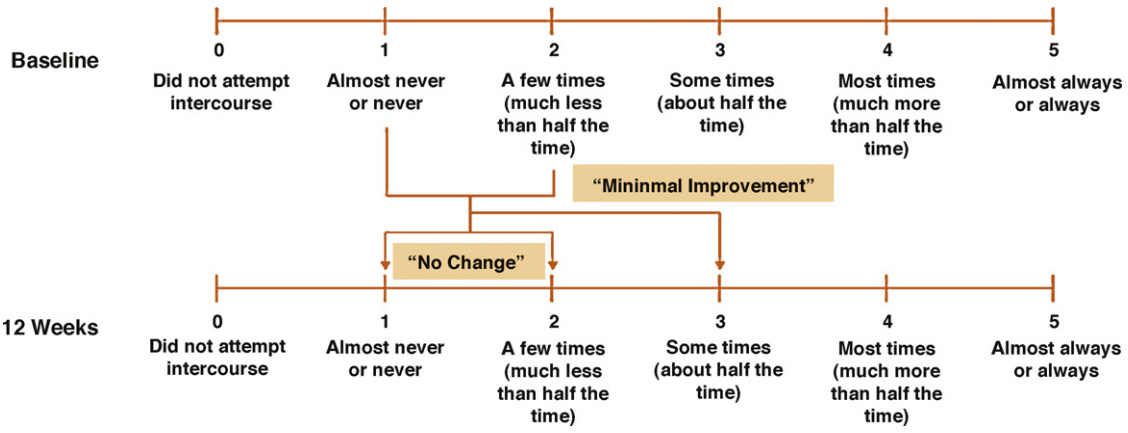

Fig. 1 – The clinical anchor or the measure of minimal improvement in question 7 of the International Index of Erectile Function: "Over the past 4 weeks, when you attempted sexual intercourse how often was it satisfactory for you?".

Of the 1240 patients in the analysis, the development and validation data sets included 863 patients (70%) and 377 patients (30%), respectively.

2.6. *Statistical analyses*

The clinical anchor for developing the MCID was the Q7MMI. The association between minimal important changes in the anchor (independent variable) and change in EF domain score (dependent variable) was assessed preliminarily by two-sample *t* test of the mean EF domain scores among patients with and without minimal important changes in the anchor (later referred to as the analysis of variance [ANOVA] approach) [8,14,15] and analysis of covariance (ANCOVA) models (adjusted for baseline EF domain score [16]), from which MCIDs were estimated. Receiver operating characteristic (ROC) analyses [15,17] were also used to estimate MCIDs, whereby the Q7MMI was regressed on a dichotomized version of the EF domain score, using logistic regression. The MCID was defined as the cut-off value for which the sum of sensitivity and specificity is maximized or, alternatively stated, where the most patients are correctly classified by the cut-off of the IIEF EF domain as having improved versus not having improved.

Convergent validity of the MCID was examined by showing that groups identified by the MCID were consistent with responses in the active treatment and placebo groups. The MCID would show convergent validity if the MCID cut-off discriminated between responses in the placebo versus active treatment groups. A figure displaying box plots of the change from baseline of the EF domain for each treatment group is presented, with reference lines for 0 (no change) and for the IIEF Q7-derived MCIDs using the ANOVA and ROC approaches.

The validation sample was used to replicate and confirm MCID values derived in the development sample. Subpopulations were defined according to (1) geographic region (North America, South America, Europe, Asia, and Australia); (2) age group (<50, 50–64, >65); etiology of ED (organic, psychogenic, mixed); (3) ED duration (3 to <6 mo, 6 mo to <1 yr, ≥1 yr); and (4) ED severity at baseline, defined as severe for EF scores 0–10, moderate for EF scores 11–16, and mild or mild to moderate for EF scores 17–25. Box plots of the change in IIEF EF domain among patients with and without minimal improvement were reviewed for each of the subpopulations.

3. Results

The MCIDs were estimated using the development sample, which was composed of 11 studies from Asia, North America, Europe, South America, and Australia. MCIDs were validated using the validation sample, composed of six studies from Asia, North America, Europe, and Africa. These samples were predicted to be similar but showed statistically significant differences in a few variables at baseline, although the samples were generally similar as middle-aged men, all with ED. Patients in the development sample had lower body mass index, consumed less alcohol, and were more likely to have psychogenic ED (Table 1). Importantly, there was no difference in baseline scores of the IIEF EF domain between the development and validation samples and no difference in the change from baseline to week 12 EF outcome measure. The EF domain values were slightly higher in the validation sample compared with the development sample among patients who had minimal improvement in the Q7MMI (8.1 vs 7.3).

We performed a preliminary assessment of the association between the Q7MMI and EF domain change using the ANOVA approach. When the model was not controlled for baseline EF domain score (ie, disease severity), the MCID was 7.3 (Table 2); however, the test of the interaction between the baseline EF score (ED severity) and the Q7MMI was significant ($p < 0.001$). MCIDs were highest among patients with more severe ED at baseline (12.4) and lowest among patients with mild ED at baseline (2.8). In the middle range, the mean change was 7.2.

Using the ROC-based approach, the calculated MCID was 4 (Table 2). The MCID using the ROC-based approach was also calculated among patients in each baseline ED severity level (p interaction <0.001); MCIDs were 2, 5, and 7 for patients with mild, moderate, and severe baseline ED, respectively. In a similar analysis evaluating the effect of age group on the choice of MCID, we did not find MCID differences across age groups using either the ANOVA or the ROC approach (p interaction ≥0.64).

The ROC-based MCID showed consistently high sensitivity and specificity in the development and validation samples. The sensitivity and specificity for the ROC-based MCID were 0.74 and 0.73 in the development sample and were similar at 0.78 and 0.69 in the validation sample (Table 2).

Table 1 – Demographic and background characteristics by study sample

	Development (n = 863)	Validation (n = 377)	p value
Therapy, n (%)			0.065
20 mg tadalafil	312 (36.2)	157 (41.6)	
10 mg tadalafil	139 (16.1)	67 (17.8)	
Placebo	412 (47.7)	153 (40.6)	
Age, mean (SD)	55.84 (11.5)	55.68 (11.4)	0.819
Age, median (IQR)	56.86 (16.4)	56.47 (15.8)	
Age, n (%)			0.590
<50	255 (29.6)	126 (33.4)	
50–64	402 (46.6)	168 (44.6)	
65–74	180 (20.9)	73 (19.4)	
≥75	26 (3.0)	10 (2.7)	
BMI, n (%)			<0.0001
<30	740 (85.8)	258 (68.4)	
≥30	123 (14.3)	119 (31.6)	
Smoking status, n (%)			0.302
No	623 (72.4)	283 (75.3)	
Yes	237 (27.6)	93 (24.7)	
Alcohol consumption, n (%)			<0.0001
No	448 (52.1)	138 (36.7)	
Yes	412 (47.9)	238 (63.3)	
Depression, n (%)			0.085
No	842 (97.6)	361 (95.7)	
Yes	21 (2.4)	16 (4.2)	
ED duration, mo, n (%)			0.576
3–6	17 (2.0)	11 (2.9)	
6 to <12	58 (6.7)	24 (6.4)	
≥12	788 (91.3)	342 (90.7)	
ED etiology, n (%)			0.015
Organic	453 (52.5)	216 (57.3)	
Psychogenic	124 (14.4)	32 (8.5)	
Mixed	286 (33.1)	129 (34.2)	
ED severity (three levels), n (%)			0.117
Mild	194 (22.5)	79 (21.0)	
Moderate	260 (30.1)	136 (36.1)	
Severe	409 (47.4)	162 (43.0)	
Baseline EF score, mean (SD)	12.04 (5.38)	12.16 (4.93)	0.393

BMI = body mass index; ED = erectile dysfunction; EF = erectile function; IQR = interquartile range; SD = standard deviation.
Note: The development and the validation samples are compared using two-sample t tests for continuous measures and the chi-square test for categorical measures.

Figure 2 indicates consistent between-group discrimination using either the ANOVA-based method (MCID: 7) or the ROC-based method (MCID: 4). The ROC-based MCID of 4 discriminates well between mean change in EF scores of the placebo group and the active groups in the development sample.

Both methods produced similar results across geographic regions (Fig. 3), ED etiology groups (Fig. 4), and age groups (Fig. 5). The mean improvement in each of the subgroup comparisons was generally consistent with both methods.

4. Discussion

Despite widespread use of the IIEF in clinical research and practice, no published data are available concerning MCID of the EF domain. In this study, we estimated anchor-based

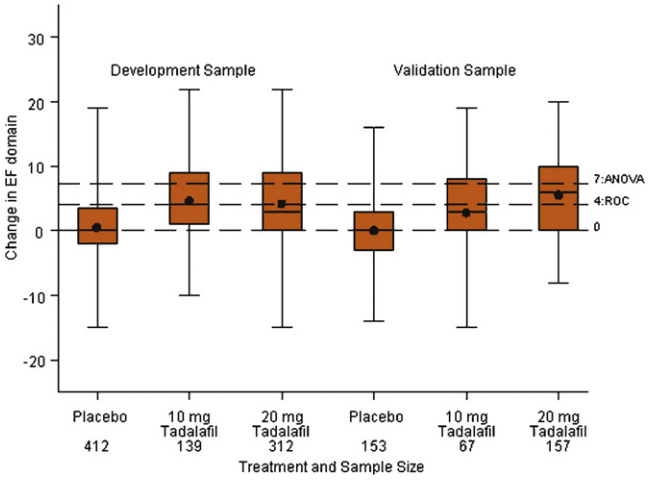

Fig. 2 – Distribution of the change in erectile function domain by treatment group in the development and validation samples. ANOVA = analysis of variance; EF = erectile function; MCID = minimal clinically important difference; ROC = receiver operating characteristic.

MCIDs based on a combination of methods. After showing a statistically significant association between EF domain scores and our selected clinical anchor (ie, IIEF Q7) by means of ANOVA, we then identified optimal cut points for MCID using a traditional ROC approach. Notably, estimated MCIDs varied according to baseline ED severity, with MCIDs increasing in magnitude with increasing ED severity. Regardless of the analytic method used to estimate the MCID, estimates generated from the development samples were generally replicable and were confirmed in the validation samples. Additionally, across geographic regions, ED etiology, and age groups, we found consistent support for the ROC-based MCID in the EF domain.

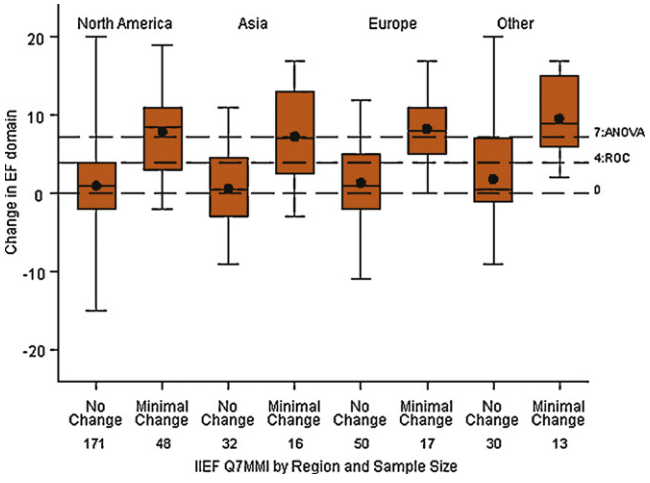

Fig. 3 – Distribution of the change in erectile function domain by question 7 measure of minimal improvement and region: validation sample. ANOVA = analysis of variance; EF = erectile function; IIEF = International Index of Erectile Function; MCID = minimal clinically important difference; Q7MMI = question 7 measure of minimal improvement; ROC = receiver operating characteristic.

Table 2 – Minimal clinically important differences of the change in International Index of Erectile Function erectile function domain

	Development sample (n = 863)				Validation sample (n = 377)			
	Mean (SD)	Minimum	Median	Maximum	Mean (SD)	Minimum	Median	Maximum
Change from baseline to week 12 IIEF EF domain								
No change in Q7MMI (n = 679)	1.27 (5.46)	−15	0	22	1.12 (5.72)	−15	1	20
Minimal improvement in Q7MMI (n = 184)	7.27 (5.93)	−7	7	22	8.13 (5.24)	−3	8	19
	MCID (95% CI)	p value	Sensitivity	Specificity		Sensitivity	Specificity	
ANOVA-based MCID [1]	7.27 (6.46–8.07)	<0.001	–	–	–	–	–	
ED severity level interaction [2]		<0.001	–	–	–	–	–	
Mild	2.79 (1.53–4.05)				3.45			
Moderate	7.21 (6.00–8.42)				8.11			
Severe	12.38 (11.04–13.72)				11.96			
ROC-based MCID [3]	4	<0.001	0.74	0.73	–	0.78	0.69	
ROC-based MCID by ED severity level [3]								
Mild	2	<0.001	0.65	0.70	–	0.68	0.77	
Moderate	5	<0.001	0.79	0.77	–	0.76	0.71	
Severe	7	<0.001	0.91	0.81	–	0.81	0.77	

ANOVA = analysis of variance; ED = erectile dysfunction; EF = erectile function; IIEF = International Index of Erectile Function; MCID = minimal clinically important difference; Q7MMI = question 7 measure of minimal improvement; ROC = receiver operating characteristic; SD = standard deviation.
[1] The p value tests the significance of the effect of the IIEF Q7MMI in predicting the change from baseline to week 12 of the IIEF EF domain. Change in EF = 1.3 + 6.0 × Q7MMI.
[2] The p value tests the interaction of the ED severity level by Q7MMI in predicting the change from baseline to week 12 of the IIEF EF domain of the analysis of covariance model.
[3] The p value tests the significance of the association between the IIEF Q7MMI and the dichotomy of the EF domain and was calculated using logistic regression.

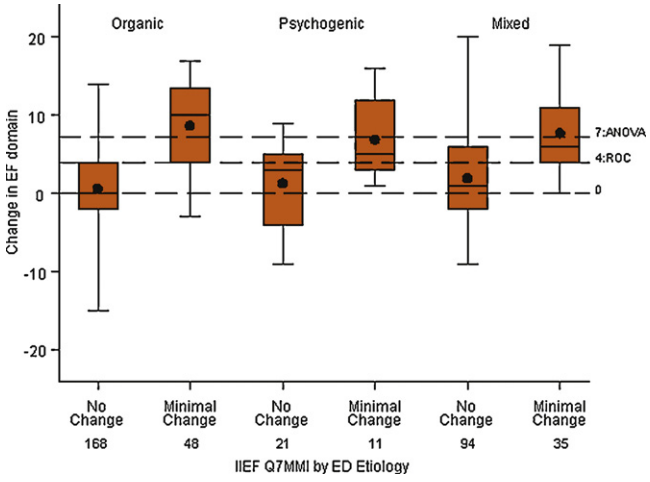

Fig. 4 – Distribution of the change in erectile function domain by question 7 measure of minimal improvement and etiology of erectile dysfunction: validation sample.
ANOVA = analysis of variance; ED = erectile dysfunction; EF = erectile function; IIEF = International Index of Erectile Function; MCID = minimal clinically important difference; Q7MMI = question 7 measure of minimal improvement; ROC = receiver operating characteristic.

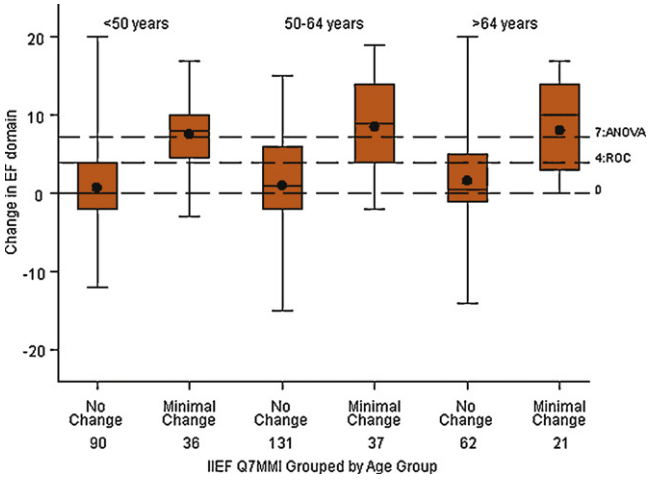

Fig. 5 – Distribution of the change in erectile function domain by question 7 measure of minimal improvement and age group: validation sample.
ANOVA = analysis of variance; EF = erectile function; MCID = minimal clinically important difference; Q7MMI = question 7 measure of minimal improvement; ROC = receiver operating characteristic.

4.1. Variation in minimal clinically important differences according to baseline erectile dysfunction severity

Estimated MCIDs of the IIEF EF domain varied according to baseline ED severity, with MCIDs increasing in size with increasing baseline ED severity. Depending on the proposed use of the MCID estimate, clinicians or researchers would be advised based on these findings to consider setting an expected change for a given individual patient based on baseline severity of ED. If the patient has a baseline EF score in the severe dysfunction range (6–10), for example, he would need to increase his EF score almost twice as much to achieve a noticeable improvement in satisfactory intercourse attempts compared with a man in the moderate ED range. Conversely, patients with mild ED (EF score: 22–25) would need to show only a 2–3 point change in EF domain score to

meet or exceed the MCID. Published reports in the literature have similarly shown greater relative improvements in patients with lower initial values in EF domain scores [9,18,19]. In contrast, no differences in MCID were observed across age groups, etiology, or geographic regions.

4.2. Variation in minimal clinically important differences according to analysis method

Not surprisingly, results differed somewhat across statistical methods used. The ANOVA-based approach is more conservative in estimating the MCID using the mean change in the EF domain for all patients who showed improvement. In contrast, the ROC-based approach is used to identify an MCID that provides optimal classification of responders and nonresponders regardless of treatment condition. Accordingly, using the ROC approach, 73% of patients were correctly classified as having improved, whereas the ANOVA approach resulted in only 47% of the patients being correctly classified as having improved. In keeping with regulatory guidelines that suggest a responder criterion be developed, we chose to emphasize the ROC-based approach as offering evidence-based cut points for classifying ED patients in clinical trials as responders or nonresponders [1].

4.3. Replication

We observed that regardless of the analytic method used to estimate the MCID, estimates generated from the development samples were generally replicable in the validation samples. The large sample size and multiple clinical trials included were major strengths of the current study and allowed cross-sample replication of the main findings.

4.4. Consistency of estimated minimal clinically important differences across subpopulations

Another strength of this analysis is the diversity of the patient base with regard to geographic region, ED etiology and duration, and age. MCIDs performed equally well across a broad range of geographic regions, ED etiology, and, importantly, age group.

4.5. Limitations

Data for the current analyses were based solely on 17 studies of tadalafil (10 mg, 20 mg) and placebo in men enrolled in ED clinical trials. These results need to be replicated in studies using other PDE5-Is or in nonpharmacologic intervention studies (eg, weight loss) [20]. All patients were heterosexual and engaged in regular sexual activity with a partner as an inclusion criterion in the studies, and this profile limits generalizability. Additional studies are also needed in special medical or surgical populations, such as men with ED secondary to radical prostatectomy, spinal cord injury, pelvic trauma, or Peyronie's disease. There were insufficient numbers of patients with these conditions in our integrated analyses for adequate replication.

