Serum Proteomic Analysis of Extracorporeal Shock Wave Therapy–Enhanced Diabetic Wound Healing in a Streptozotocin-Induced Diabetes Model

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Background: Previous studies have demonstrated that extracorporeal shock wave therapy has a significant positive effect on accelerating diabetic wound healing. However, the systemic effect after therapy is still unclear.
Methods: This study investigated the plasma protein expression in the extracorporeal shock wave therapy group and diabetic controls using proteomic study. A dorsal skin defect (6 × 5 cm) in a streptozotocin-induced diabetic Wistar rat model was used. Diabetic rats receiving either no therapy or extracorporeal shock wave therapy after wounding were analyzed. The spots of interest were subjected to in-gel trypsin digestion and matrix-assisted laser desorption ionization time-of-flight mass spectrometry to elucidate the peptide mass fingerprints. The mass spectrometric characteristics of the identified proteins, including their theoretical isoelectric points, molecular weights, sequence coverage, and Mascot score, were analyzed. Protein expression was validated using immunohistochemical analysis of topical periwounding tissues.
Results: The proteomic study revealed that at days 3 and 10 after therapy rats had significantly higher abundance of haptoglobin and significantly lower levels of the vitamin D-binding protein precursor as compared with the diabetic controls. Immunohistochemical staining of topical periwounding tissue also revealed significant upregulation of haptoglobin and downregulation of vitamin D-binding protein expression in the extracorporeal shock wave therapy group, which was consistent with the systemic proteome study.
Conclusion: Proteome analyses demonstrated an upregulation of haptoglobin and a downregulation of vitamin D-binding protein in extracorporeal shock wave therapy–enhanced diabetic wound healing. (*Plast. Reconstr. Surg.* 133: 59, 2014.)

Nonhealing foot ulcers in patients with diabetes are the leading cause of complications such as infection and amputations. Studies have described various treatment modalities that produce controversial results with regard to wound healing in diabetic patients.[1] Appropriate wound management varies according to the cause of the wound and the principles of good wound care, such as aggressive débridement, adequate infection control, pressure off-loading, angioplasty or bypass surgery for ischemic ulcers, hyperbaric oxygen therapy, and topical wound dressings.[1–4] Although poor wound healing is a major complication in diabetic patients and could result in morbidity or death, tissue ischemia induced by leukocyte-mediated inflammation and inadequate neovascularization are believed to be the principal factors that predispose a patient to poor wound healing. There

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Received for publication March 28, 2013; accepted June 13, 2013.

DOI: 10.1097/01.prs.0000439050.08733.cf

Disclosure: *None of the authors has a financial interest in any of the products, devices, or drugs mentioned in this article.*

are many adjunctive therapeutic approaches; however, wounds can be highly resistant to treatment in some cases or even slowly progressive.

Shock waves are sonic pulses generated by an underwater high-voltage condenser spark discharge and then focused at the diseased area, using an elliptical reflector.[5] Extracorporeal shock wave therapy is a widely accepted technique for treating patients with urinary stones to disintegrate urolithiasis. It has recently been applied extensively in orthopedics for soft-tissue repair and regeneration, and has shown a positive influence on calcifying tendinitis of the shoulder, epicondylitis of the elbow, and plantar fasciitis.[6,7] Our previous studies reported that topical extracorporeal shock wave therapy enhances the survival of ischemic flap tissue in the rat model,[8,9] and an extracorporeal shock wave therapy strategy is feasible and well tolerated with chronic wound healing in the rodent diabetic model.[10] We have applied extracorporeal shock wave therapy in human diabetic ulcer clinical trials and obtained promising results.[11] This technique represents a feasible method for enhancing wound healing or improving compromised tissue circulation. Our past studies attempted to elucidate the mechanical effects of extracorporeal shock wave therapy in diabetic wound healing, and we found that it significantly increased expression of vascular endothelial growth factor, endothelial nitric oxide synthase, and proliferating cell nuclear antigen, and enhanced topical blood perfusion of wound edges.[10,11] A recent study also showed that extracorporeal shock wave therapy induced endothelial nitric oxide synthase at the wound tissues and accelerated the wound healing through promoting vascular endothelial growth factor expression and neovascularization.[12] Therefore, it was proposed that the potential mechanisms of extracorporeal shock wave therapy include initial neovascularization with ensuing durable and functional angiogenesis.[13] Recruitment of mesenchymal stem cells, stimulated cell proliferation and differentiation, and anti-inflammatory effects are also considered important facets of the biological responses to extracorporeal shock wave therapy.[13,14] These studies indicated that the therapy increased the tissue neovascularization and regeneration associated with suppression of the topical tissue inflammatory response. However, the exact biomechanisms of extracorporeal shock wave therapy in wound healing are still unclear.