Another potential limitation is our selection of the clinical anchor for our analyses (ie, IIEF Q7). Anchor-based approaches to defining MCIDs should ideally use patient ratings of change administered at different periods of time or on exit from a clinical trial [1]. This anchor was selected as the best of the available options. Of the items available in the integrated data set, Q7 of the IIEF represented the clinical intent and spirit of MCID analyses by allowing us to select an item that could quantify the minimally important difference and that was directly relevant to the NIH definition of ED (ie, inability to perform satisfactory intercourse) [12]. Other items were less optimal. GAQ, which required patients to rate the overall success of treatment, was administered in all randomized clinical trials with tadalafil; however, this item was asked in a simple "yes/no" (binary) response format in each case, limiting its utility as a sensitive anchor or index of minimal improvement. The SEP measure has a similar binary rating of intercourse success and is rated on every intercourse event rather than for the overall treatment period. For these and other reasons, we selected IIEF Q7, which we noted has been used previously as an anchor item in the development and validation of severity cut points on the EF domain of the IIEF [3,13].

5. Conclusions

This analysis considered the estimation of MCIDs using anchor-based approaches for an end point (the EF domain of the IIEF) that is commonly used in ED efficacy trials. The anchoring of changes in the scoring of the EF domain of the IIEF in clinically meaningful terms is critical to understanding efficacy, to interpreting study results, and to managing patients. This analysis provides, for the first time, anchor-based estimates of MCIDs in IIEF. Based on these results, responder rates can be calculated more accurately and used as study end points in terms of normalization of function, as traditionally defined (ie, EF score >25) [4], or as the percent of subjects who achieve MCID, correcting for baseline severity of ED. This evidence-based end point is recommended for future studies of ED.

***Author contributions*:** Raymond C. Rosen had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Rosen, Allen, Ni, Araujo.
Acquisition of data: Rosen, Allen, Ni, Araujo.
Analysis and interpretation of data: Rosen, Allen, Ni, Araujo.
Drafting of the manuscript: Rosen, Allen, Araujo.
Critical revision of the manuscript for important intellectual content: Rosen, Allen, Ni, Araujo.
Statistical analysis: Allen.
Obtaining funding: Rosen, Araujo.
Administrative, technical, or material support: None.
Supervision: Rosen, Araujo.
Other (specify): None.

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Extracorporeal Cardiac Shock Wave Therapy Markedly Ameliorates Ischemia-Induced Myocardial Dysfunction in Pigs in Vivo

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Background—Prognosis of ischemic cardiomyopathy still remains poor because of the lack of effective treatments. To develop a noninvasive therapy for the disorder, we examined the in vitro and vivo effects of extracorporeal shock wave (SW) that could enhance angiogenesis.

Methods and Results—SW treatment applied to cultured human umbilical vein endothelial cells significantly upregulated mRNA expression of vascular endothelial growth factor and its receptor Flt-1 in vitro. A porcine model of chronic myocardial ischemia was made by placing an ameroid constrictor at the proximal segment of the left circumflex coronary artery, which gradually induced a total occlusion of the artery with sustained myocardial dysfunction but without myocardial infarction in 4 weeks. Thereafter, extracorporeal SW therapy to the ischemic myocardial region (200 shots/spot for 9 spots at 0.09 mJ/mm^2) was performed (n=8), which induced a complete recovery of left ventricular ejection fraction (51±2% to 62±2%), wall thickening fraction (13±3% to 30±3%), and regional myocardial blood flow (1.0±0.2 to 1.4±0.3 mL · min^{-1} · g^{-1}) of the ischemic region in 4 weeks (all $P<0.01$). By contrast, animals that did not receive the therapy (n=8) had sustained myocardial dysfunction (left ventricular ejection fraction, 48±3% to 48±1%; wall thickening fraction, 13±2% to 9±2%) and regional myocardial blood flow (1.0±0.3 to 0.6±0.1 mL · min^{-1} · g^{-1}). Neither arrhythmias nor other complications were observed during or after the treatment. SW treatment of the ischemic myocardium significantly upregulated vascular endothelial growth factor expression in vivo.

Conclusions—These results suggest that extracorporeal cardiac SW therapy is an effective and noninvasive therapeutic strategy for ischemic heart disease. **(*Circulation*. 2004;110:3055-3061.)**

Key Words: angiogenesis ■ contractility ■ hibernation ■ ischemia ■ regional blood flow

Prognosis of ischemic cardiomyopathy without an indication for coronary intervention or coronary artery bypass grafting still remains poor because medication is the only therapy to treat the disorder.[1] Thus, it is imperative that an effective and noninvasive therapy for ischemic cardiomyopathy be developed. Although no medication or procedure used clinically has shown efficacy in replacing myocardial scar with functioning contractile tissue, it could be possible to improve the contractility of the hibernating myocardium by inducing angiogenesis.

It recently has been suggested that shock wave (SW) could enhance angiogenesis in vitro.[2] SW is a longitudinal acoustic wave, traveling with the speed in water of ultrasound through body tissue. It is a single pressure pulse with a short needle-like positive spike <1 μs in duration and up to 100 MPa in amplitude, followed by a tensile part of several microseconds with lower amplitude.[3] SW is known to exert the "cavitation effect" (a micrometer-sized violent collapse of bubbles inside and outside the cells)[3] and recently has been demonstrated to induce localized stress on cell membranes that resembles shear stress.[4] If SW-induced angiogenesis could be reproduced in vivo, it would provide a unique opportunity to develop a new angiogenic therapy that would not require invasive procedures such as open-chest surgery or catheter intervention. Therefore, the present study was designed to examine the possible beneficial effects of SW on ischemia-induced myocardial dysfunction in a porcine model of chronic myocardial ischemia in vivo.

Methods

This study was reviewed by the Committee on Ethics in Animal Experiments of Kyushu University and was carried out under the

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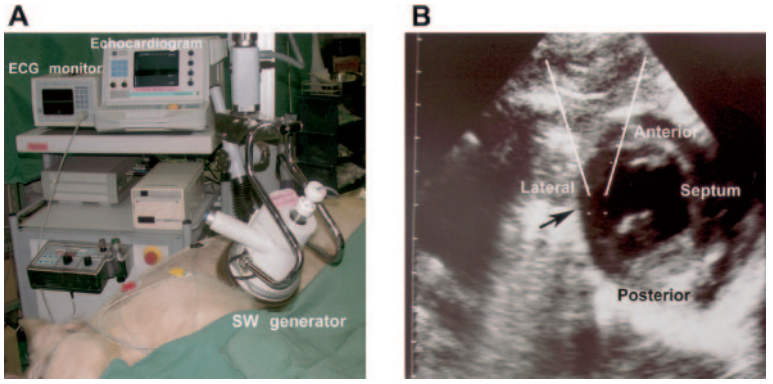

Figure 1. Extracorporeal cardiac SW therapy in action in a pig chronically instrumented with an ameroid constrictor. A, The machine is equipped with a SW generator and in-line echocardiography. The SW generator is attached to the chest wall when used. B, The SW pulse is easily focused on the ischemic myocardium under the guidance of echocardiography (black arrow).

Guidelines for Animal Experiments of Kyushu University and the Law (No. 105) and Notification (No. 6) of the Japanese Government.

Effect of SW on Human Umbilical Vein Endothelial Cells in Vitro

We purchased single-donor human umbilical vein endothelial cells (HUVECs) (Clonetics, Walkersville, Md) and cultured them in a complete endothelial medium (EBM-2 BulletKit, Clonetics). HUVECs were subcultured and used at passages 3 to 5 and were maintained in EBM-2. Twenty-four hours before the SW treatment, HUVECs (1×10^5) were resuspended in a 2-mL tube with EBM (Clonetics). We treated the HUVECs with 500 shots of SW at 4 different energy levels (0 [control], 0.02, 0.09, 0.18, and 0.35 mJ/mm^2) and stored them for 24 hours in the same medium before RNA extraction.

Ribonuclease Protection Assay

We analyzed equal amounts of mRNA by ribonuclease protection assay by means of the RiboQuant multiprobe template (PharMingen). Briefly, we hybridized RNA overnight with a ^{32}P-labeled RNA probe, which previously had been synthesized from the template set. We digested single-stranded RNA and free probe by ribonuclease A and T1. We then analyzed protected RNA on a 5% denaturing polyacrylamide gel. We analyzed several angiogenic factors, including vascular endothelial growth factor (VEGF) and its receptor, *fms*-like tyrosine kinase (Flt)-1, and angiopoietin and its receptor, tie-1, either by means of an NIH image or by means of autoradiography and subsequent quantification by densitometry (Alpha Innotech). For quantification, we normalized the signals for each sample of the blot with the corresponding signals of the housekeeping genes GAPDH and L32.

Porcine Model of Chronic Myocardial Ischemia

A total of 28 domestic pigs (25 to 30 kg in body weight) were used in this study. We anesthetized the animals with ketamine (15 mg/kg IM) and maintained anesthesia with an inhalation of 1.5% isoflurane for implantation of an ameroid constrictor, SW treatment, and euthanization. We opened the chest, suspended the pericardium and the left atrial appendage, revealed the left circumflex coronary artery (LCx), and put an ameroid constrictor around the proximal LCx to gradually induce a total occlusion of the artery in 4 weeks without causing myocardial infarction.[5,6] We also confirmed histologically that no myocardial necrosis had developed in the present porcine model (data not shown). This model is widely used to examine the effect of an angiogenic therapy in the ischemic hibernating myocardium.[5,6]

Extracorporeal Cardiac SW Therapy to Chronic Ischemic Myocardium

On the basis of the in vitro experiment, we applied a low energy of SW (0.09 mJ/mm2, ≈10% of the energy for the lithotripsy treatment) to 9 spots in the ischemic region (200 shots/spot) with the guidance of an echocardiogram equipped within a specially designed SW generator (Storz Medical AG) (Figure 1A). We were able to focus SW in any part of the heart under the guidance of echocardiography (Figure 1B). We applied SW to the ischemic myocardium in an R-wave–triggered manner to avoid ventricular arrhythmias. We performed the SW treatment (n=8) at 4 weeks after the implantation of an ameroid constrictor 3 times within 1 week, whereas animals in the control group (n=8) received the same anesthesia procedures 3 times a week but without the SW treatment. Because the SW treatment only requires the gentle compression of the generator to the chest wall, it is unlikely that this handling itself enhances angiogenesis in the ischemic myocardium.

Coronary Angiography and Left Ventriculography

After systemic heparinization (10 000 U/body), we performed coronary angiography and left ventriculography in a left oblique view with the use of a cineangiography system (Toshiba Medical). We semiquantitatively evaluated the extent of collateral flow to the LCx by the graded Rentrop score (0, no visible collateral vessels; 1, faint filling of side branches of the main epicardial vessel without filling the main vessel; 2, partial filling of the main epicardial vessel; 3, complete filling of the main vessel).[7] We also counted the number of visible coronary arteries in the LCx region. To compare the extent of collateral development at a given time, we selected the frame in which the whole left anterior descending coronary artery was first visualized.

Echocardiographic Evaluation

We performed epicardial echocardiographic studies at ameroid implantation (baseline) and at 4 and 8 weeks after the implantation of the constrictor (Sonos 5500, Agilent Technology). We calculated wall thickening fraction (WTF) by using the following formula: WTF = 100 × (end-systolic wall thickness − end-diastolic wall thickness) /end-diastolic wall thickness. We measured WTF when pigs were sedated, with and without dobutamine loading (15 $\mu g \cdot kg^{-1} \cdot min^{-1}$). Dobutamine was infused continuously from the ear vein, and WTF was measured after the hemodynamic condition was stabilized (in ≈5 minutes).

Measurement of Regional Myocardial Blood Flow

We evaluated regional myocardial blood flow (RMBF) with colored microspheres (Dye-Trak, Triton Technology) at ameroid implantation (baseline) and at 4 and 8 weeks after implantation.[8] We injected microspheres through the left atrium and aspirated a reference arterial blood sample from the descending aorta at a constant rate of 20 mL/min for 60 seconds using a withdrawal pump. We extracted microspheres from the left ventricular (LV) wall and blood samples by potassium hydroxide digestion, extracted the dyes from the spheres with dimethylformamide (200 μL), and determined their concentrations by spectrophotometry.[8] We calculated myocardial blood flow ($mL \cdot min^{-1} \cdot g^{-1}$) of the endocardial and epicardial lateral LV wall (the LCx region).

Analysis of Cardiac Enzymes

We measured serum concentrations of cardiac troponin T and creatinine kinase (CK)–MB by using chemiluminescence immuno-

assay before the SW treatment and at 4, 5 (2 hours after the SW treatment), and 8 weeks after ameroid implantation.

Factor VIII Staining

We treated paraffin-embedded sections with a rabbit anti–factor VIII antibody (N1505, Dako, Copenhagen, Denmark). We counted the number of factor VIII–positive cells in the endocardial and epicardial wall in 10 fields of the LCx region in each heart at 400× magnification.

Real-Time Polymerase Chain Reaction

To examine the effect of SW treatment on the ischemic myocardium in vivo, the animals with an ameroid constrictor were euthanized 1 week after the SW treatment. Total RNA was isolated from rapidly frozen ischemic LV wall (LCx region) after 3 SW treatments and was reverse transcribed. Quantification of VEGF and its receptor Flt-1 was performed by amplification of cDNA with an ABI Prism 7000 real-time thermocycler.

Western Blot Analysis for VEGF

We performed Western blot analysis for VEGF. Western blot analysis for VEGF was performed with and without 3 SW treatments. Three sections from the ischemic LV wall (LCx region) were measured. The regions containing VEGF proteins were visualized by electrochemiluminescence Western blotting luminal reagent (Santa Cruz Biotechnology). The extent of the VEGF was normalized by that of β-actin.

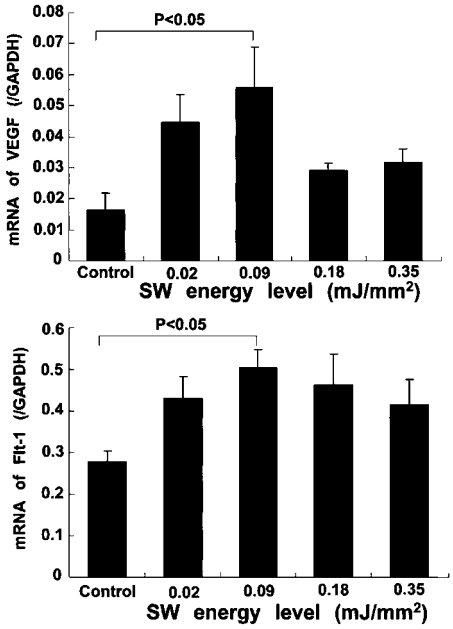

Figure 2. SW treatment upregulated mRNA expression of VEGF (A) and Flt-1 (B) in HUVECs in vitro with a maximum effect noted at 0.09 mJ/mm^2. Results are expressed as mean±SEM (n=10 each).

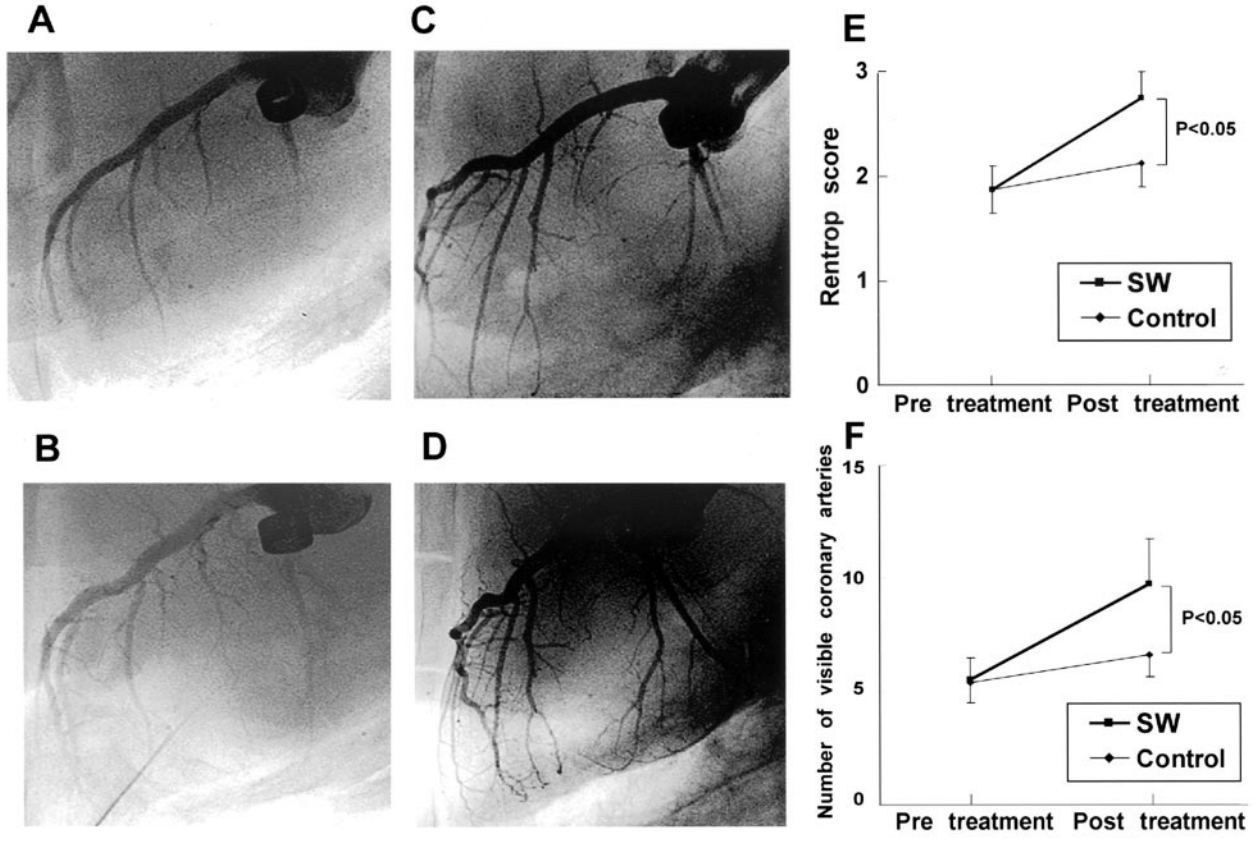

Figure 3. Extracorporeal cardiac SW therapy enhances coronary angiogenesis in vivo. A and C, Four weeks after the implantation of an ameroid constrictor, LCx was totally occluded and was perfused via collateral vessels with severe delay in both the control group (A) and the SW group (before SW therapy) (C). B and D, Four weeks after the first coronary angiography, no significant change in coronary vessels was noted in the control group (B), whereas a marked development of visible coronary vessels was noted in the SW group (D). E and F, Four weeks after the first coronary angiography, no significant increase in the Rentrop score (E) or visible coronary arteries from LCx (F) was noted in the control group, whereas increased Rentrop score and a marked development of visible coronary vessels were noted in the SW group. Results are expressed as mean±SEM (n=8 each).