Proteomic technology is an emerging tool for detecting the simultaneous expression of various proteins in serum, fluid, cell cultures, and tissue in pathological conditions. The use of proteomic technologies to detect changes in systemic serum protein and topical wound edge tissue expression in diabetic wound healing after extracorporeal shock wave therapy has not been tested. Therefore, in this study, we extended our previous studies and performed serum proteome analysis to elucidate the biosignals of extracorporeal shock wave therapy in the enhancement of the wound healing process.

MATERIALS AND METHODS

Streptozotocin-Induced Diabetes Mellitus

Rats with diabetes were induced by a single intraperitoneal injection of streptozotocin (50 mg/kg; Sigma-Aldrich, St. Louis, Mo.). One week after injection, blood sugar was measured from tails. Rats with a blood sugar level greater than 300 mg/dl were defined as having successful induction of diabetes and were then used for subsequent experiments. To equalize the blood sugar level at 200 mg/dl, diabetic rats were subcutaneously administered intermittent-acting insulin (1 to 2 unit/kg; Montards Novo Nordisk A/S, Bagsvaerd, Denmark) until the animals were killed with an overdose of sodium pentobarbital.

Animal Model

The animal model followed our previous studies.[11,15] The wounding operation was performed 4 weeks after streptozotocin injection and made sure the blood sugar level was greater than 200 mg/dl. The skin flap tissue of the dorsum of the Wistar rats was excised to create a skin defect with an area of 6 × 5 cm. The entire skin was undermined below the level of the dorsal fascia, and the margin of the wound defect was sutured in place with 4-0 silk sutures to prevent wound contracture. The wound was temporarily covered with transparent Tegaderm (3M HealthCare, Borken, Germany) until extracorporeal shock wave therapy was initiated. Animals were treated humanely according to the guidelines provided in the *Guide for the Care and Use of Laboratory Animals* (National Institute of Health, Bethesda, Md.). The Division of Laboratory Animal Resources at Kaohsiung Chang Gung Memorial Hospital administered veterinary care to the rodents. This study was approved by the Institutional Animal Care and Use Committee at Kaohsiung Chang Gung Memorial Hospital.

Experimental Design and Serum Sample Collection

Twelve 4-month-old male Wistar rats (National Experimental Animals Production Center, Taipei,

Taiwan) with streptozotocin-induced diabetes were divided into two groups (six rats in each group). The dorsal skin defect was created on all rats, but only the extracorporeal shock wave therapy group was treated with two sessions of defocused shock waves (Reflector Type CP155; MTS GmbH, Konstanz, Germany) using 800 impulses at 10 kV, equivalent to an energy flux density of 0.09 mJ/mm^2, on days 3 and 7 after wounding. The shock waves were applied to eight areas along the margin of the dorsal wound (100 impulses/area × 8 areas in all wound edges; each area had about a 2-cm radius). Peripheral blood samples were collected from tail veins of these animals at 3 and 10 days after treatment or the similar days after wounding (10 and 17 days) in control diabetic groups, processed to obtain serum, and then stored at −80°C until analysis.

Isoelectric Focusing, Gel Electrophoresis, and Silver Staining

Isoelectric focusing, two-dimensional electrophoresis, and sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis of serum proteins were performed on an Ettan IPGphor II/3 IEF system and SE 600 Ruby gel apparatus (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Briefly, 250 μg of serum protein in 250 μl of rehydration buffer was loaded onto Immobiline strips (pH 4 to 7, 13 cm; GE Healthcare Bio-Sciences AB) and isoelectric focused at 32,000 V/hr at 20°C. After isoelectric focusing, the strips were equilibrated in a buffer containing 50 mM Tris-HCl (pH 8.8), 30% glycerol, 2% sodium dodecyl sulfate, 0.25% iodacetamide, and 8 M urea, and loaded onto the 10% sodium dodecyl sulfate–polyacrylamide gels. Two-dimensional electrophoresis was performed at 110 V at 4°C for 16 hours, and then the gels were silver-stained using the PlusOne Silver Staining Kit (GE Healthcare Bio-Sciences AB) according to the manufacturer's instructions. Each specimen was subjected to isoelectric focusing and gel electrophoresis in duplicate.