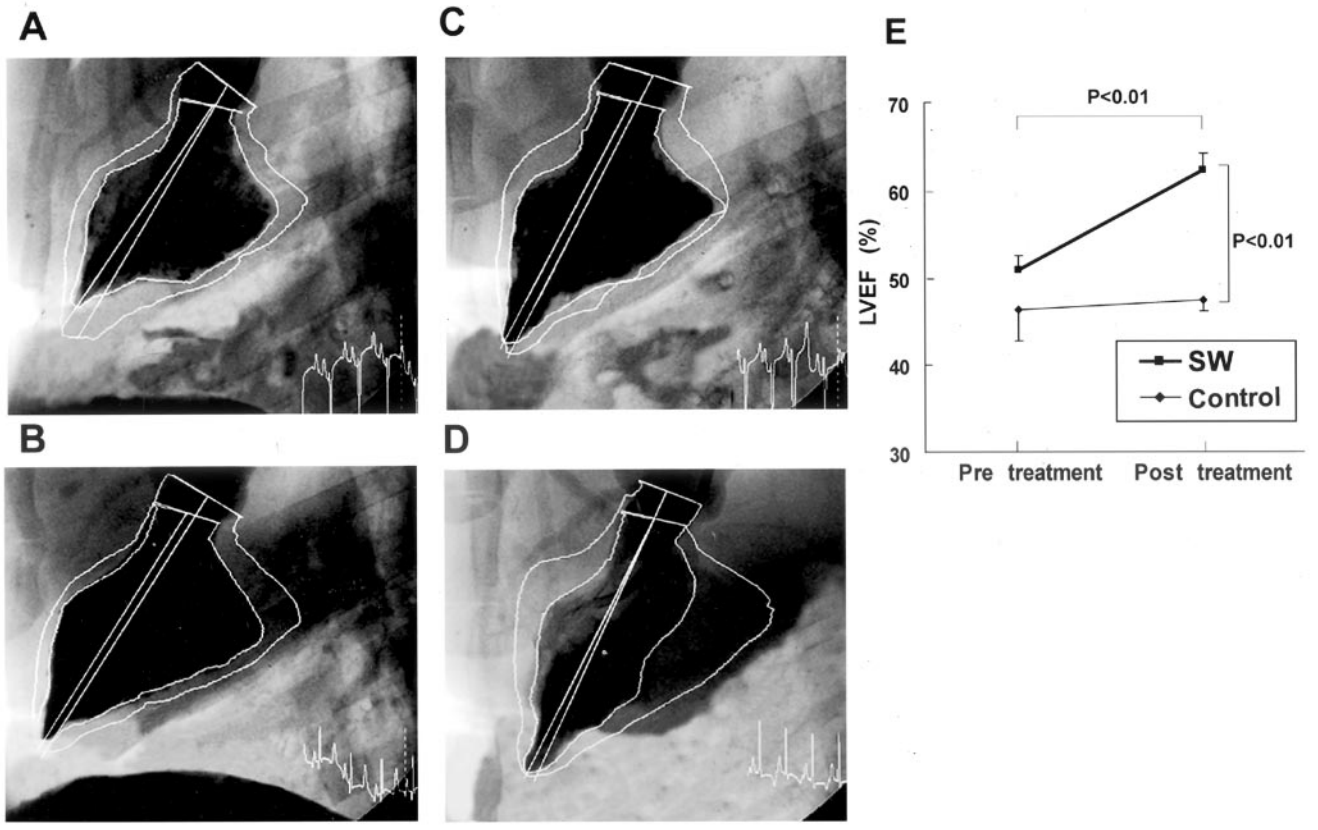

Figure 4. Extracorporeal cardiac SW therapy improves ischemia-induced myocardial dysfunction in vivo. A and C, Four weeks after the implantation of an ameroid constrictor, LV wall motion of the LCx (posterolateral) region was reduced in both the control (A) and the SW group (before the SW therapy) (C). B and D, Four weeks after the first left ventriculography, no significant change in LV wall motion was noted in the control group (B), whereas marked recovery was noted in the SW group (D). E, The SW therapy normalized left ventricular ejection fraction in the SW group but not in the control group. Results are expressed as mean±SEM (n=8 each).

Statistical Analysis

Results are expressed as mean±SEM. We determined statistical significance by analysis of variance for multiple comparisons. A value of $P<0.05$ was considered to be statistically significant.

Results

Effect of SW on mRNA Expression of VEGF and Flt-1 in HUVECs

SW treatment significantly upregulated mRNA expression of VEGF and its receptor Flt-1 in HUVECs, with a maximum effect noted at 0.09 mJ/mm^2 (Figure 2).

Effects of Extracorporeal Cardiac SW Therapy on Angiogenesis and Ischemia-Induced Myocardial Dysfunction

Four weeks after ameroid implantation, coronary angiography demonstrated a total occlusion of the LCx, which was perfused via collateral vessels with severe delay in both the control (Figure 3A) and the SW groups (Figure 3C). At 8 weeks after ameroid implantation (4 weeks after SW therapy), the SW group (Figure 3D), but not the control group (Figure 3B), had a marked development of coronary collateral vessels in the ischemic LCx region, an increased Rentrop score (Figure 3E), and an increased number of visible coronary arteries in the region (Figure 3F). Similarly, at 4 weeks, left ventriculography demonstrated an impaired left ventricular ejection fraction in both groups (Figure 4A, 4C, and 4E), whereas at 8 weeks, left ventricular ejection fraction was normalized in the SW group but remained impaired in the control group (Figure 4B, 4D, and 4E).

Effects of Extracorporeal Cardiac SW Therapy on Regional Myocardial Function and Myocardial Blood Flow

We serially measured WTF of the LCx region (lateral wall of the LV) by epicardiac echocardiography. At 4 weeks, we observed a significant reduction in WTF (%) in both groups (13±2 in the control group and 13±3 in the SW group; Figure 5A). At 8 weeks, however, the SW treatment markedly improved WTF in the SW group (30±3) but not in the control group (9±2) under control conditions (Figure 5A). Under dobutamine-loading conditions, which mimicked exercise conditions, WTF was further reduced at 4 weeks after the ameroid implantation in both groups (16±3 in the control and 18±2 in the SW groups), however, at 8 weeks, WTF was again markedly ameliorated only in the SW group (31±2) but not in the control group (16±4) (Figure 5B).

At 4 weeks, RMBF in the endocardium and epicardium (mL · min^{-1} · g^{-1}) was equally decreased in both groups (1.0±0.3 and 0.9±0.2 in the control group and 1.0±0.2 and 0.9±0.2 in the SW group, respectively). The SW treatment again improved RMBF in the endocardium (0.6±0.1 in the

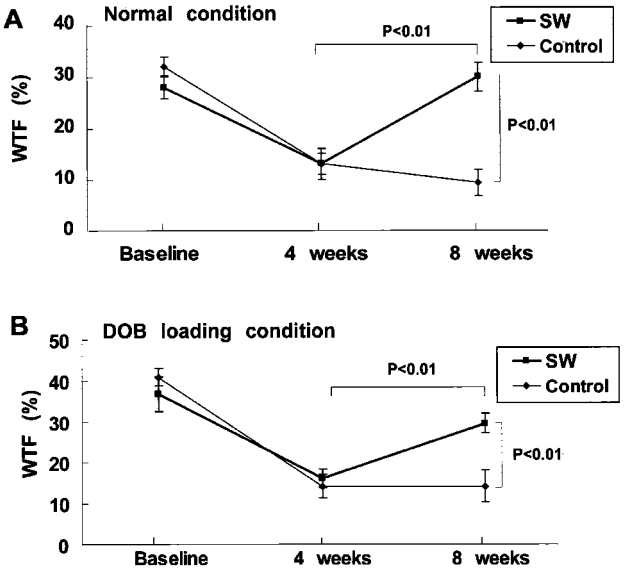

Figure 5. Extracorporeal cardiac SW therapy improves regional myocardial function in vivo. SW therapy induced a complete recovery of WTF of the ischemic lateral wall under control conditions (A) and under dobutamine (DOB) loading conditions (B). Results are expressed as mean±SEM (n=8 each).

control group and 1.4±0.3 in the SW group, $P<0.05$; Figure 6A) as well as in the epicardium (0.7±0.2 in the control group and 1.5±0.2 in the SW group, $P<0.05$; Figure 6B).

Effects of Extracorporeal Cardiac SW Therapy on Capillary Density and VEGF Expression in the Ischemic Myocardium

Factor VIII staining showed that the number of factor VIII–positive capillaries was increased in the SW group compared with the control group (Figure 7A and 7B). Quantitative analysis demonstrated that the number of capillaries was significantly higher in the SW group in both the endocardium (840±26 in the control group and 1280±45 in the SW group, $P<0.05$; Figure 7C) and the epicardium (820±30 in the control group and 1200±22 in the SW group, $P<0.05$; Figure 7D). RT-PCR analysis and Western blotting demonstrated a significant upregulation of VEGF mRNA expression (8.0±6 in the control group and 32±8 in the SW group, $P<0.05$; Figure 8A) and protein expression (2.23-fold increase in the SW groups, $P<0.05$; Figure 8B) after the SW treatment to the ischemic myocardium in vivo.

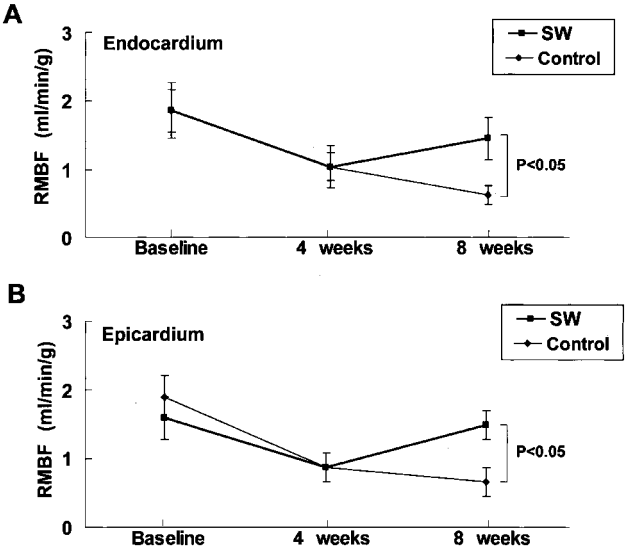

Figure 6. Extracorporeal cardiac SW therapy improves RMBF in vivo. SW therapy significantly increased RMBF, assessed by colored microspheres in both the endocardium (A) and the epicardium (B). Results are expressed as mean±SEM (n=8 each).

Side Effects of Extracorporeal Cardiac SW Therapy

All animals treated with the SW therapy were alive and showed no arrhythmias as assessed by 24-hour Holter ECG during and after the treatment (n=3; data not shown). There also was no myocardial cell damage as assessed by serum concentrations of CK-MB (ng/mL); the values before the SW treatment and at 4, 5 (2 hours after the SW treatment), and 8 weeks after the ameroid implantation were 5.0±0.6, 6.2±0.5, 5.5±0.2, and 7.1±0.9 in the control group and 5.1±0.2, 7.7±0.6, 6.1±0.6, and 6.4±0.4 in the SW group, respectively (n=6 each). The serum concentrations of troponin T were not detected in most cases in both groups. No significant differences were noted in hemodynamic variables (eg, heart rate or blood pressure) between the 2 groups (data not shown).

Discussion

The novel finding of the present study is that the extracorporeal cardiac SW therapy enhances angiogenesis in the ischemic myocardium and normalizes myocardial function in a porcine model of chronic myocardial ischemia in vivo. To the best of our knowledge, this is the first report that demonstrates the potential usefulness of extracorporeal cardiac SW therapy as a noninvasive treatment of chronic myocardial ischemia.

Extracorporeal Cardiac SW Therapy as a Novel Strategy for Ischemic Cardiomyopathy

Because of the poor prognosis of ischemic cardiomyopathy,[1,9] it is crucial to develop an alternative therapy for ischemia-induced myocardial dysfunction. To accomplish effective angiogenesis, it is mandatory to upregulate potent angiogenesis ligands, such as VEGF, and their receptors.[9,10] Furthermore, in the clinical setting, the goal for the treatment of ischemic cardiomyopathy should include not only enhancement of angiogenesis but also recovery of ischemia-induced myocardial dysfunction. In the present study, we were able to demonstrate that SW treatment (1) normalized global and regional myocardial functions as well as RMBF of the chronic ischemic region without any adverse effects in vivo, (2) increased vascular density in the SW-treated region, and (3) enhanced mRNA expression of VEGF and its receptor Flt-1 in HUVECs in vitro and VEGF production in the ischemic myocardium in vivo. Thus, SW-induced upregulation of the endogenous angiogenic system may offer a novel and promising noninvasive strategy for the treatment of ischemic heart disease.

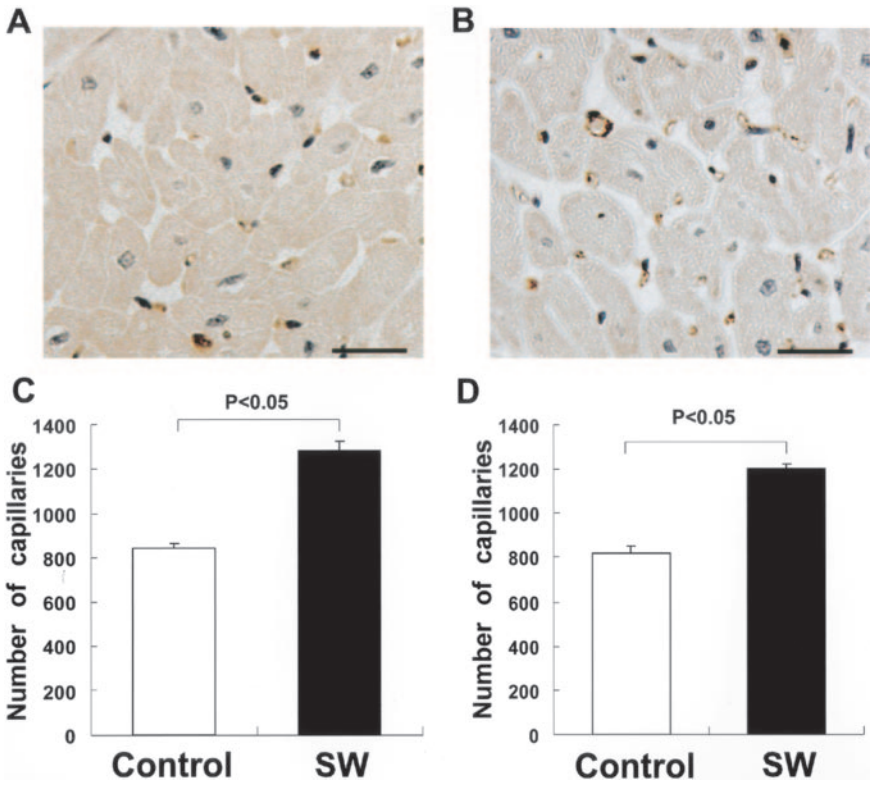

Figure 7. Extracorporeal cardiac SW therapy increases the density of factor VIII–positive capillaries in the ischemic myocardium. A and B, Factor VIII staining of the LCx region from the control (A) and the SW group (B). Scale bar represents 20 μm. C and D, Capillary density was significantly greater in the SW group (SW) than in the control group (Control) in both the endocardium (C) and the epicardium (D). Results are expressed as mean±SEM (n=6 each).

Advantages of Extracorporeal Cardiac SW Therapy

Recent attempts to enhance angiogenesis in the ischemic organs include gene therapy and bone marrow cell transplantation therapy. The main purpose of gene therapy is to induce overexpression of a selected angiogenic ligand (eg, VEGF) that leads to angiogenesis in the ischemic region. Although phase 1 trials of gene transfer of plasmid DNA encoding VEGF demonstrated safety and clinical benefit for the treatment of ischemic limb and heart,[11–13] gene therapy for ischemic cardiomyopathy is still at a preclinical stage. Bone marrow cell transplantation therapy, which depends on adult stem cell plasticity, also may be a useful strategy for angiogenesis because endothelial progenitor cells could be isolated from circulating mononuclear cells in humans and could be shown to be incorporated into neovascularization.[14] However, the need for invasive delivery of those cells to the ischemic myocardium may severely limit its usefulness in clinical situations.

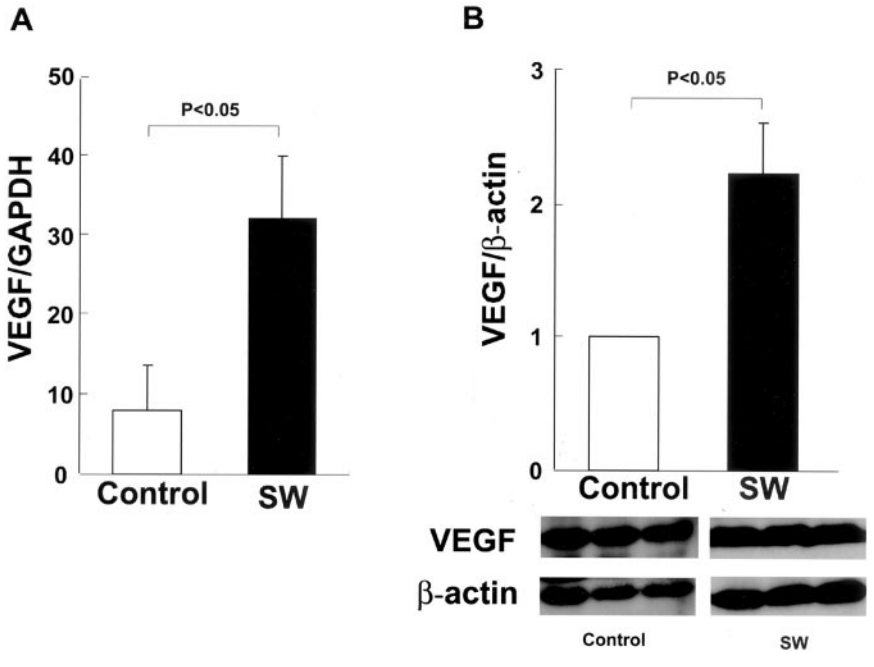

Figure 8. SW treatment upreglated mRNA (A) and protein (B) expression of VEGF in the ischemic myocardium (n=5 each).

A major advantage of the extracorporeal cardiac SW therapy over these 2 strategies is shown by the fact that it is quite noninvasive and safe, without any adverse effects. If necessary, we could repeatedly treat patients (even outpatients) with SW therapy because no surgery, anesthesia, or even catheter intervention is required for the treatment. This is an important factor in determining the clinical usefulness of angiogenic therapies in patients with ischemic cardiomyopathy. Thus, the extracorporeal cardiac SW therapy appears to be an applicable and noninvasive treatment for ischemic heart disease. Indeed, the SW treatment itself already has been clinically established as an effective and safe treatment for lithotripsy and chronic plantar fasciitis.[15,16] Our present results indicate that SW therapy, at ≈10% of the energy needed for lithotripsy treatment, is effective for in vivo angiogenesis in the ischemic heart.