Gel Imaging

The silver-stained polyacrylamide gels were scanned using an ImageScanner (GE Healthcare Bio-Sciences AB). The gel images and spot patterns were matched and analyzed using Bio-Rad Proteoweaver 2-D Analysis Software version 4.0 (radius limit, 4; intensity limit, 2000; contrast limit, 50; border contrast, 0.2; active spots intensity warning limit, 5000) (Bio-Rad Laboratories Inc., Hercules, Calif.).

Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry

The spots of interest were manually excised, washed with deionized water, destained, rehydrated, reduced, and then trypsin digested. The digestion products were extracted with 1% trifluoroacetate in acetonitrile. Aliquots of the extracted digestion products were then loaded onto AnchorChip (Burker Daltonics, Leipzig, Germany) followed by matrix-assisted laser desorption ionization time-of-flight assessment using an Ultraflex TOF/TOF mass spectrometer (Bruker Daltonik GmbH, Leipzig, Germany). The peptide mass data were submitted to the National Center for Biotechnology Information and SWISS-PROT databases using Mascot (Matrix Science, Boston, Mass.) search engines for peptide matching. The matched peptides that were considered as potent candidates had the highest Mascot score (≥65) and a peptide sequence coverage of 20 percent of the matched peptide.

Immunohistochemical Staining

Polyclonal antibodies against haptoglobin (GeneTex, Irvine, Calif.) and vitamin D-binding protein (LifeSpan BioSciences, Seattle, Wash.) were used as the primary antibodies. The tissue sections were incubated with primary antibodies (1:200 dilutions) for 1 hour, and then incubated with biotinylated goat anti-rabbit antibodies for 30 minutes. The specific binding of the secondary antibodies to the primary antibodies was visualized using a horseradish peroxidase–diaminobenzidine staining kit (R&D Systems, Minneapolis, Minn.). After staining, the sections were mounted, cleared, cover-slipped, and examined using a Zeiss microscope (Zeiss, Gottingen, Germany).

Statistical Analysis

All values are expressed as mean ± standard error. A paired *t* test was used to detect the differences between two groups of each protein level. The test was two-sided, with statistical significance set at 0.05, and all computations were made using SPSS for Windows release 13.0 software (SPSS, Chicago, Ill.).

RESULTS

Two-Dimensional Electrophoresis Profiles

Two-dimensional electrophoresis was performed using serum samples obtained from rats in the diabetic control group and the group that received two sessions of extracorporeal shock wave therapy at days 3 and 10 after shock

wave treatment. We chose these two time periods because the histological examination of our previous study revealed significant suppression of the proinflammatory reaction and increased tissue regeneration. Image analysis showed that approximately 180 spots (178.5 ± 7.8 spots in the control diabetic group versus 181.2 ± 7.5 spots in the extracorporeal shock wave therapy group)

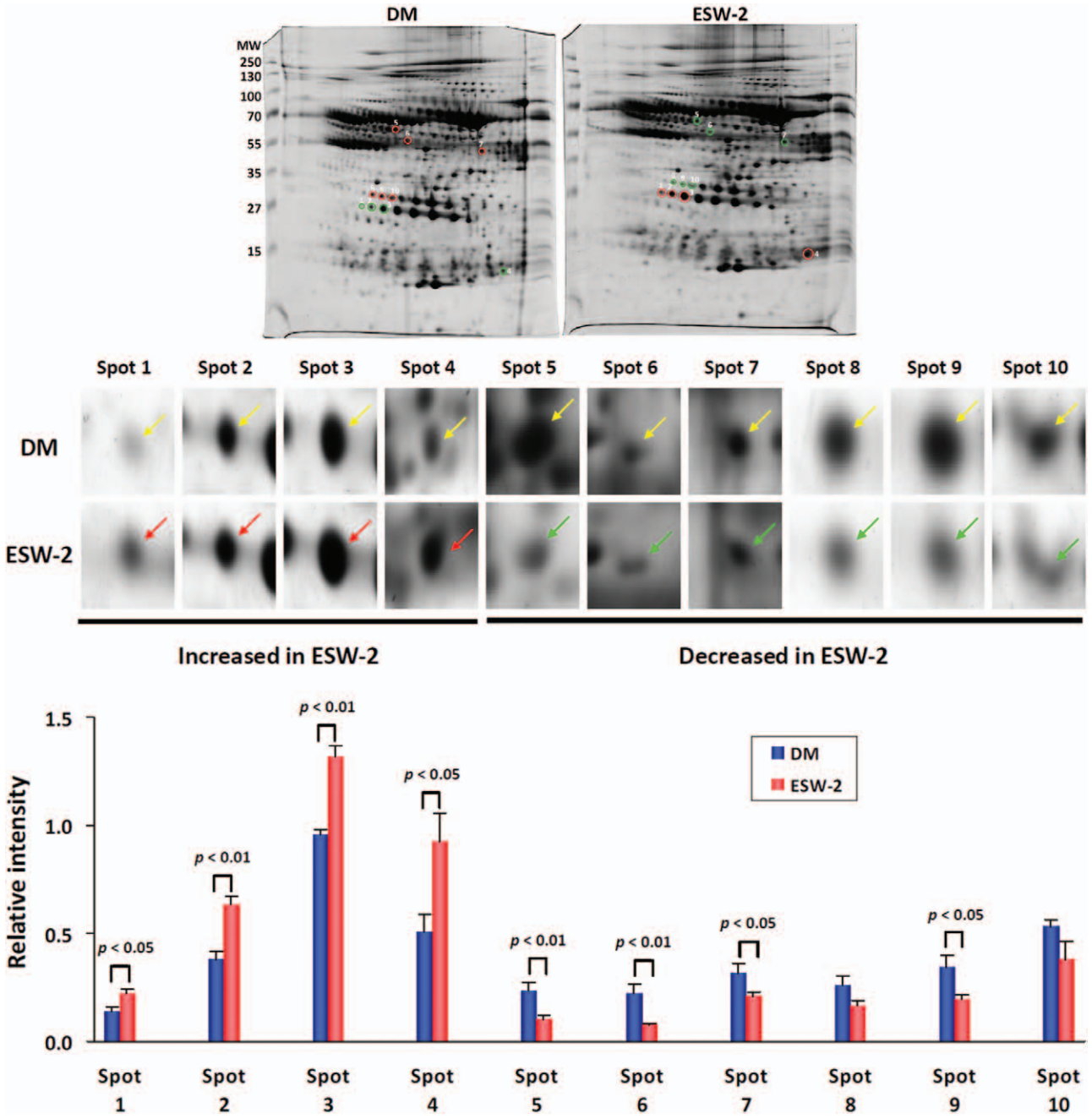

Fig. 1. Protein spots of interest in two-dimensional gel electrophoretograms of serum proteins obtained from a control streptozotocin-induced diabetes mellitus rat (*DM*) and a streptozotocin-induced diabetic rat at day 3 after two sessions of extracorporeal shock wave therapy (*ESW-2*). (*Above*) Representative two-dimensional gel electrophoretograms of serum proteins. The serum samples (200 μg) were subjected to isoelectric focusing (pH 4 to 7), sodium dodecyl sulfate–polyacrylamide gel separation, and silver staining. The numbers on the left indicate the molecular weight (*MW*), in kilodaltons. The serum samples were taken on day 10 after wounding in the DM group and on day 3 (day 10 after wounding) in the ESW-2 group. (*Center*) Enlarged regions of the 10 spots of interest in the silver-stained sodium dodecyl sulfate–polyacrylamide gels. The spots in the gels of DM and ESW-2 rats were matched using Bio-Rad Proteoweaver 2-D Analysis Software version 4.0. The *arrows* indicate the spots of interest. (*Below*) Relative intensities of the positively identified proteins. Relative density was calculated by dividing the density of the matched spot by the density of all the matched spots in the respective gel.

($p = 0.810$) could be detected in the gels (Figs. 1, *above*, and 2, *above*). Serum protein spots in each rat of the group that received two sessions of shock wave therapy were further matched and compared with those in control diabetic group, and the relative density of each spot in each matched gel was calculated. In the serum samples obtained from rats on day 10 after wounding (day 3 after extracorporeal shock wave therapy for the group that received two sessions of shock therapy), the intensities of 10 protein spots were significantly different (Fig. 1). Among these 10 protein spots, the intensities of four of the spots were increased and six were decreased in shock wave treatment rats, as compared with that in controls without shock wave therapy (Fig. 1). In the serum samples obtained from rats on day 17 after wounding (day 10 after extracorporeal shock wave therapy for the group treated with two sessions of shock wave therapy), the intensities of seven protein spots were significantly different (Fig. 2). Among the seven protein spots, the intensities of two of the spots were increased in shock wave therapy rats and five were decreased in these rats, as compared with the control diabetic rats (Fig. 2).