Mechanisms for SW-Induced Angiogenesis

When a SW hits tissue, cavitation (a micrometer-sized violent collapse of bubbles) is induced by the first compression by the positive pressure part and the expansion with the tensile part of a SW.[3] Because the physical forces generated by cavitation are highly localized, SW could induce localized stress on cell membranes, as altered shear stress affects endothelial cells.[17] Recent reports have demonstrated the biochemical effects of SW, including hyperpolarization and Ras activation,[18] nonenzymatic nitric oxide synthesis,[19] and induction of stress fibers and intercellular gaps.[20] Although precise mechanisms for the SW-induced biochemical effects remain to be examined, these mechanisms may be involved in the underlying mechanisms for SW-induced angiogenesis. Indeed, Wang et al[21] reported that SW induces angiogenesis of the Achilles tendon–bone junction in dogs.

We were able to demonstrate that the SW treatment upregulated mRNA expression of VEGF and its receptor Flt in HUVECs in vitro and VEGF expression in the ischemic myocardium in vivo. Because the VEGF-Flt system is essential in initiating vasculogenesis and/or angiogenesis,[22] this effect of SW could explain, at least in part, the underlying mechanisms for SW-induced angiogenesis. It should be noted, however, that we showed only the upregulation of VEGF and Flt and that the effect of SW on signal transduction after receptor–ligand interaction still remains to be clarified. In addition, we need to fully elucidate the mechanisms for the SW-induced complete recovery of ischemia-induced myocardial dysfunction, although the increased myocardial blood flow caused by the SW treatment appears to play a primary role for the improved myocardial function. Further studies are required to determine the precise molecular mechanism for SW-induced angiogenesis and recovery of myocardial function.

In summary, we were able to demonstrate that noninvasive extracorporeal cardiac SW therapy effectively increases RMBF and normalizes ischemia-induced myocardial dysfunction without any adverse effects. Thus, extracorporeal cardiac SW therapy may be an effective, safe, and noninvasive therapy for ischemic cardiomyopathy.

Acknowledgments

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RAPID COMMUNICATION

Double-Blind and Placebo-Controlled Study of the Effectiveness and Safety of Extracorporeal Cardiac Shock Wave Therapy for Severe Angina Pectoris

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Background: Low-energy shock wave (SW) therapy has improved myocardial ischemia in both a porcine model and in patients with severe angina pectoris.

Methods and Results: To further confirm the effectiveness and safety of SW therapy, 8 patients with severe angina pectoris were treated with SW therapy in a double-blind, placebo-controlled and cross-over manner. SW therapy, but not placebo, significantly improved chest pain symptoms and cardiac function without any complications or adverse effects.

Conclusions: Extracorporeal cardiac SW therapy is an effective, safe and non-invasive therapeutic option for severe angina pectoris. (*Circ J* 2010; **74**: 589–591)

Key Words: Angina pectoris; Angiogenesis; Myocardial ischemia; Shock wave

The number of patients with severe angina pectoris without indications for coronary artery bypass grafting (CABG) or percutaneous coronary intervention (PCI) is rapidly increasing worldwide and their prognosis still remains poor.[1,2] Thus, it is crucial to develop new therapeutic strategies for these patients. We have previously demonstrated that extracorporeal cardiac shock wave (SW) therapy with low-energy SW (≈10% of the energy density used for urolithiasis) ameliorates myocardial ischemia and dysfunction in a porcine model of chronic myocardial ischemia in vivo.[3,4] We subsequently demonstrated in an open trial that our SW therapy effectively improved chest pain symptoms and exercise tolerance without any adverse effects in 9 patients with severe angina pectoris.[3,5] In the present study, to further confirm the effectiveness and safety of our SW therapy, we performed a double-blind placebo-controlled trial in patients with severe angina pectoris.

Methods

We enrolled 8 consecutive patients with severe angina pectoris who already had undergone CABG or PCI, but who no longer had further indications for these therapies even though they still suffered from stable effort angina under intensive medication (M/F, 5/3; age, 70±3 years) (**Table**).

The patients were treated with one series of placebo and the SW therapy in a double-blind and cross-over manner with an interval of 3 months. One series of therapy comprised 3 sessions per week. Throughout the study, the patient and the doctor in charge were not informed of the type of therapy. We performed the SW therapy (200 shoots/spot at 0.09 mJ/mm^2 for 40–60 spots per session; Modulith SLC, Storz Medical, Kreuzlingen, Switzerland) as described previously.[3,5] As placebo, the patients underwent the procedure of SW therapy but without irradiation. The patients were followed-up for 3 months after completion of the therapy. We evaluated symptoms using the Canadian Cardiovascular Society (CCS) class score, the patient's requirement for nitroglycerin,[5] exercise tolerance in a 6-min walk, and a cardiopulmonary exercise test, and cardiac function assessed by MRI (Achieva 1.5 T, Philips, Eindhoven, Netherlands). The left ventricular ejection fraction (LVEF) was measured using contiguous short-axis slices obtained by cine MRI; end-diastolic and end-systolic endocardial traces were used to determine end-diastolic and end-systolic left ventricular (LV) volumes, respectively. We also evaluated the number of circulating progenitor cells in peripheral blood by FACS analysis 2 days before the 1st session and 1 h after the 3rd session in 7 of the 8 patients

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Table. Baseline Characteristics of the 8 Patients With Severe Angina Pectoris

Patient no.	Age (years)	Gender	CAD	Previous treatment	OMI	ASO	HT	DM	HL
1	69	M	3VD	CABG, PCI	+	+	+	+	+
2	61	M	3VD	CABG, PCI	+	−	+	+	+
3	70	M	3VD	CABG	+	+	+	−	+
4	78	F	3VD	CABG, PCI	+	−	+	−	+
5	80	M	3VD	CABG, PCI	+	−	+	+	+
6	60	F	3VD	CABG, PCI	+	−	+	+	+
7	72	F	3VD	CABG, PCI	+	−	−	−	+
8	70	M	3VD	CABG, PCI	+	+	+	+	+

CAD, coronary artery disease; OMI, old myocardial infarction; ASO, arteriosclerosis obliterans; HT, hypertension; DM, diabetes mellitus; HL, hyperlipidemia; VD, vessel disease; CABG, coronary artery bypass grafting; PCI, percutaneous coronary intervention.

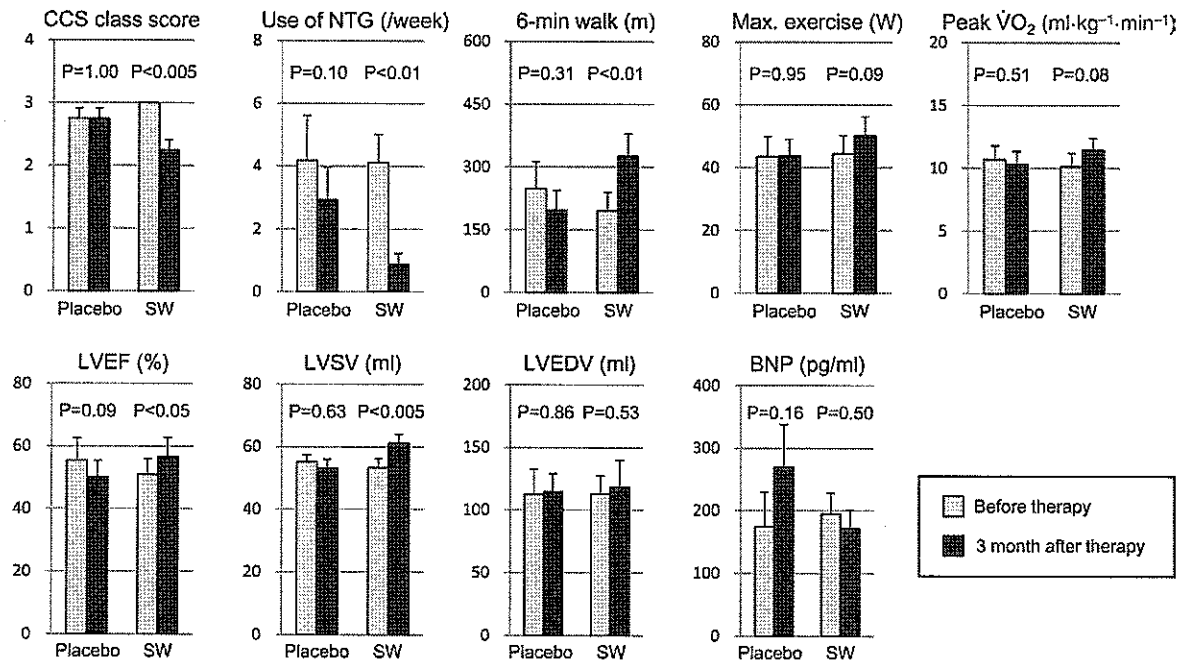

Figure. Effects of shock wave (SW) therapy. Results are mean ± SE (n=8 each). CCS, Canadian Cardiovascular Society; NTG, nitroglycerin; Max. exercise, maximum exercise capacity in watts (W); Peak $\dot{V}O_2$, peak oxygen uptake; LVEF, left ventricular (LV) ejection fraction; LVSV, LV stroke volume; LVEDV, LV end-diastolic volume; BNP, brain natriuretic peptide.

(technical problem in the remaining one patient).

The present study was approved by the Ethical Committees of Tohoku University in 2005, and informed consent was given by each patient.

Results are expressed as mean±SEM. Comparisons during the time course after SW therapy were made by repeated measure ANOVA followed by Bonferroni/Dunn post hoc test. All statistical analyses were performed using StatView (SAS Institute, Cary, NC, USA), and $P<0.05$ was considered to be statistically significant.

Results

The SW therapy, but not placebo, significantly improved symptoms (CCS class score) and nitroglycerin use (Figure). The SW therapy also significantly improved the 6-min walking distance and tended to improve both maximum exercise capacity and peak oxygen uptake (peak $\dot{V}O_2$). LVEF and LV stroke volume evaluated by MRI were significantly improved only with the SW therapy, although LV end-diastolic volume and plasma brain natriuretic peptide level remained unchanged. The number of $CD34^+/KDR^+$ and $CD34^+/KDR^+/c\text{-}kit^+$ cells in peripheral blood also remained unchanged with both therapies (data not shown). No procedural complications or adverse effects were noted during or after either therapy as in the previous studies.[3–7]

Discussion

We have previously demonstrated that low-energy SW

therapy enhanced angiogenesis and improved myocardial ischemia in a pig model of chronic myocardial ischemia,[3,4] and that SW therapy improved the symptoms and myocardial perfusion in patients with severe angina pectoris in an open trial.[3,5] The present double-blind and placebo-controlled study further demonstrates that our extracorporeal cardiac SW therapy is an effective therapeutic option for severe angina pectoris, providing convincing evidence for its effectiveness and safety.

During the past 2 decades, regenerative therapies using genes, cytokines, and progenitor cells have been under investigation for ischemic cardiovascular diseases.[8] However, these therapies have not been consistently effective in humans, despite promising results in early preclinical studies.[9–12] A potential explanation for these inconsistent results is the complex crosstalk among multiple pathways, in which enhancement of only 1 factor among numerous angiogenic factors may not be enough to achieve clinical benefit. Furthermore, animal studies of cell therapy have revealed that the number of newly generated vascular cells is too low to induce any functional improvement, suggesting that the paracrine action of transplanted cells stimulates intrinsic angiogenic capacity.[13] In contrast, low-energy SW upregulates multiple angiogenic pathways (eg, VEGF, flt-1, SDF-1, and nitric oxide synthase).[4,7,14]

There are several limitations to the present study. First, the number of patients is small. Although more than 150 patients with severe angina pectoris were reviewed as potential candidates for this study, most of them were excluded due to insufficient midication, potential indications of CABG or PCI, and co-existence of malignant tumor. However, we were able to reconfirm the beneficial effects of SW therapy in the present double-blind and placebo-control study, as we had observed in a previous open study.[5] A future large-scale trial would validate the present results. Second, maximum exercise capacity and pVO_2 were not significantly improved while the symptoms and 6-min walking distance were significantly improved by the SW therapy. In 6 of the 8 patients, exercise was stopped because of leg pain or fatigue before reaching the anaerobic threshold. Thus, exercise tolerance might have been underestimated because of arteriosclerosis obliterans and/or physical deconditioning. Another parameter, such as arteriovenous difference in lactate concentration under overdrive pacing, might have been a better index of ischemia. Third, the number of circulating progenitor cells in peripheral blood was not increased in the present study. Thus, it remains to be examined whether our SW therapy promotes recruitment of bone marrow-derived cells by the ischemic myocardium of humans.[14,15] Fourth, the detailed molecular mechanisms of the beneficial effects of SW in humans remain to be clarified in future studies.[3–7]

In conclusion, the present double-blind, placebo-controlled study further confirmed the effectiveness and safety of our extracorporeal cardiac SW therapy for the treatment of severe angina pectoris, although large-scale multi-center study is needed.

Acknowledgments

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Extracorporeal Shock Wave Therapy Ameliorates Hindlimb Ischemia in Rabbits

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We have recently demonstrated that the low-energy extracorporeal cardiac shock wave (SW) therapy improves myocardial perfusion and cardiac function in a porcine model of chronic myocardial ischemia and also ameliorates myocardial ischemia in patients with severe coronary artery disease. The present study was designed to examine whether our SW therapy also is effective to ameliorate hindlimb ischemia in rabbits. Hindlimb ischemia was made by surgical excision of the entire unilateral rabbit femoral artery. One week after the operation, we performed the SW ($n = 9$) or sham-therapy ($n = 9$) to the ischemic region 3 times a week for 3 weeks. Three weeks after the SW therapy, the development of collateral arteries, the flow ratio of the ischemic/non-ischemic common iliac arteries, the blood pressure ratio of the ischemic/non-ischemic hindlimb, and the capillary density in the ischemic muscles were all significantly increased in the SW group compared with the control group, indicating that the SW therapy induced therapeutic angiogenesis. Importantly, no adverse effect, such as muscle damage, hemorrhage, or thrombosis, was noted with the therapy. Finally, we examined the role of endothelial nitric oxide synthesis (eNOS) and vascular endothelial growth factor (VEGF) in the mechanisms of SW-induced angiogenesis on day 28. The expression levels of eNOS and VEGF proteins in ischemic hindlimb muscles tended to be increased in the SW group compared with the control group. These results suggest that our low-energy SW therapy also is effective and safe for the treatment of peripheral artery disease. ——— shock wave therapy; angiogenesis; peripheral artery disease; blood flow; capillary density.
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The severity of peripheral arterial disease (PAD) is closely associated with the risk of myocardial infarction, ischemic stroke, and death from vascular causes (Caro et al. 2005). The current management of PAD has three major therapeutic options, including medical treatment, percutaneous transluminal angioplasty, and bypass surgery. However, prognosis of patients with severe PAD still remains poor when there is no indication of bypass surgery or percutaneous transluminal angioplasty (Hirsch et al. 2006). Angiogenesis is a new promising therapeutic strategy for severe

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PAD, however, gene or cell therapy is invasive in nature, difficult to repeat, and still at preclinical stage (Isner et al. 1996; Leschke et al. 1996; Losordo et al. 1998; Tateishi-Yuyama et al. 2002).

We have recently demonstrated that low-energy shock wave (SW) could induce various angiogenic factors and enhance angiogenesis in vitro (Nishida et al. 2004). SW is a longitudinal acoustic wave, traveling with the speed in water of ultrasound through body tissue, which is a single pressure pulse with a short needle-like positive spike of less than 1 microsecond duration and amplitude up to 100 MPa followed by a tensile part of several microseconds with lower amplitude (Apfel 1982). SW is known to exert "cavitation effect" (a micrometer sized violent collapse of bubbles inside the cells) and has recently been demonstrated to induce localized stress on cell membranes that resembles shear stress (Apfel 1982; Maisonhaute et al. 2002). We have recently demonstrated that low-energy extracorporeal SW therapy effectively induces angiogenesis and ameliorates myocardial perfusion and cardiac function in a porcine model of chronic myocardial ischemia and in patients with severe coronary artery disease without any adverse effects (Nishida et al. 2004; Fukumoto et al. 2006; Uwatoku et al. 2007). Furthermore, it has been recently demonstrated that low-energy SW therapy improves recruitment of circulating endothelial progenitor cells via enhanced expression of chemoattractant factors in hindlimb ischemia of rat model (Aicher et al. 2006). The present study was thus designed to examine whether our SW therapy also is effective to ameliorate severe hindlimb ischemia in rabbits.

Methods

All procedures were approved by the Institutional Animal Care and Use Committee and were conducted in conformity with the institutional guidelines of Kyushu University. The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the article as written.

Animal preparation

Male New Zealand White rabbits (weight, 3.0 to 3.2 kg) were anesthetized with an intramuscular injection of xylazine (5 mg/kg) and ketamine (50 mg/kg). Rabbit unilateral hindlimb ischemia model was made as previously described (Takeshita et al. 1994). Briefly, under sterile surgical conditions, we performed a longitudinal incision in the left hindlimb, where the femoral artery was completely excised from its proximal origin to the point where it bifurcates into the popliteal and saphenous arteries including all branches of the femoral artery (Takeshita et al. 1994).

Extracorporeal cardiac SW therapy

One week after the induction of left hindlimb ischemia, we performed the SW therapy (0.09 mJ/mm^2, about 10% of the energy for lithotripsy treatment, 30 spots in the ischemic muscle, 200 shots/spot) (Nishida et al. 2004) to the animals with a guidance of ultrasonic echogram 3 times a week for 3 weeks ($n = 9$). In the previous study, we confirmed that the current energy level of shock wave exerts maximum angiogenic effects) (Nishida et al. 2004).

Study protocol

The SW therapy was performed 3 times a week, starting at day 7 for 3 weeks, while the control group received the same procedures 3 times a week without the SW therapy ($n = 9$ each). Angiography was performed at day 7 and 28, and blood flow, histological, and immunoblotting analyses were done at day 28 of sacrifice.

Angiographic analysis

Selective angiography of the ischemic limb was performed at day 7 and 28 after the surgery ($n = 6$ each), using the quantitative cineangiography (QCA) system (Toshiba Medical, Tokyo) (Nishida et al. 2004; Uwatoku et al. 2007). A 4F end-hole infusion catheter was inserted into the left common iliac artery of the ischemic limb via the common carotid artery. Then, angiography was performed with 4 mL of contrast media (1 mL/sec) for 8 sec. To quantitatively assess collateral vessel development, a grid with 2.5-mm-diameter squares was placed over the angiogram in the region of middle thigh. The number of squares crossed by contrast-pacified arteries was counted and angiographic score was calculated as the ratio of the number of total squares (Nishida et al. 2004; Uwatoku et al. 2007). To quantitatively assess collateral vessel flow, the number of frame from beginning of infusion of contrast media to filling to distal popliteal and saphenous artery were calculated by recorded angiography (Nishida et al. 2004; Uwatoku et al. 2007).

Common iliac artery blood flow ratio

Common iliac artery blood flow was directly measured by ultrasonic transit-time flow meter (Transonic Systems Inc, NYC, NY, USA) at the time of sacrifice (day 28, $n = 9$ each). We performed a longitudinal incision in the lower abdomen, where lower abdominal aorta and bilateral common iliac artery was carefully isolated, and bilateral common iliac artery blood flow was simultaneously measured at least for 5 min. The ratio of ischemic to non-ischemic hindlimb blood flow was defined in each rabbit as the ratio of mean common iliac artery flow (ml/min) of the ischemic limb to that of the non-ischemic limb.

Lower limb calf blood pressure ratio

Calf blood pressure was measured with auto-detected blood pressure monitor (BP100D, Fukuda ME Kogyo Co., Ltd., Tokyo) at day 28 ($n = 9$, each). The ratio of ischemic to non-ischemic hindlimb blood flow was defined for each rabbit as the ratio of systolic blood pressure (mmHg) of the ischemic limb to that of the non-ischemic limb.

Lower limb muscle weight

We measured the weight of the adductor muscle and the semimembranous muscle of the ischemic and non-ischemic limbs of each animal ($n = 7$-8 each) at the time of sacrifice (day 28).

Capillary density and capillary /muscle fiber ratio

Tissue specimens were obtained as transverse sections from the adductor muscle and the semimembranous muscle of the ischemic limb of each animal ($n = 6$ each) at the time of sacrifice (day 28). Muscle samples were embedded in OCT compound and snap-frozen in liquid nitrogen. Multiple frozen sections were then sliced (5 μm in thickness) from each specimen on a cryostat (Nishida et al. 2004; Uwatoku et al. 2007). The tissue sections were stained for alkaline phosphatase using an indoxyl-tetra-zolium method to detect capillary endothelial cells, as previously described, and were counterstained with eosin (Nishida et al. 2004; Uwatoku et al. 2007). A total of 20 different fields from the two muscle were randomly selected, and capillaries were counted under a x100 objective to determine the capillary density (number of capillaries/mm^2) (Nishida et al. 2004; Uwatoku et al. 2007). In order to ensure that analysis of capillary density was not overestimated because of muscle atrophy, capillary density was also evaluated as a function of the number of muscle fibers in the section (capillaries/muscle fibers ratio) (Nishida et al. 2004; Uwatoku et al. 2007).

Western blot analysis for eNOS and VEGF protein expression

Whole cell proteins were extracted from the adductor muscle and the semimembranous muscle of the ischemic limb of each group ($n = 6$ each) at day 28. Mouse monoclonal antibody to human endothelial nitric oxide synthesis (eNOS, pS633, BD Transduction Laboratories, San Jose, CA, USA) and human vascular endothelial growth factor (VEGF, G143-850, BD Pharmingen, San Jose, CA, USA) were used. The same amount of extract protein (50 μg) was loaded for SDS-PAGE/ immunoblotting analysis. The regions containing eNOS and VEGF proteins were visualized by ECL Western blotting luminal regent (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The levels of Western blot for eNOS and VEGF were normalized to those for β-actin as an internal control.

Statistical analysis

Continuous variables were expressed as mean ± S.E.M. Comparisons of all parameters were evaluated by unpaired *t*-test. All statistical analyses were performed using Stat View (SAS Institute, Cary, NC, USA), and P values of less than 0.05 were considered to be statistically significant.

Results

Hemodynamic variables

At day 7 and day 28 after the surgery, systemic arterial pressure and heart rate were comparable between the control and the SW groups (data not shown).

Angiogenic effects of the SW therapy

The selective internal iliac angiography showed that both of control and SW-treated rabbits did not have well-developed collateral arteries at day 7 (Fig. 1A, C), while SW therapy increased the collateral fillings at day 28 as compared with the control group (Fig. 1B, D). The evaluation by angiographic score and filling time to distal popliteal and saphenous artery demonstrated that the SW therapy significantly increased the angiographic collateral fillings at day 28 (Fig. 2).

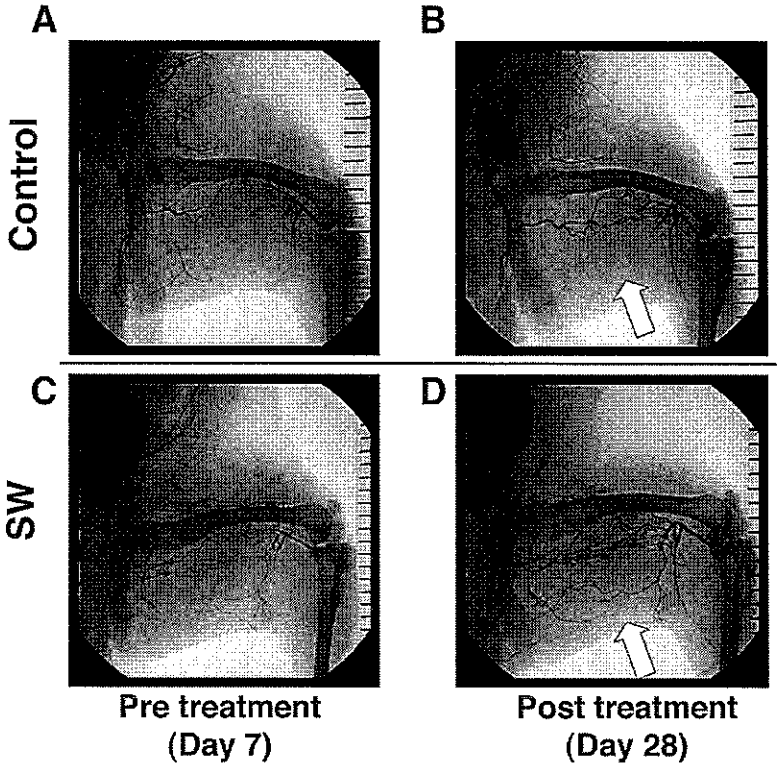

Fig. 1. Representative selective internal iliac angiography of control rabbit at day 7 (A) and day 28 (B), and shock wave (SW)-treated rabbit at day 7 (C, before SW therapy) and day 28 (D, 3 weeks after SW therapy). Note that increased collateral arteries were noticed in the SW-treated animal at day 28 (D).

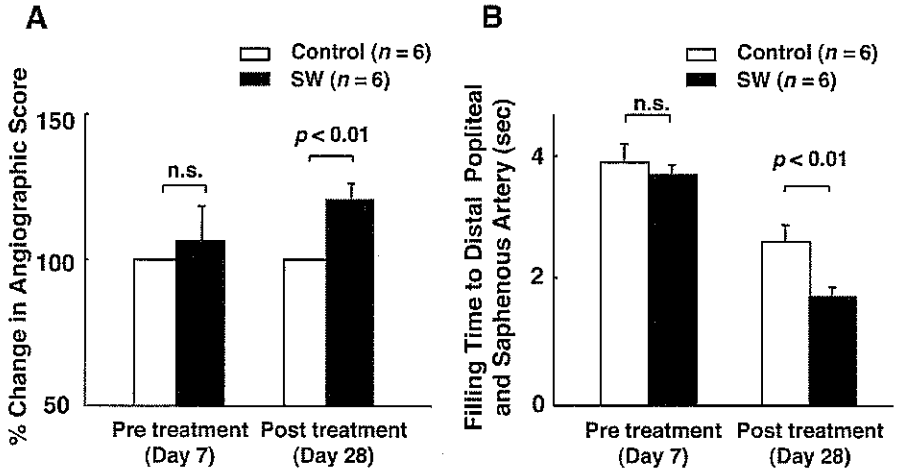

Fig. 2. Effects of the SW therapy in the ischemic limbs on collateral vessel growth and collateral blood flow by angiography. (A) The SW therapy significantly increased the angiographic score expressed as percent change compared with control group at day 28. (B) Filling time to distal popliteal and saphenous artery was significantly decreased in the SW group compared with the control group at day 28. Results are expressed as means ± S.E.M.

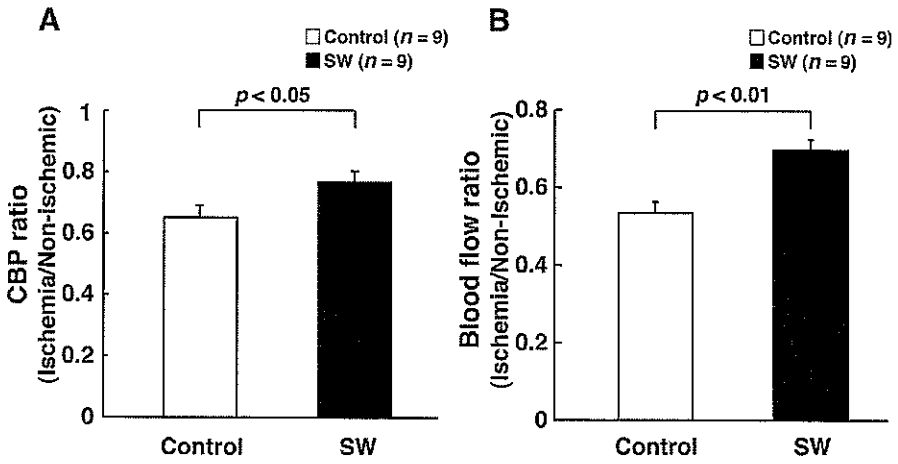

Fig. 3. Effects of the SW therapy in the ischemic limb on hindlimb blood pressure and common iliac arterial blood flow. The SW therapy significantly improved the calf blood pressure (CBP) ratio (ischemic/non-ischemic hindlimb) (A) and the common iliac flow ratio (ischemic/normal hindlimb) (B) at day 28. Results are expressed as means ± S.E.M.

Furthermore, the SW therapy to the ischemic hindlimb significantly improved the calf blood pressure ratio and the common iliac flow ratio (ischemic/non-ischemic hindlimb) (Fig. 3). Although the SW therapy tended to increase the weight of the ischemic lower limbs, the effect did not reach the statistically significant level (Fig. 4). It increased capillary density in the ischemic muscle as compared with the control group at day 28 (Fig. 5). No adverse effect (e.g. muscle damage, hemorrhage, thrombosis) was noted with the SW therapy.

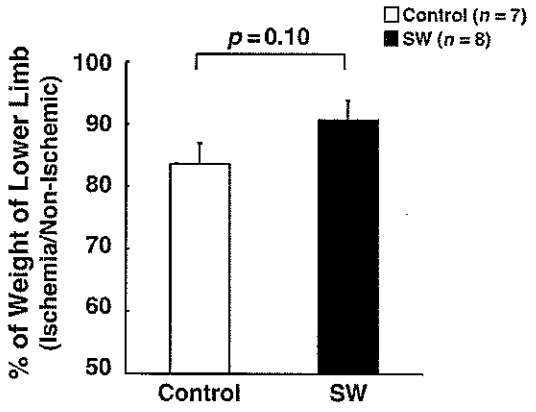

Fig. 4. Effects of the SW therapy in the ischemic/non-ischemic limbs on the weight of the adductor muscle and the semimembranous muscle. The SW therapy tended to increase the weight of ischemic lower limb muscle at day 28. Results are expressed as means ± S.E.M.

Effects of the SW therapy on eNOS and VEGF expression

The SW therapy tended to increase eNOS and VEGF protein expression in the ischemic limb at day 28 (Fig. 6).

Discussion

The novel finding of the present study is that our non-invasive SW therapy increased angiographic collaterals and blood flow in rabbits with ischemic limb without any procedural complications or adverse effects. The present result is in accordance with our previous findings that our non-invasive extracorporeal cardiac SW therapy ameliorates myocardial ischemia in a porcine model of chronic myocardial ischemia and in patients with severe coronary artery disease (Nishida et al. 2004; Fukumoto et al. 2006; Uwatoku et al. 2007).

Mechanism of the SW therapy-induced angiogenesis

The precise mechanism of a low-energy SW to induce angiogenesis remains to be fully eluci-

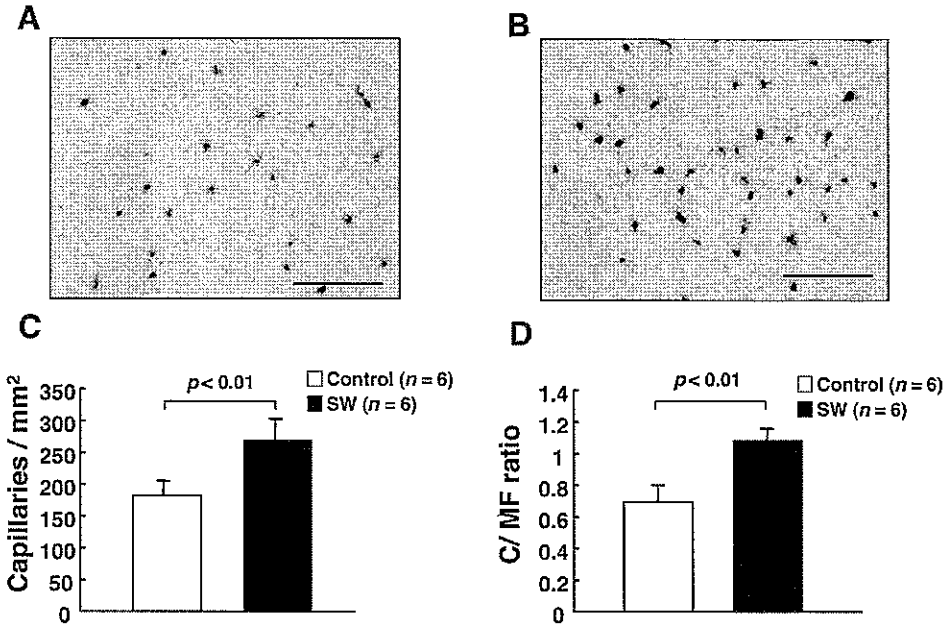

Fig. 5. Effects of the SW therapy on capillary density in the ischemic skeletal muscle. Capillary density in the ischemic muscle was increased in the SW group (B) compared with the control group (A) at day 28. Dark blue dots indicate capillaries that are positive for alkaline phosphatase staining. Bars indicate 100 μm. Quantitative analysis of capillary density indicates that capillaries/mm^2 (C) and capillaries/muscle fiber ratio (D) in the ischemic muscle were both significantly greater in the SW group than in the control group. Results are expressed as means ± S.E.M.

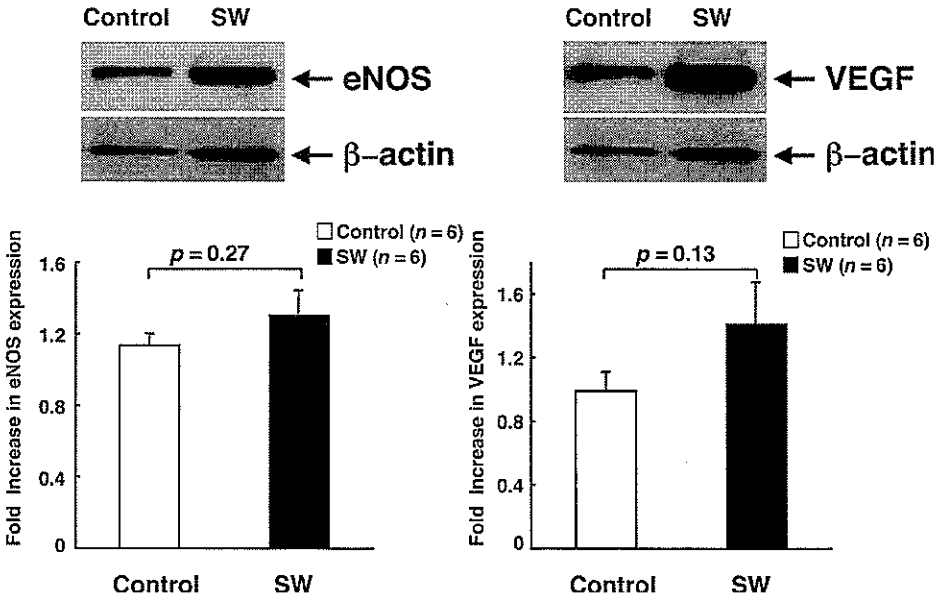

Fig. 6. Effects of the SW therapy on the protein expression of eNOS (A) and VEGF (B) in the ischemic skeletal muscle. The SW therapy tended to increase eNOS and VEGF expression in the ischemic limb. Results are expressed as means ± S.E.M.

dated; however, it has been demonstrated that SW can cause non-enzymatic nitric oxide (NO) synthesis from L-arginine and hydrogen peroxide (Gotte et al. 2002), and that SW induces neovascularization at tendon (Rompe et al. 1995; Wang et al. 2003) and up-regulates the expression of eNOS, VEGF, and proliferating cell antigen (PCNA) (Wang et al. 2003). We have previously confirmed that low-energy SW up-regulates VEGF and its receptor, Flt-2, in endothelial cells in vitro and VEGF in the ischemic porcine myocardium in vivo (Nishida et al. 2004). Recently, Aicher et al. (2006) have demonstrated that low-energy SW up-regulates mRNA expression of the chemoattractant stromal cell-derived factor 1 and the number of VEGF-positive endothelial cells at various energy levels, resulting in significantly enhanced recruitment and homing of endothelial progenitors that were intravenously administered 24 hrs after SW therapy in a rat model of hindlimb ischemia. However, in the present study, we were unable to demonstrate the significant up-regulation of eNOS or VEGF in the ischemic limb although there noted such a tendency. It is conceivable that the timing of the examination (day 28) might not be adequate to detect the up-regulation because the VEGF-Flt system could mainly affect the initiation of vasculogenesis and/or angiogenesis in ischemic limb (Nishida et al. 2004). It also is possible that endogenous angiogenic systems other than eNOS or VEGF are involved in the effect of the SW therapy. These points remain to be examined in future studies.

Advantage of the non-invasive SW therapy

Although angiogenesis by gene or cell therapy may be effective in patients with severe PAD, it is invasive in nature and difficult to repeat (Isner et al. 1996; Leschke et al. 1996; Losordo et al. 1998; Tateishi-Yuyama et al. 2002). A major advantage of our SW therapy is shown by the fact that it is quite non-invasive and safe, without any procedural complications or adverse effects (Nishida et al. 2004; Fukumoto et al. 2006; Uwatoku et al. 2007). If necessary, we can repeatedly treat patients with our SW therapy because no surgery, anesthesia, or even catheter intervention is required for the therapy. This is an important factor in determining the clinical usefulness of angiogenic therapies in elderly patients with severe PAD. Therefore, our non-invasive SW therapy appears to be a useful treatment for ischemic PAD.