Protein Identification

To elucidate the peptide mass fingerprints, the protein spots of interest (days 3 and 10 after extracorporeal shock wave therapy) were subjected to in-gel trypsin digestion and matrix-assisted laser desorption ionization time-of-flight mass spectrometry. The peptide mass data of each spot were submitted to the National Center for Biotechnology Information and SWISS-PROT bioinformation stations using Mascot search engines. Ten spots of interest were positively identified as haptoglobin (spots 1, 2, and 3), albumin (spot 4), T-kininogen 1 (spot 5), vitamin D-binding protein (spot 6), serotransferrin (spot 7), and serine protease inhibitor A3N (spots 8, 9, and 10) in rats at day 3 after shock wave treatment, as compared with those in controls. In shock wave–treated rats at day 10 after therapy, the seven spots of interest were positively identified as immunoglobulin lambda light chain (spot 1), haptoglobin (spot 2), vitamin D-binding protein (spots 3, 4, and 5), apolipoprotein A-IV (spot 6), and immunoglobulin kappa chain C region (spot 7). The mass spectrometric characteristics of the identified proteins are summarized in Tables 1 and 2.

Rats treated with extracorporeal shock wave therapy had significantly higher levels of haptoglobin ($p < 0.01$) and albumin ($p < 0.05$) and significantly lower levels of T-kininogen 1 ($p < 0.01$), vitamin D-binding protein ($p < 0.01$), serotransferrin ($p < 0.05$), and serine protease inhibitor A3N at day 3 after treatment (Fig. 1, *below*). At day 10 after treatment, they had significant upregulation of immunoglobulin lambda light chain ($p < 0.05$) and haptoglobin ($p < 0.05$) and downregulation of vitamin D-binding protein precursor ($p < 0.05$), apolipoprotein A-IV precursor, and immunoglobulin kappa chain C region (Fig. 2, *below*).

Confirmation of Haptoglobin and Vitamin D-Binding Protein Expression Using Immunohistochemical Staining

Since upregulated haptoglobin and downregulated vitamin D-binding protein were observed in shock wave–treated rats at both days 3 and 10 after treatment using two-dimensional gel electrophoresis, we further confirmed the differential protein expression by immunohistochemical staining using biopsy samples obtained from the wound margin of rats in the normal control group, the diabetic control group, and the group treated with two sessions of shock wave therapy (days 3 and 10 after shock wave therapy or days 10 and 17 after wounding). The immunohistochemical staining analysis revealed that haptoglobin expression was significantly increased and vitamin D-binding protein expression significantly decreased in shock wave–treated rats at both days 3 and 10 after treatment (days 10 and 17 after wounding) as compared with that in the control group ($p < 0.001$) (Fig. 3). Furthermore, we also demonstrated a higher expression level of haptoglobin and a lower expression level of vitamin D-binding protein in normal control rats than in diabetic rats. These results were consistent with our finding observed in two-dimensional gel electrophoresis of serum samples.

DISCUSSION

Recent studies have indicated treatment with an optimal session of extracorporeal shock wave therapy represents a feasible method for enhancing wound healing associated with increased neoangiogenesis and tissue regeneration, and topical anti-inflammatory response.[10,16] However, the biomechanism by which this treatment modality exerts its therapeutic effects remains unclear. To further elucidate the effects of extracorporeal shock wave therapy in wound healing, we investigated the differential plasma protein expressions