Limitations of the study

Several limitations should be mentioned for the present study. First, the present rabbit model lacks atherosclerotic lesions in the systemic or peripheral vasculature. Future studies are needed to demonstrate whether SW therapy is effective in animals or patients complicated with systemic and/or peripheral atherosclerosis. Second, as we discussed above, the up-regulation of eNOS and VEGF by the SW therapy at day 28 did not reach a statistically significant level. The precise mechanism of SW-induced angiogenesis remains to be examined in future studies. Third, it remains to be examined whether our SW therapy is also effective to ameliorate hindlimb ischemia with greater or lesser extent than that in the present rabbit model (~50% reduction of blood flow). Fourth, the best strategy of SW delivery still remains to be elucidated. Our strategy was to shoot 200 shots/spot in 30 spots, 3 times a week for 3 weeks in the ischemic muscle in the present study (Nishida et al. 2004), while Aicher et al. (2006) have shot 500-2,000 shocks in one session. Further studies are required to determine the best treatment strategy. Finally, it was difficult to distinguish vasculogenesis from angiogenesis in this model, because we were unable to recognize which vessels were de novo formation of blood vessels and which were from pre-existing vascular network.

Conclusions

We were able to demonstrate that our low-energy non-invasive SW therapy effectively induces angiogenesis in ischemic limbs, resulting in the increase in collateral flow, hindlimb blood pressure, and arterial blood flow without any adverse effects. Thus, our SW therapy could be a useful strategy for the treatment of PAD.

Acknowledgments

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ORIGINAL PAPER

Angiogenic response to extracorporeal shock wave treatment in murine skin isografts

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Abstract Skin grafts are commonly utilized and proven effective methods of open wound coverage. Revascularization through neoangiogenesis is a pivotal mechanism for skin graft integration and durability. Extracorporeal shockwave treatment (ESWT) has been demonstrated to accelerate wound repair; however, its mechanism-of-action is unclear. We investigated the role of ESWT in early revascularization of full-thickness skin isografts in a murine model. Cohorts of mice were euthanized and skin grafts were harvested 6 h, 2, 4, and 7 days post grafting ± ESWT. Various aspects of graft neovascularization were measured including gross morphology, quantitative microscopy (vessel number, density), immunohistochemistry (CD31), cDNA SuperArrays for 84 angiogenesis-specific genes, and custom-designed 'Wound Repair' TaqMan® Low Density Array (TLDA) cards to assess expression of 188 wound repair genes. We demonstrate that a single administration of ESWT immediately following skin grafting significantly enhances recipient graft revascularization (increased vessel number, size, and density). An augmented early pro-angiogenic and suppressed delayed pro-inflammatory response to ESWT was accompanied by significantly increased expression of both skin graft CD31 and angiogenesis pathway-specific genes, including ELR-CXC chemokines (CXCL1, CXCL2, CXCL5), CC chemokines (CCL2, CCL3, CCL4), cytokines (IL-1β, IL-6, G-CSF, VEGF-A), matrix metalloproteinases (MMP3, MMP9, MMP13), hypoxia-inducible factors (HIF-1α), and vascular remodeling kinase (Mst1), as early as 6 h and up to 7 days post grafting and treatment. These findings suggest that early pro-angiogenic and anti-inflammatory effects of ESWT promote tissue revascularization and wound healing by augmenting angiogenesis and dampening inflammation.

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Keywords Angiogenesis · Extracorporeal shock wave therapy · Skin graft · Wound healing

Abbreviations
ESWT Extracorporeal shock wave treatment

Introduction

Wound healing is a complex, sequential, local cellular, and molecular response encompassing overlapping biological processes generally defined by inflammation, epithelialization, angiogenesis, and matrix deposition [1]. During early wound healing, a vigorous angiogenic response supports the increased metabolic demands of rapidly proliferating and migrating leukocytes, keratinocytes, fibroblasts, and endothelial cell (EC) precursors [2–6]. Ultimately, these early angiogenic and inflammatory responses diminish as wound closure, fibrosis, and remodeling predominate [7, 8]. In chronic wounds, this orderly and intricate biological response to wounding is disrupted. The inability to progress through the inflammatory phase of wound healing to complete epithelialization is characteristic of chronic wounds, where prolonged and aberrant accumulation of matrix metalloproteinases (MMPs) impedes cell migration and downregulation of tissue inhibitors of MMPs, hinders neovascularization essential to restoration of local oxygen delivery (O_2), and interferes with extra-cellular matrix (ECM) remodeling [9–12].

Non-healing wounds can pose difficult challenges to timely recovery, rehabilitation, quality of life and cost-effective medical care. Skin grafts remain a time-tested reliable means of efficient and definitive open wound closure. The ultimate survival of skin grafts is dependent on the ability of the graft to receive essential metabolic nutrients (serum imbibition) and vascular in-growth from the recipient wound bed [13]. The underlying mechanisms of skin graft angiogenesis and vasculogenesis have recently been elucidated in depth and include: recipient bed vascular in-growth, donor graft vascular endothelial regression or involution, and inosculation of donor and recipient vascular endothelium, driven in part by recipient bone marrow-derived endothelial progenitor cells responding to hypoxic signaling pathways [2, 8].

Various therapeutic strategies have been implemented to accelerate epidermal cell proliferation and angiogenesis within the open wound bed; however, attempts to use soluble mediators and growth factors have been unfulfilling [14, 15]. Recent studies have emphasized the importance of the wound stimulatory effects of micromechanical forces ("cellular mechanotransduction") [16, 17]. One application of this principal, micro-deformational wound therapy, utilizes local negative pressure- or vacuum-assisted closure to promote neovascularization, granulation tissue formation, and epithelial cell proliferation and migration within the open wound [18, 19]. Innovative approaches to accelerated wound healing and tissue regeneration have broadened the potential clinical ability of other biomechanical therapeutic modalities [20–22].

One such biomechanical modality, extracorporeal shock wave treatment (ESWT), generates an acoustic pressure wave that penetrates tissue and produces favorable biological responses. The target tissue response occurs through complex and incompletely understood cellular biochemical pathways. We have recently shown in a mouse model of severe full-thickness burn injury that ESWT has significant anti-inflammatory properties associated with a significant decrease in pro-inflammatory chemokine and cytokine synthesis at the wound site coupled with a marked reduction in inflammatory leukocyte recruitment to the treated burn [23]. Furthermore, preliminary findings from earlier studies suggest that local delivery of shock waves may stimulate early expression of angiogenesis-related growth factors including endothelial nitric oxide synthase (eNOS), vascular endothelial growth factor (VEGF), and proliferating cell nuclear antigen (PCNA), associated with vasculogenesis, improved local blood flow, EC proliferation, and accelerated soft tissue repair [24–27].

As skin graft survival relies on neoangiogenesis to revascularize ischemic tissue, and the underlying mechanisms of shock wave-derived molecular effects are poorly understood, the present study evaluates the angiogenic response to ESWT in an established ischemic tissue model of murine full-thickness skin isografting. We demonstrate that a single shock wave treatment immediately after skin grafting significantly augments recipient graft revascularization. This pro-angiogenic response to ESWT is associated with significantly increased CD31-positive EC proliferation and early post treatment VEGF-A expression, upregulation of angiogenesis pathway-specific genes in the skin graft, as well as reduced and delayed inflammatory response to ESWT. The early pro-angiogenic and anti-inflammatory effects of ESWT in ischemic tissue make it an attractive therapeutic modality for promoting wound healing.

Materials and methods

Animals

Seven- to eight-week-old female BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained at the Walter Reed Army Institute of Research (WRAIR, Silver Spring, MD) animal facility,

which is certified by the Association for the Assessment and Accreditation of Laboratory Animal Care International. All procedures were conducted using facilities and protocols approved by the Animal Care and Use Committee of WRAIR (protocols #K01-08 and #K06-05). Mice were housed five animals per cage prior to any study intervention, and caged individually post skin transplantation in standard micro-isolator polycarbonate cages. Mice, age 10–12 weeks, weighing 18–22 g were used for skin transplantation. Animal rooms were maintained at a temperature 21 ± 2°C with 50 ± 10% humidity on a 12-h light/dark cycle. Commercial rodent ration (Harlan Teklad Rodent Diet 8604) was available freely, as was acidified (pH = 2.5) water to prevent opportunistic infections.

Skin transplantation

Skin transplantation was conducted as previously described [28]. Briefly, full-thickness donor skin (3 cm × 3 cm) was harvested from the dorsum of euthanized and shaved donor BALB/c mice, and the underside of the donor skin was gently scraped with a scalpel to remove fat and muscle. Donor skin sections were placed in a petri dish containing sterile normal saline and maintained at 4°C until grafted. The dorsal surface of an anesthetized (Ketamine 100 mg/kg and Xylazine 5 mg/kg injected intra-peritoneally; Reckitt Benckiser Pharmaceuticals, Richmond, VA) recipient BALB/c mouse was shaved and skin washed with 70% ethanol. An oval graft bed ~2 cm in maximum diameter was prepared with fine curved scissors by removing an area of epidermis and dermis down to the level of the underlying musculature. Full-thickness skin grafts, 3 cm^2 in surface area, were fitted to the prepared recipient bed without suturing, covered with a liberal amount of Bacitracin ointment (Pharmaderm, Melville, NY), shock wave- or sham-treated, then covered with an adhesive plastic bandage. Once the mice were recovered from anesthesia, they were placed alone in separate cages and maintained under standard conditions in the animal facility. Buprenorphine (Reckitt Benckiser Pharmaceuticals, Richmond, VA), 0.1 mg/kg SC BID, was given on post-operative days 1 and 2 for pain management. No topical wound care was provided aside from the aforementioned Bacitracin.

A total of 90 full-thickness skin grafts, including 46 shock wave-treated and 44 sham-treated skin transplants, were included in this study. Cohorts of mice (4–5 per group at each time point for each experiment) were euthanized 6 h, 2, 4, and 7 days post grafting and treatment. Following euthanasia, the skin grafts and adjacent normal tissue were excised carefully. The underside of the skin was photographed 2, 4, and 7 days post grafting for macroscopic examination of blood vessel formation. Next, the graft and a small amount of peripheral recipient skin tissue were divided into three segments. One segment was processed by formalin fixation and paraffin embedding for histological evaluation, the second section was embedded in Tissue-Tek OCT cryopreservation medium and snap-frozen (in 2-methyl butane using dry ice bath) for immunohistochemistry staining; and, the third segment was dissected into small pieces and stored in RNAlater® (Ambion, Austin, TX) for gene transcript expression studies.

Extracorporeal shock wave treatment

Immediately post grafting, Bacitracin ointment was applied directly to the unfocused lens of the DermaGold™ (Tissue Regeneration Technologies, LLC, Woodstock, Georgia) shock-wave applicator. The ESWT applicator is comprised of parabolic reflector, was gently placed directly on the dorsal skin isograft, which was treated with 200 impulses (energy level = 0.1 mJ/mm^2, frequency = 3 pulses per second). The parabolic reflector permits a large treatment area to be stimulated by the acoustical field. ESWT lasted ~45 s. Sham-treated grafts were treated identically; however, no shock wave impulses were administered. The dose response (ESWT energy level and frequency) is well established for this modality and based on extensive animal and human use testing [22, 23, 29, 30].

Quantitative angiogenesis assessment

At the gross macroscopic level, documentation of new vessel formation on the underside of full-thickness dorsal skin grafts was captured by photographic image analysis using a digital Fuji Finepix Camera (Fuji Imaging, Valhalla, NY) in the presence of a scaled ruler (to ensure standardized assessment at consistent magnification). Images were imported into Adobe Photoshop CS2 (Adobe Systems, San Jose, CA) for reproduction. Images were assessed for visible alterations in graft vascularity under magnification at 4×. Visible vessels with and without ramifications and branches were counted in accordance with the criteria described by Isenberg et al. [31]. For quantification of skin graft microvessels, 5-μm graft cross-sections made from the mid portion of each full-thickness skin graft were made using a Leica CM 1900 cryostat (Leica Microsystems). All graft sections and washes were performed at room temperature. Cryosections were fixed in acetone, endogenous peroxidase inactivated (0.03% H_2O_2, 0.15 mol/l NaN_3), and non-specific binding sites blocked with a 1:10 dilution of normal mouse serum (Sigma Chemical Company, St Louis, MO) in phosphate buffered saline (PBS) for 30 min. Central graft cross-sections were incubated with 1.0 μg/ml rat anti-mouse CD31 antibody (anti-PECAM-1, clone MECA-133; Pharmingen,

San Diego, CA). Graft cross-sections were washed, incubated for 30 min with biotinylated mouse anti-rat IgG (1:250 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA), and visualized using a Streptavidin–Biotin–Peroxidase staining kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. The sections were developed using horseradish peroxidase substrate, 3,3′-diaminobenzidine (Sigma Chemical Company, St Louis, MO) for 10 min. Slides were counterstained with Harris Hematoxylin (Sigma Chemical Company, St Louis, MO) to stain nuclei and coverslipped with a permanent mounting medium (Permount, Fisher Scientific, Pittsburg, PA). Digital images of CD31 (PECAM-1)-stained skin graft sections were captured at ×100, ×200, and ×400 magnification using a BX50 Olympus microscope equipped with an Insight Firewire Spot Color Camera and Spot 4.6 photographic and analysis software (Diagnostic Instruments, Inc., Sterling Heights, MI). Two independent observers (blinded to study group assignment) assessed microvessel density in the graft sections by enumerating the number of CD31-positive vessels in consecutive 8–16 high-power fields (at 400× magnification) across each central graft section. Standardized analysis of pixel density using digital analysis software (ImageJ, NIH) was used to enumerate the CD31 (PECAM)-positive area(s) within each skin graft section and the overall percent vascularization.

RNA extraction

Total RNA was extracted from the excised skin graft and adjacent marginal tissue and stored in RNAlater® (Ambion, Austin, TX). Briefly, skin tissue was homogenized using Trizol reagent (Invitrogen, Carlsbad, CA) and total RNA was isolated using Qiagen RNeasy Lipid Tissue Mini Kit (QIAGEN Inc. Valencia, CA) according to manufacturer's instructions. Isolated RNA was resuspended in 30 µl of 10 mM Tris buffer, pH 7.5. Sample purity, quantity, and quality were assessed by determining the $A_{260/280}$, $A_{260/230}$ ratio on a Nanodrop Spectrophotometer (NanoDrop Technologies Inc. Wilmington, DE), by measuring 28S/18S ribosomal RNA ratio and ascertaining RNA integrity number (RIN) using an Agilent 2100 Bio-Analyzer (Agilent Technologies Inc. Santa Clara, CA). Agilent RNA integrity values for all sampled wound specimens in this study were $\geq$8.5.

Gene expression profiling for angiogenic transcripts

Gene expression profiles were created using the RT^2-Profiler PCR Array: Mouse Angiogenesis Gene Array (SuperArray, Rockville, MD). The total RNA was isolated as described above, and subsequently converted to cDNA using SuperArray's RT^2 First Strand Kit per the manufacturer's instructions (SuperArray, Rockville, MD). The expression of each gene tested at multiple time points (6 h, 2, 4 and 7 days post skin grafting ± ESWT) was expressed as relative gene transcript expression level, normalized to the averaged value of the set of multiple internal controls (housekeeping genes). A gene was considered to be differentially up- or downregulated by shock wave treatment if it was differentially expressed by at least twofold compared with the sham-treated control group.

Quantitative real-time PCR (RT-PCR) gene profiling for wound healing-repair gene transcripts

Quantitative real-time polymerase chain reaction (RT-PCR) was performed using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Custom-designed 'Wound Repair' TaqMan® Low Density Array (TLDA) cards (Applied Biosystems, Foster City, CA) were used to assess skin graft transcript gene expression. The set of TLDA cards comprised 188 individual target assays including respective forward and reverse primers and a dual-labeled probe (5′-6-FAM; 3′-MGB) in quadruplicate on a 384-well card (96 genes per card, including housekeeping genes). Amplification parameters were as follows: one cycle of 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 30 s and 60°C for 1 min. Two tissue samples were processed on each card.

RT-PCR data analysis

RT-PCR data were analyzed using the Sequence Detection System version 2.1 included with the ABI Prism 7900HT SDS (Applied Biosystems, Foster City, CA) or using Microsoft Excel. The threshold cycle (C_t) for each sample was manually set to 0.2 and the baseline was set between 3 and 15 cycles. 18S ribosomal RNA was used as an endogenous housekeeping control for normalization and the comparative C_t method was used to calculate the relative fold expression by$2^{-\Delta\Delta C_t}$. Assays with C_t values greater than 35 cycles were excluded from analysis.

Statistics

Data were analyzed using GraphPad Prism version 4.01 software (GraphPad Software, San Diego, CA, USA). Gene expression is shown as fold change in average C_t value relative to expression levels of naïve skin. A greater than twofold increase in specific gene expression was considered significant. SuperArray (SuperArray, Rockville, MD) statistical analyses were performed using the manufacturer's software, setting $P < 0.05$ for significant differential gene expression between sham- and ESWT-treated tissues. All other results are expressed as mean ± SD. Statistical

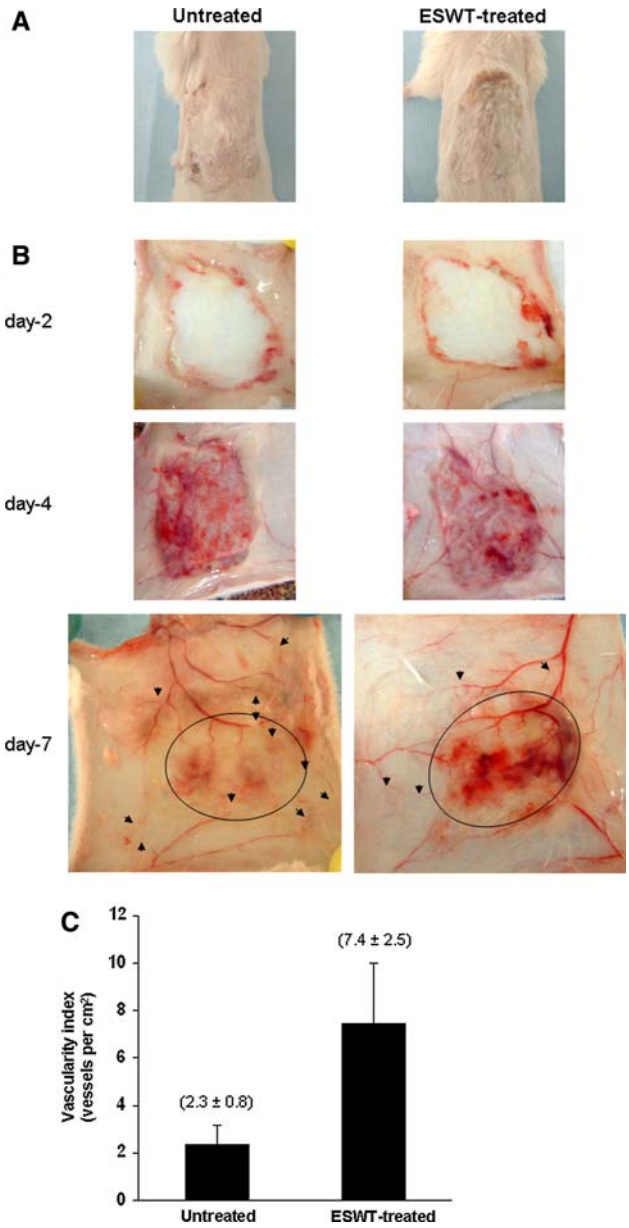

Fig. 1 ESWT stimulates angiogenesis and tissue revascularization in a murine skin grafting tissue ischemia model. Full-thickness skin from syngeneic donor mice was grafted onto the dorsum of adult recipient BALB/c mice treated with either a single ESWT application (200 impulses of ESWT, energy level = 0.1 mJ/mm^2, frequency = 3 pulses per second) or sham-treated at the time of graft placement over the wound bed. **A** Representative photographs of sham-treated and ESWT-treated isografts on BALB/c recipient mice 7 days post grafting ($n = 16$ mice per group) demonstrate no gross macroscopic differences in either isograft appearance, graft acceptance, or the overall healing process. **B** Macroscopic analysis of the vessels in the underside of ESWT-treated and sham-treated isografts reveals more pronounced angiogenesis in ESWT-treated isografts. Representative images ($n = 12$ mice per group) of the underside of isografts surgically excised from sham-treated and ESWT-treated mice on days 2, 4, and 7 post skin transplantation. The margin of the graft edge is delineated with the *elliptical outline*. The *black arrow heads* point to those vessels that are obviously undergoing vascular regression as determined by microscopic assessment of the images at ×4 magnification. **C** Vascularity of the donor skin graft and the adjacent recipient tissue at the peripheral graft edge was measured on day 7 post skin transplantation. Results represent the mean ± SD number of visible vessels (×4 magnification) per cm^2 ($P < 0.001$, $n = 12$ mice per group)

analysis of variance was used to compare mean values of specific covariates between study groups, and Mann–Whitney U test was used to determine the level of significance of differences between sample means. Values of $P < 0.05$ were considered statistically significant.