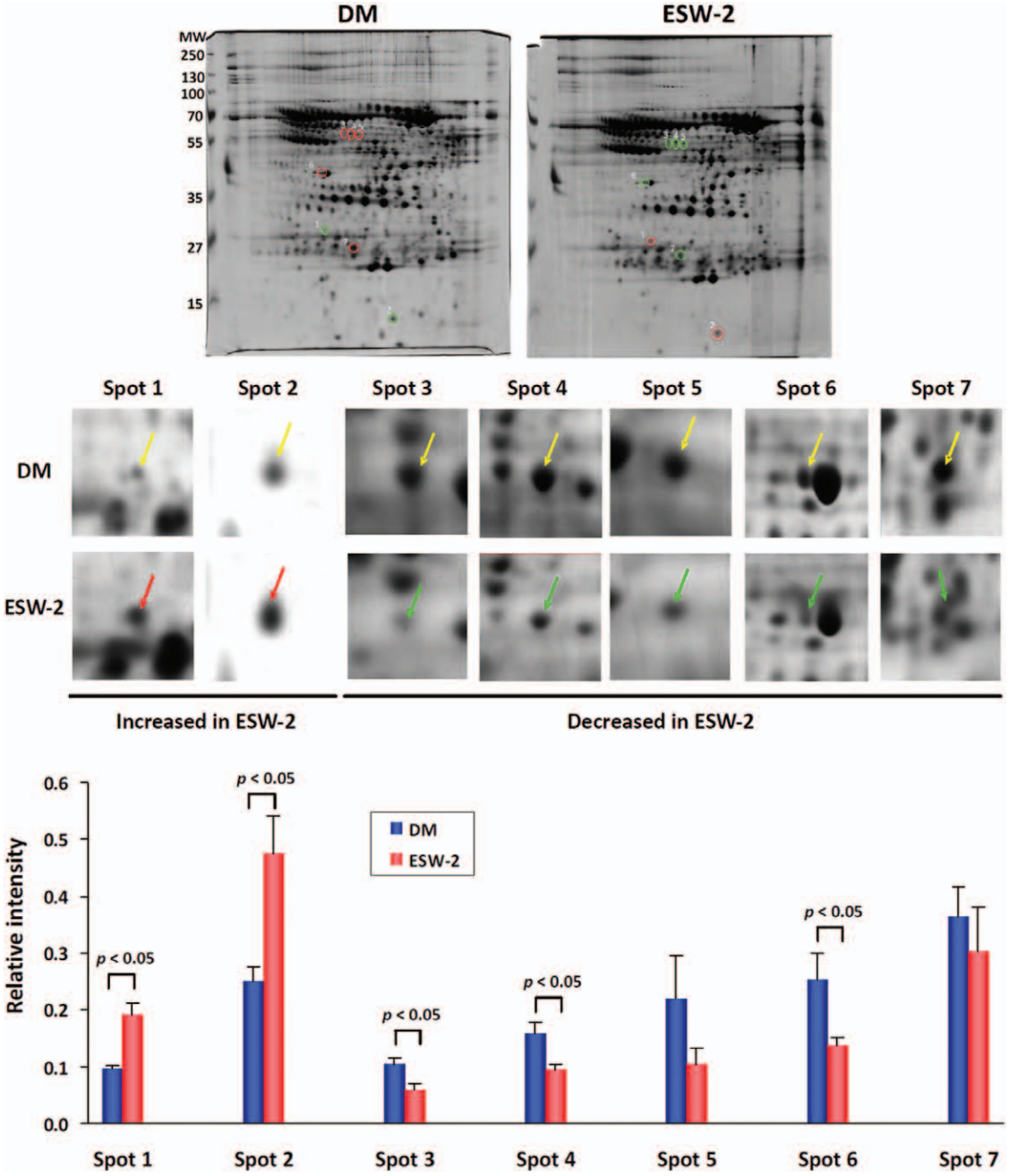

Fig. 2. Protein spots of interest in two-dimensional gel electrophoretograms of serum proteins obtained from control streptozotocin-induced diabetes mellitus rat (*DM*) and streptozotocin-induced diabetic rat at day 10 after two sessions of extracorporeal shock wave therapy (*ESW-2*). (*Above*) Representative two-dimensional gel electrophoretograms of serum proteins. Serum samples (200 μg) were subjected to isoelectric focusing (pH 4 to 7), sodium dodecyl sulfate–polyacrylamide gel separation, and silver staining. The numbers on the left indicate molecular weight (*MW*), in kilodaltons. The serum samples were taken on day 17 after wounding in the DM group and on day 10 (day 17 after wounding) in the ESW-2 group. (*Center*) Enlarged regions of the seven spots of interest in the silver-stained sodium dodecyl sulfate–polyacrylamide gels. The spots in the gels of DM and ESW-2 rats were matched using Bio-Rad Proteoweaver 2-D Analysis Software version 4.0. The *arrows* indicate the spots of interest. (*Below*) Relative intensities of the positively identified proteins. Relative density was calculated by dividing the density of matched spot by the density of all the matched spots in the respective gel.