Results

ESWT enhances ischemic tissue angiogenesis and revascularization

We evaluated the effects of ESWT on ischemic tissue angiogenesis and revascularization using a well-established murine model of skin transplantation. Full-thickness skin isografts were transplanted from syngeneic BALB/c donor mice onto the dorsum of adult recipient BALB/c mice. At the time of placement of the skin graft over the open recipient bed (devoid of overlying dermis), isografts were treated with 200 impulses of ESWT (energy level = 0.1 mJ/mm^2, frequency = 3 pulses per second) or identical sham treatment absent the delivery of shock waves. No gross macroscopic differences in isograft appearance, graft acceptance, or overall healing were noted in the short-term (6 h to 1 week post transplant; Fig. 1A; $n = 16$) or over a 30-day observational period. Cohorts of treated mice were euthanized at 6 h and on days 2, 4, and 7 post transplantation, at which time isografts were excised surgically and graft angiogenesis–revascularization quantified. Figure 1B ($n = 12$) shows the vascular anatomy of the central skin isograft undersurface demonstrating a marked macroscopic neovascular response in both ESWT- and sham-treated mice. The undersurface of all grafts demonstrated gross morphology consistent with viability and well-perfused appearance. Histological examination of multiple hematoxylin and eosin (H&E)- or Masson's trichrome-stained sections from the central cross-section portion of each graft on day 7 post transplantation failed to reveal any significant degree of hemorrhage or necrosis (data not shown). Gross inspection revealed marked granulation tissue development on day 4 post skin grafting followed by significant granulation tissue regression, which was evident on day 7 post skin grafting. Macroscopic analysis of the vessels in the underside of ESWT-treated and sham-treated isografts on day 4 and 7 post grafting revealed more pronounced graft edge vascularity (neovascularization, which we interpret as new blood vessel formation and vessel

in growth) in ESWT-treated isografts (Fig. 1B). In addition, day 7 ESWT-treated grafts demonstrated a greater number and more prominent blood vessels visible at the periphery (less vessel regression observed, black arrows), which progressed centrally in the treated skin isografts (Fig. 1C; 7.4 ± 2.5 vessels/cm^2 vs. 2.3 ± 0.8 vessels/cm^2; $n = 12$; $P < 0.001$). It is important to note that full-thickness skin grafts revascularize primarily from the graft edges, unlike split-thickness grafts, which have been shown to revascularize not only from the graft edges but also from the underlying recipient wound bed [8, 32]. Also, the hypodermal/adipose dermis/fascial fat layer (adipose dermis) and panniculus carnosus were all removed from the donor skin graft prior to transplantation.

Next, quantitative immunohistochemical analysis of ESWT effects on microvessel revascularization of ischemic skin tissue was conducted. Two independent observers (blinded to treatment assignment) assessed microvessel density on CD31-stained frozen tissue sections by counting the number of CD31-positive vessels per high-power field (×400 magnification). Eight to sixteen consecutive high-power fields across each graft section were enumerated (Fig. 2A, B). In addition, digital analysis software (ImageJ, NIH) was used to quantitate the total CD31-positive area(s) within each skin section, and the overall percent vascularization (Fig. 2C). Representative images of anti-CD31 immunohistochemical staining in Fig. 2A revealed significantly more newly

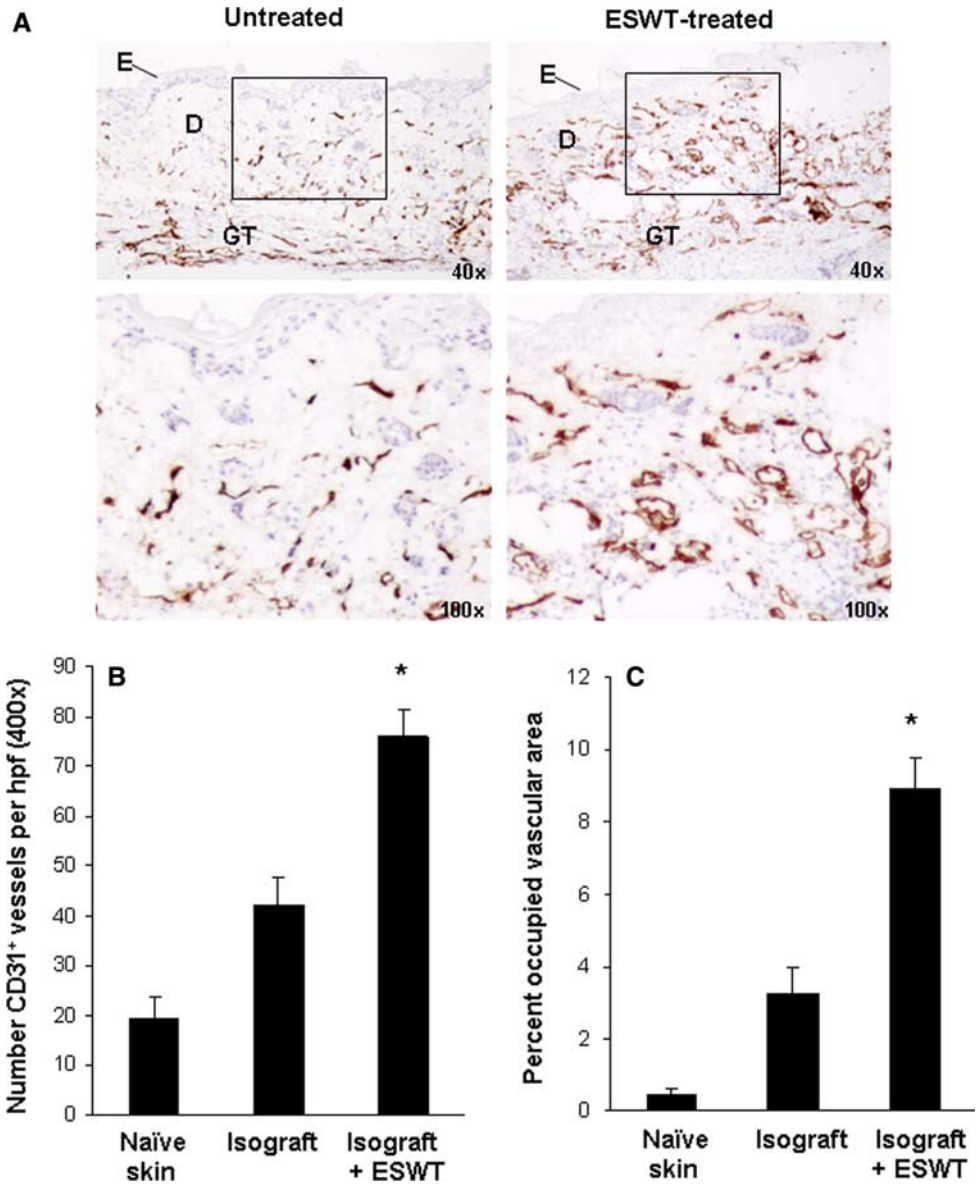

Fig. 2 **A** Microscopic immunohistochemical analysis (×40 and ×100 magnification) of CD31-stained tissue sections on 7 days post skin grafting shows a significant 1.8-fold increase in the capillary-vessel density (**B**) and a 2.7-fold increase in vascular area (**C**) in the graft-wound bed in ESWT-treated isografts compared with sham-treated isografts. Representative images from multiple sections from three independent animals in each group are shown. *E* epithelium; *D* dermis; *GT* granulation tissue. Vessel density measurements were determined by two independent observers that counted the number of CD31-positive vessels in consecutive 8–16 high-power fields (×400 magnification) across each graft section. The percent vascular areas were determined as CD31-positive areas within the graft bed using digital analysis software (ImageJ NIH). All values represent the mean ± SD ($n = 6$ animals). * $P < 0.01$, ESWT-treated compared to sham-treated isografts

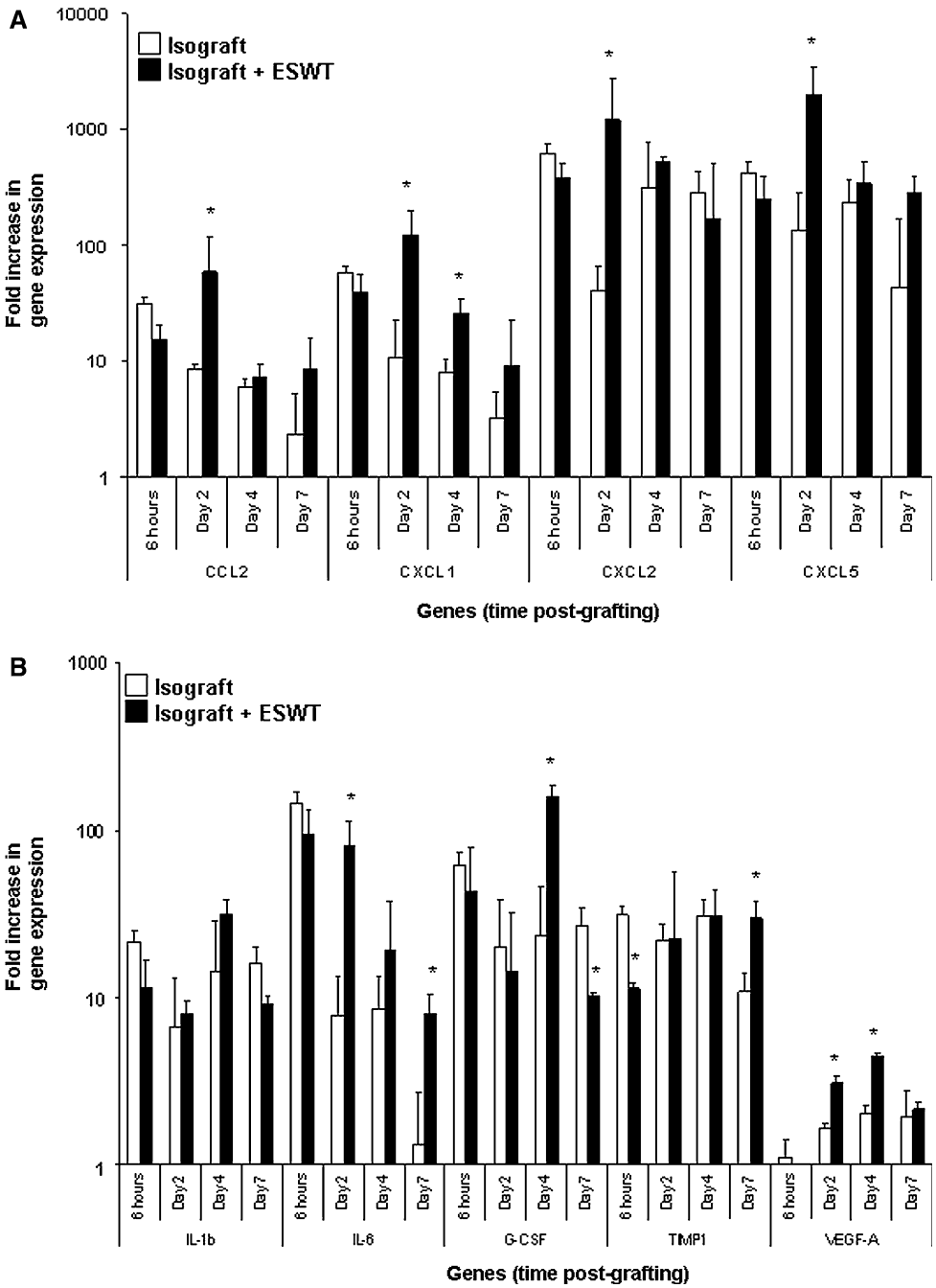

Fig. 3 ESWT enhances local pro-angiogenic gene expression in full-thickness murine skin isografts. Analysis of pro-angiogenic ELR$^+$-CXC and CC chemokines and pro-angiogenic cytokine gene expression at skin graft margins of untreated and ESWT-treated BALB/c mice. Quantitative RT-PCR on angiogenesis pathway-specific SuperArrays was performed as described in the "Materials and methods" and representative results from two independents experiments are shown at study points: 6 h, 2, 4, and 7 days post grafting ± ESWT ($n = 4$ mice per treatment group at each time point). Gene expression profiles were generated. **A**, **B** Fold change in the expression of genes within transplanted skin, including a small rim of marginal native recipient tissue, when normalized to gene expression levels in control-untreated naïve skin [untreated (□) and ESWT-treated (■) isografts]. Note differences of scales on *y* axis. Data illustrated are from one of two independent experiments with similar results and are expressed as the mean ± SD of two technical repeats per tissue sample. * $P < 0.05$, ESWT-treated compared to sham-treated isografts

formed CD31-positive blood vessels within the wound bed of ESWT-treated isografts in comparison with sham-treated isografts (Fig. 2B; 76.1 ± 5.2 vs. 42.4 ± 5.2 vessels per hpf; $n = 6$ per group; $P < 0.01$). In addition, change in vessel size during revascularization in ESWT-treated isografts was more pronounced than that of sham-treated skin grafts. In accordance with this finding, quantitative analysis 1 week post grafting revealed that the relative graft wound bed area occupied by blood vessels (as measured by the percentage of CD31-positive area in the wound bed per hpf) was 2.7-fold greater (Fig. 2C; 8.9 ± 0.8 vs. 3.3 ± 0.7; $n = 6$ per group; $P < 0.001$) in ESWT-treated grafts than in sham-treated control isografts.

ESWT enhances early expression of pro-angiogenic genes in ischemic skin grafts

In order to identify pro-angiogenic and wound repair genes that may mediate the effects of ESWT on angiogenesis, we analyzed the expression profiles of isolated mRNA transcripts in tissue digests by quantitative RT-PCR analysis. Native recipient skin at the periphery of the graft was tested at 6 h. Transplanted skin including a small rim of marginal

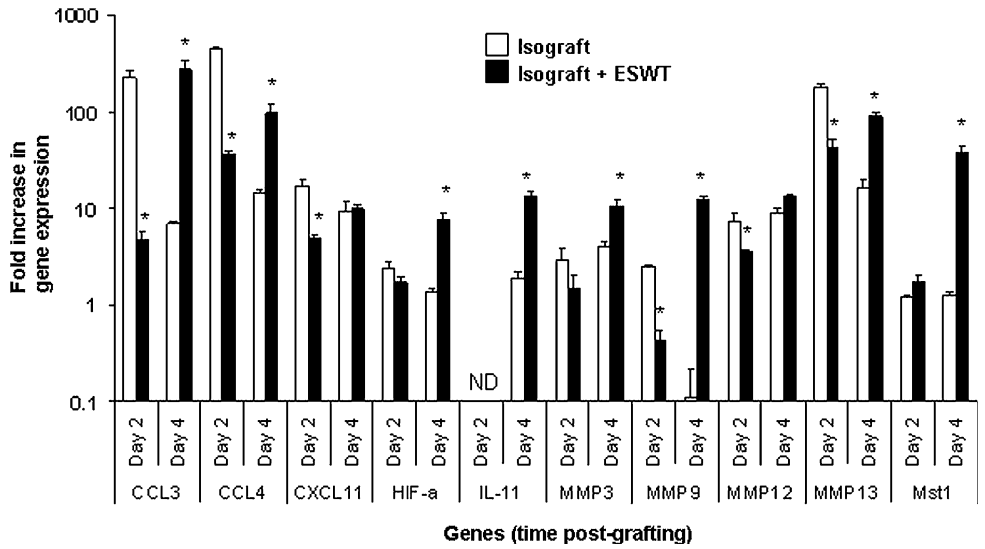

Fig. 4 The analysis of additional candidate wound repair genes utilizing custom-designed 188-gene TaqMan® Low Density Array (*TLDA*) cards by quantitative RT-PCR was performed 2 and 4 days post full-thickness skin grafting ± ESWT. ESWT-treated isografts demonstrated significantly increased expression of CC chemokines, pro-inflammatory cytokines, matrix metalloproteinases, hypoxia-inducible factor-1α, and Mst1. Gene expression profiles were generated. Fold change in the expression of genes within transplanted skin, including a small rim of marginal native recipient tissue, when normalized to gene expression levels in control-untreated naïve skin [untreated (□) and ESWT-treated (■) isografts]. Data illustrated are from one of two independent experiments with similar results and are expressed as the mean ± SD of two technical repeats per tissue sample. * $P < 0.05$, ESWT-treated compared to sham-treated isografts

native recipient tissue was tested 2, 4, and 7 days post ESWT or sham treatment.

Ischemic stress produced marked increases, relative to naïve skin, in pro-angiogenic ELR$^+$-CXC chemokines (CXCL1, CXCL2, CXCL5) and cytokines (IL-1β, IL-6, and G-CSF) within 6 h post skin grafting (Fig. 3A, B; $n = 6$ per group per time point; $P > 0.05$). Although pro-angiogenic chemokine and cytokine expression peaked early after treatment in either study treatment group, transcript levels for these angiogenic factors in ESWT-treated isografts persisted or increased significantly. Consistent with these findings, VEGF-A signals were significantly increased, albeit at relatively lower levels than other pro-angiogenic signals, on 2 and 4 days post skin grafting in ESWT-treated versus sham-treated grafts.