Table 1. Mass Spectrometric Characteristics of Positively Identified Spots in Serum of Rats That Had Undergone Two Sessions of Extracorporeal Shock Wave Therapy at Day 3 after Treatment

Spot	Identified Protein	Nominal Mass	Theoretical Isoelectric Point	Sequence Coverage (%)	Matched Peptides	Searched Peptides	Mascot Score
1	Haptoglobin	38539	6.10	21	11	35	61
2	Haptoglobin	38539	6.10	37	18	46	118
3	Haptoglobin	38539	6.10	43	20	41	147
4	Albumin	68686	6.09	20	11	30	72
5	T-kininogen 1	47745	6.08	26	9	31	93
6	Vitamin D-binding protein	53509	5.65	30	12	27	70
7	Serotransferrin	76346	7.14	15	11	21	78
8	Serine protease inhibitor A3N	46622	5.33	37	15	33	123
9	Serine protease inhibitor A3N	46622	5.33	26	9	39	62
10	Serine protease inhibitor A3N	46622	5.33	22	10	32	76

Table 2. Mass Spectrometric Characteristics of Positively Identified Spots in Serum of Rats That Had Undergone Two Sessions of Extracorporeal Shock Wave Therapy at Day 10 after Treatment

Spot	Identified Protein	Nominal Mass	Theoretical Isoelectric Point	Sequence Coverage (%)	Matched Peptides	Searched Peptides	Mascot Score
1	Immunoglobulin lambda light chain	22621	5.45	50	8	42	82
2	Haptoglobin, isoform CRA_alpha	16094	5.37	63	15	43	134
3	Vitamin D-binding protein precursor	53482	5.76	41	15	37	71
4	Vitamin D-binding protein precursor	53482	5.76	51	25	52	128
5	Vitamin D-binding protein precursor	53482	5.76	38	13	38	76
6	Apolipoprotein A-IV	44429	5.12	69	30	58	239
7	Immunoglobulin kappa chain C region	11725	4.99	87	8	52	87

in the diabetic rats treated with or without extracorporeal shock wave therapy, using proteomic study and matrix-assisted laser desorption ionization time-of-flight mass spectrometry to characterize the identified proteins.

The results of this current study revealed that wounded diabetic rats that received extracorporeal shock wave treatment had significantly higher levels of haptoglobin and albumin and significantly lower levels of T-kininogen 1, vitamin D-binding protein, serotransferrin, and serine protease inhibitor A3N at day 3 after treatment. At day 10 after treatment, significantly upregulated immunoglobulin lambda light chain and haptoglobin and downregulated vitamin D-binding protein precursor, apolipoprotein A-IV precursor, and immunoglobulin kappa chain C region were observed. Since the expression of serum haptoglobin and vitamin D-binding protein was up- and downregulated, respectively, at both 3 and 10 days after extracorporeal shock wave treatment, immunohistochemical staining was performed to confirm this observation. Results of immunohistochemical analysis also revealed higher haptoglobin and decreased vitamin D-binding protein expression in periwounding tissue at 3 days and 10 days after extracorporeal shock wave treatment (Fig. 3). These results indicate that upregulated haptoglobin and downregulated vitamin D-binding protein may be involved in extracorporeal shock wave treatment–accelerated diabetic wound healing.

Haptoglobin is an abundant hemoglobin-binding protein in plasma whose major function is to prevent heme-iron–mediated oxidation. Haptoglobin transcription is increased in response to inflammatory stimuli, and its anti-inflammatory activities are important for the maintenance of redox homeostasis while augmenting tissue repair.[17,18] The haptoglobin-hemoglobin complex (CD163) is recognized by the CD163 receptor on monocytes and macrophages.[19,20] The formation of haptoglobin-hemoglobin complexes-1 rapidly stimulates the production of anti-inflammatory cytokines, such as interleukin 10 and interleukin 6.[18] Haptoglobin directly promotes the migration of fibroblasts needed for tissue regeneration by inhibiting the activities of the matrix metalloproteinases (matrix metalloproteinases 2 and 9) required for the breakdown of gelatin.[21] In our previous study, we have demonstrated that extracorporeal shock wave therapy exerts its anti-inflammatory effect by suppressing tumor necrosis factor-α expression and decreased CD45-positive leukocyte cells in topical wounding tissue.[8] In this study, the significant increase of haptoglobin