In addition, we observed significant changes in the differential expression of CC chemokines (CCL2, CCL3, CCL4), pro-inflammatory cytokines (IL-11), MMPs (MMP-3, MMP-9, MMP-13), hypoxia-inducible factor-1α (HIF-1α), and the mammalian sterile 20-like kinase 1 (Mst1) vascular remodeling-related gene in skin samples obtained from ESWT-treated isografts on 2 and 4 days post skin grafting (Fig. 4; $n = 4$ per group per time point). Compared with sham-treated skin grafts on day 2, ESWT-treated isografts had a 7.4-fold increase in expression of monocyte chemoattractant protein-1 (MCP-1, CCL2), a known pro-angiogenic chemokine produced by endothelial cells (ECs), smooth muscle cells, epithelial cells, and monocytes. Furthermore, ESWT-treated skin grafts on day 2 had significantly decreased local inflammatory responses as assessed by marked lower expression of macrophage-derived inflammatory protein-1α (MIP-1α, CCL3) and macrophage inflammatory protein-1β (MIP-1β, CCL4) (48.6-fold and 12.3-fold reduction, respectively) in comparison with sham-treated grafts.

Note, in comparison with sham-treated isografts, the early enhanced angiogenic response in ESWT-treated skin grafts on day 2 was accompanied by a delayed but marked increased gene expression in wound-graft macrophage-derived factors including MIP-1α (CCL3), MIP-1β (CCL4), and MMP-13 on day 4. These macrophage-derived factors along with the upregulated expression of Mst-1 are known to be pivotal in wound healing and vascular-tissue remodeling.

Discussion

The current study harnesses low-energy unfocused shock waves to illustrate positive angiogenic effects of physical energy (in the form of an acoustic pressure wave) in a murine model of tissue ischemia. The understanding of the stimulatory effects of shock waves on ischemic full-thickness skin revascularization has been extended through characterization of specific changes and early differential expression of specific pro-inflammatory genes. Our findings

suggest that a single treatment of 200 shock wave impulses at an energy flux density 0.1 mJ/mm^2 applied immediately following isogenic full-thickness skin transplantation in a murine model results in significantly enhanced graft revascularization whilst suppressing inflammation and enhancing extracellular matrix remodeling.

Various targeted therapeutic approaches have been explored to address the problem of threatened soft tissue viability, particularly in chronic wounds and remote aspects of skin flaps compromised by ischemic necrosis. Topical growth factor and cytokine therapy involving single pro-angiogenic proteins as well as adenovirus-mediated VEGF, TGF-β1, and angiopoietin-1 gene therapy have had partial therapeutic neoangiogenic effects in ischemic flaps and grafts [15, 16, 33–37]. In an effort to improve the therapeutic index, biomechanical approaches using non-invasive modalities have been utilized to stimulate angiogenesis in ischemic tissue. These include shock wave therapy, electrical stimulation, and ultrasound [26, 29, 30, 38–46]. However, the clinical decision algorithm specifying type, timing, sequence, combination, and dose intensity of these adjunctive modalities for a given wound remains to be defined.

Neovascularization of ischemic tissue is central to wound healing and is dependent both on local resident EC activation, invasion, migration, and proliferation (angiogenesis), and on formation of new vasculature stemming in part from differentiation of bone marrow-derived endothelial precursor cells (vasculogenesis) [3, 6, 47]. The regulation of angiogenesis is complex and multi-dimensional, reflecting the balance between pro-angiogenic and angiostatic factors expressed by different cell types in response to hypoxic signals [7, 33].

Angiogenesis and inflammation have been shown to be distinct, though inter-related processes; however, the temporal dominance of one biological process over the other may profoundly influence tissue repair and overall healing outcome [48]. Whereas most factors that stimulate angiogenesis have a concomitant pro-inflammatory effect [1, 48, 49], a single ESWT administration at the time of skin grafting in this study was associated with a predominant early pro-angiogenic response and a delayed inflammatory response. Relative to sham-treated grafts, shock wave-treated grafts showed early and significantly enhanced increases in pro-angiogenic gene expression of ELR$^+$-CXC chemokines (CXCL1, CXCL2, CXCL5), CC chemokines (MCP-1/CCL2), and cytokines (IL-1β, IL-6, IL-11, G-CSF). In contrast, sham-treated skin grafts showed enhanced early local inflammatory responses exceeding observed pro-angiogenic effects. The dominant pro-inflammatory response in sham-treated grafts is evident by marked (>100-fold) tissue gene expression of MIP-1α (CCL3) and MIP-1β (CCL4) when compared with ESWT-treated grafts. During the same study time points, sham-treated animals showed a 10- to 100-fold lower level of pro-angiogenic gene expression (CXCL1, CXCL2, CXCL5). Conversely, ESWT-treated skin grafts demonstrated markedly delayed as well as reduced local inflammatory responses evidenced by significantly lower expression of macrophage-derived factors, MIP-1α and MIP-1β, relative to sham-treated skin. ESWT was associated with marked differential expression of interleukin-1β (IL-1β) and interleukin-6 (IL-6), cytokines shown previously to indirectly stimulate angiogenesis through VEGF-A induction [50, 51]. Collectively, our observations suggest that an important shift in the balance of pro-angiogenic chemokines–cytokines and pro-inflammatory mediators, supporting an enhanced and prevailing early angiogenic response to ESWT. These results are consistent with those of others reporting angiogenesis, wound healing progression, formation of new vascular granulation tissue, and tissue regeneration. These findings have particular relevance when one considers the fact that scarless healing is impeded by on-going inflammation [1, 49].

In experimental models of repair, overt inflammation has been shown to delay healing and contribute to increased wound scarring. The importance of pro-inflammatory mediators and leukocyte infiltration in the overall healing and angiogenic response is evident in skin wounds of mice deficient for the TNF receptor TNF-Rp55 (decreased PMN and macrophage infiltration resulting in increased angiogenesis and accelerated healing-wound closure) or antagonist for IL-1 (increased leukocyte infiltration resulting in decreased angiogenesis and delayed healing-wound closure) [52, 53]. Consistent with our findings, recently published studies demonstrate the ability of ESWT to stimulate angiogenesis and suppress pro-inflammatory responses in ischemic skin flap segments [29, 30] and acute burn wounds [23]. Single application of low-energy ESWT immediately after extended dorsal rodent epigastric island skin flap elevation significantly enhances flap perfusion and healing of distal ischemic skin necrosis [29, 30]. This effect of ESWT appears to occur through upregulation of endothelial and fibroblast VEGF-A, upregulation of basal epidermal and fibroblast PCNA, downregulation of ischemic tissue TNF-α, and reduction in leukocyte infiltration into the distal skin flap zone of ischemia [29, 30]. A recent study of rodent hind limb ischemia demonstrated a preconditioning effect of ESWT through upregulation of chemoattractant SDF-1 mRNA and quantitative increase in VEGF$^+$ myocytes in non-ischemic limbs [38]. Applying the same ESWT dose regimen used in this study, we demonstrated previously that ESWT of burn wounds 1 h post wounding significantly blunts PMN and macrophage infiltration into the wound [23]. ESWT treatment potently attenuates both CC- and CXC-chemokine

expression, acute pro-inflammatory cytokine expression, and extracellular matrix proteolytic activity at the wound margin [23]. Major sources of these mediators at the wound edge include infiltrating PMNs and macrophages. Of note, a large full-thickness burn wound without eschar excision is extremely inflammatory in nature in comparison with smaller or equal in size incisional or excisional wounds. Additionally, several groups have reported a beneficial effect of ESWT in the reversal of ischemic heart disease in large animal models thought to be secondary to a pro-angiogenic effect [25, 54]. However, the biological mechanism of therapeutic shock waves in wound healing remains incompletely understood, and an area of active translational research.

Sequential and spatial regulation of MMPs is critical for rapid neovascularization and normal wound healing. ECs from chronic wounds have been shown to be deficient in enzyme and growth factor production; these cells demonstrate impaired migration, proliferation, and formation of new capillaries [9–11]. Similarly, keratinocytes in non-healing wounds have functional impairment apparent in reduced ability to migrate, proliferate, and synthesize cytokines, provisional matrix, and basement membrane. During ischemic injury, EC interactions with neighboring ECs are disrupted and ECM elements are digested; this is facilitated by soluble collagen-cleaving proteolytic enzymes such as MMPs [4]. Activated ECs along with monocytes/macrophages, fibroblasts, and smooth muscle cells release VEGF-A, transforming growth factor-β (TGF-β), and platelet-derived growth factor (PDGF). These paracrine pro-angiogenic cytokines enable EC mobilization, ECM infiltration, and migration along with cytokine gradients, proliferation, and neovessel formation [3, 50].

Correspondingly, we show here differential expression of VEGF-A and MMPs in ESWT-treated isogenic skin grafts early post grafting consistent with these defined mechanisms. Collectively, the findings from the present study contribute to a clearer understanding of pro-angiogenic chemokine, cytokine, and MMP responses of ischemic tissue to shock wave treatment at a molecular level. We speculate that the early pro-angiogenic and anti-inflammatory effects of ESWT may promote wound healing not only by augmenting angiogenesis and dampening early inflammation but also by inducing EC and keratinocyte cell proliferation and migration. Conversely, the early differential expression of transcripts for angiogenic cytokines and chemokines in ESWT-treated grafts may act to sustain a prolonged physiological inflammatory response that favors persistent neovascularization and delays hemodynamic remodeling, which is governed in part by other mediators (“angiogenesis-associated inflammation”). Follow-on molecular and cellular studies to elucidate definitively these mechanisms are warranted.

Although not the principal aim of our current study, sufficient work preceding ours exists to address the role of host versus donor vasculature in ischemic skin graft revascularization [8, 29]. Early revascularization is attributable to newly formed vascular anastomoses between graft and recipient wound bed vessels; this occurs mainly in the central portion of the recipient bed and graft [32]. It would also be important to distinguish the participation of preexisting versus newly formed vessels in this model. This is beyond the scope of the current study and is the focus of future investigations using GFP recipient/donor mice. It is important to re-emphasize that in our studies we used full-thickness skin grafts which, unlike split-thickness skin grafts known to revascularize from the periphery (graft edges) and underlying recipient wound bed, revascularize primarily from the peripheral graft edges [8]. This is a very important distinction between graft types.

The rationale for this experiment is based on a number of hypothesis-generating though provisional findings in the literature using shock waves to treat ischemic tissue in preliminary undeveloped studies. The aim of our study was to conclusively determine whether ESWT has a pro-angiogenic effect on ischemic tissue wound healing and whether ESWT could augment recipient skin graft revascularization. A skin graft is an ideal model for such an inquiry, as it is devascularized prior to grafting and its subsequent survival is dependent upon revascularization. The finding that ESWT augments angiogenic processes formed the basis of gene expression networks that are differentially regulated in ESWT-treated grafts when compared with untreated grafts, which may be contributory to enhanced vascular in-growth. Follow-on localization studies to determine precisely the source of the gene products and molecular signals will help to define mechanistically relevant target cells.

Therapeutic shock wave application has unique characteristics making it a suitable wound healing adjunct including non-invasiveness, ease of application, minimal risk and drug interactions, cost effectiveness, and comparable efficacy to existing treatments. We have found low-energy ESWT at energy flux densities of 0.5–0.1 mJ/mm^2 to be safe clinically, as this level of stimulation is not associated with apoptosis or tissue necrosis [22]. Although the mechanisms of therapeutic shock waves are being elucidated and are currently incompletely understood, data accumulated thus far indicate that ESWT is a feasible and clinically relevant approach to a variety of acute and chronic ischemic conditions. Therapeutic shock waves are currently undergoing clinical evaluation as a means to facilitate cutaneous tissue repair (chronic and difficult to heal acute wounds), and regeneration in thermal injury, and as a means of accelerating healing in autologous skin graft donor sites.

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EB2001 symposium report

Endothelial cellular response to altered shear stress

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Fisher, Aron B., Shu Chien, Abdul I. Barakat, and Robert M. Nerem. Endothelial cellular response to altered shear stress. *Am J Physiol Lung Cell Mol Physiol* 281: L529–L533, 2001.—Endothelial cells are normally exposed constantly to mechanical forces that significantly influence their phenotype. This symposium presented recent information concerning endothelial cell responses to shear stress associated with blood flow. Endothelial cell shear stress mechanosensors that have been proposed include membrane receptor kinases, integrins, G proteins, ion channels, intercellular junction proteins, membrane lipids (e.g., those associated with caveolae), and the cytoskeleton. These sensors are linked to signaling cascades that interact with or result in generation of reactive oxygen species, nitric oxide, and various transcription factors among other responses. Endothelial cells adapt to sustained shear stress, and either an increase or decrease from normal shear leads to signaling events. In vitro models for the study of endothelial cell responses must consider the pattern of shear stress (e.g., steady vs. oscillatory flow), the scaffold for cell growth (e.g., basement membrane or other cell types such as smooth muscle cells), and the extent of flow adaptation. These cellular responses have major relevance for understanding the pathophysiological effects of increased shear stress associated with hypertension or decreased shear stress associated with thrombotic occlusion.

mechanosensors; cell signaling; hypertension; ischemia

IT IS WIDELY APPRECIATED that cells sense and respond to a broad range of chemical stimuli. Recent evidence (10, 12) has indicated that mechanical factors can also markedly influence cell structure, growth, and function. Cells in situ are subjected to varied mechanical stresses including gravitational force, mechanical stretch or strain, and shear stress. The endothelial cells lining blood vessels are subjected to each of these mechanical forces but, in particular, are exposed to a relatively elevated shear stress associated with blood flow. How do cells, and the endothelium in particular, sense a change in shear stress, and what are the signaling pathways for the cellular response? From a simplistic standpoint, changes in fluid shear stress could be sensed directly by cell membrane components such as membrane proteins, ion channels, or caveolae or by alterations of the cellular cytoskeleton; subsequent cellular signaling cascades through phosphorylation events or generation of reactive oxygen species (ROS) can lead to diverse effects such as the release of cytokines and other mediators, activation of transcription factors, altered gene and protein expression, and cell division or death (7, 8, 14, 18, 22, 31). These issues that are summarized in this report were considered at a symposium held during the Experimental Biology Meeting in Orlando, FL, on April 4, 2001. The symposium was sponsored by the Respiration Section of the

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to cessation of flow, followed within the first minute by ROS generation, increased intracellular Ca^{2+}, and NO generation. Thus these events represent a rapid cellular response to the loss of shear. The use of various inhibitors suggested that membrane depolarization results from inactivation of a shear stress-sensitive K^+ channel (3, 26), that generation of ROS occurs through activation of endothelial membrane NADPH oxidase (lung ROS generation with ischemia was lost in mice with "knockout" of $gp91^{phox}$, the flavoprotein component of the oxidase) (2), that increased intracellular Ca^{2+} is due to intracellular release followed by influx from extracellular sources (1, 26, 29), and that the generation of NO is due to activation of endothelial NO synthase (1).

To extend the results obtained in the intact lung to an in vitro system, Manevich et al. (20) developed a cell culture model that permitted the direct spectroscopic evaluation of cells during the initial phase of simulated ischemia. Bovine pulmonary artery endothelial cells were grown in a parallel plate chamber constructed to allow its insertion into a standard spectrophotometer or fluorometer chamber. Cells that were adapted to laminar flow (5 dyn/cm^2 for 24 h) exhibited the characteristic structural reorganization, with their long axis in the direction of flow. We confirmed that oxygenation of the medium remained adequate during the initial 3 min of simulated ischemia because Po_2 was >40 mmHg and the observed initial effects of ischemia were not altered by preequilibration of the medium with 100% oxygen. Flow-adapted (but not control) cells subjected to simulated ischemia demonstrated cell membrane depolarization, ROS generation, increased intracellular Ca^{2+}, and NO generation as observed for the endothelium in the intact lung. As expected for the membrane-bound NADPH oxidase, generation of $O_2^-\cdot$ was demonstrated to be extracellular by reduction of exogenous cytochrome *c* and its inhibition with superoxide dismutase; H_2O_2 formed from $O_2^-\cdot$ would diffuse freely into cells during continued ischemia, serving as a mechanism for signal transduction. An additional in vitro study (31) utilized bovine pulmonary artery endothelial cells grown in an artificial capillary system where the oxygenation of cells during simulated ischemia could be maintained for prolonged periods. These studies demonstrated that ROS production led to activation of the transcription factors nuclear factor-κB and c-Jun after 1 h of ischemia and increased cell division at 24 h of ischemia.

Our interpretation of these results is that the loss of shear stress associated with flow cessation in flow-adapted endothelial cells leads to a complex signaling response, possibly initiated by flow-sensitive K^+ channels, leading to membrane depolarization and propagated by ROS generation and Ca^{2+} release. The physiological significance of these signaling events may be the net generation of vasoactive mediators and signals for capillary angiogenesis in an attempt to restore the compromised circulation.

ROLE OF ION CHANNELS IN SHEAR STRESS SENSING IN VASCULAR ENDOTHELIUM[3]

The ability of arterial endothelial cells to sense and respond to changes in fluid mechanical shear stress is essential for vasoregulation and for vascular wall remodeling and may play a role in the development and localization of early atherosclerotic lesions. Exposure to shear stress elicits humoral, metabolic, and structural responses in endothelial cells. A virtually immediate endothelial response to shear stress is the activation of flow-sensitive ion channels. Because of their very rapid response to flow, these ion channels have been hypothesized to play a role in shear stress sensing and transduction.

The activation of flow-sensitive ion currents in endothelial cells was first reported by Olesen et al. (23), who used whole cell patch-clamp recordings to demonstrate that steady shear stress stimulates inward rectifying K^+ channels, the activation of which leads to cell membrane hyperpolarization. More recently, Barakat et al. (4) used whole cell patch-clamp recordings and measurements from membrane potential-sensitive fluorescent dyes to demonstrate that steady shear stress also induces an outward rectifying Cl^- current in endothelial cells and that activation of this current reverses the K^+ channel-mediated hyperpolarization to depolarization within ~100 s of the onset of flow. On cessation of flow, the membrane potential returns to preflow baseline levels, but this process is relatively slow. The K^+ and Cl^- currents are independently activated; pharmacologically blocking either current does not interfere with the activation of the other by flow. Although both of these currents are activated very rapidly on the initiation of flow, the fact that the cell membrane initially hyperpolarizes and then depolarizes suggests that flow-sensitive K^+ channels attain full activation and/or desensitize more rapidly than the Cl^- channels. Differences in the dynamics of activation and desensitization between the two channel types may have important implications for the vascular endothelial responsiveness to a sustained flow stimulus.

Endothelial cells in arterial regions prone to the development of early atherosclerosis are cuboidal (or round), whereas in athero-resistant regions, they are elongated. Therefore, there is a need for understanding how the shape of endothelial cells may regulate their function. Toward this goal, endothelial cells were exposed for 24 h to a steady shear stress of 19 dyn/cm^2 that led to extensive cytoskeletal remodeling and cellular elongation and alignment in the direction of flow. Subsequent whole cell patch-clamp recordings on flow-elongated endothelial cells revealed that, as in previously unsheared cells, flow results in cell membrane hyperpolarization followed by depolarization and hence activates both the flow-sensitive K^+ and Cl^- channels. These results may be interpreted in one of two ways: either cell shape does not play a role in

[3] Presented by Abdul I. Barakat.

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