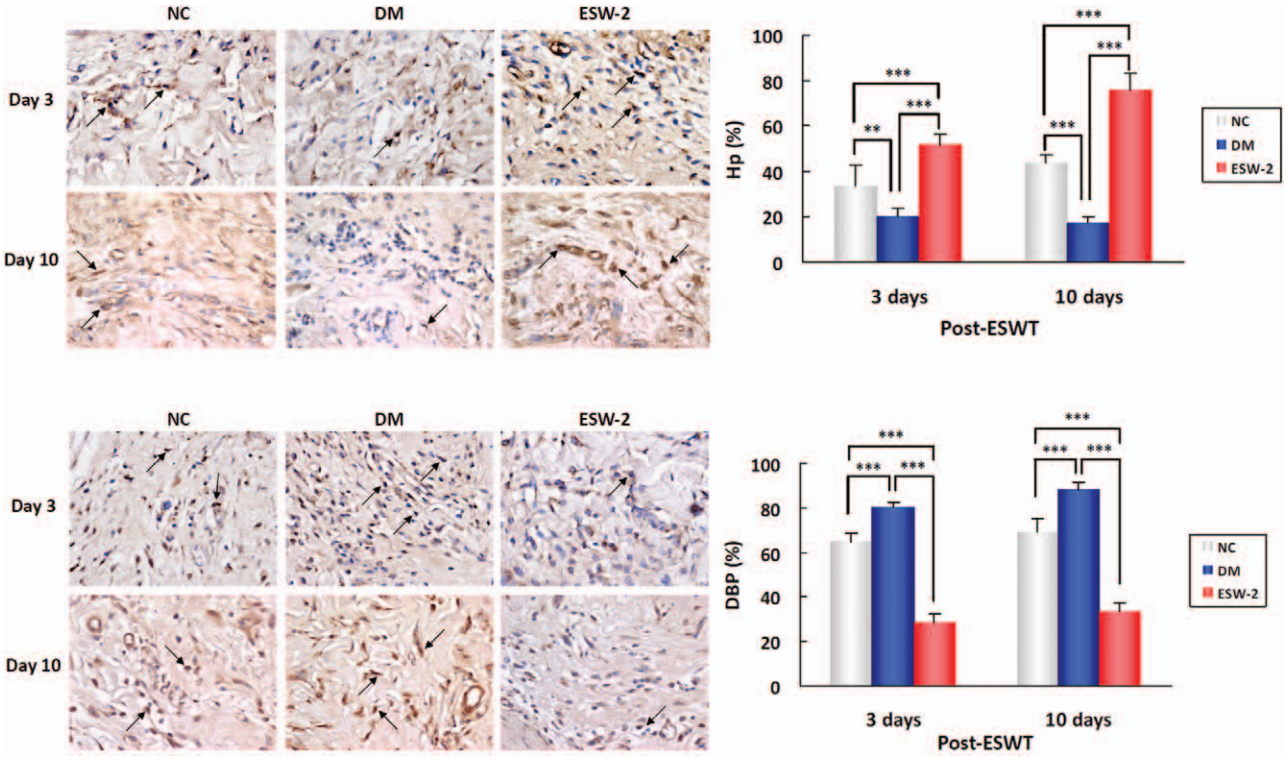

Fig. 3. Extracorporeal shock wave therapy (*ESWT*) upregulated haptoglobin (*Hp*) and vitamin D-binding protein (*DBP*) expression in diabetic wound edges. Immunohistochemical staining was performed on biopsy samples obtained from the transitional zone of the wound edge at days 10 and 17 after wounding of normal control (*NC*) rats, control diabetic (*DM*) rats, and diabetic rats at days 3 and 10 after two sessions of extracorporeal shock wave therapy (*ESW-2*). The original magnification is 400× and representative microscopic fields are shown. (*Above*) Cells positively stained for haptoglobin antibody are shown. $^{**}p < 0.005$. $^{***}p < 0.001$. (*Below*) Cells positively stained for vitamin D-binding protein antibody are shown. $^{***}p < 0.001$.

observed at both day 3 and day 10 after shock wave therapy further suggests that haptoglobin was at least partly involved in the anti-inflammatory effect and increased tissue regeneration of shock wave therapy–induced wound healing.

Vitamin D-binding protein is a multifunctional, highly expressed, 458-amino acid polymorphic serum protein.[22] It is the major plasma carrier of vitamin D3 and its metabolites.[23] It has been shown to enhance the leukocyte chemotactic activity of activated complement peptides, which are the precursor of the macrophage-activating factor.[23] Studies have also shown that vitamin D-binding protein has antiproliferative effects and can activate apoptotic pathways, and has the characterization of antiendothelial activity and inhibition of angiogenesis.[24,25] High levels of vitamin D-binding protein have been demonstrated in proteomics of myocardial infarction.[26] These studies indicated that serum vitamin D-binding protein plays a role in enhancing inflammatory cytokine and suppression of angiogenesis and is potentially antiproliferative. In this study, vitamin D-binding protein was significantly decreased at day 3 and day 10 after extracorporeal shock wave therapy, demonstrating that enhanced wound healing by shock wave therapy might be correlated with the suppression of serum vitamin D-binding protein and an increased anti-inflammatory effect and angiogenesis.

In addition to haptoglobin and vitamin D-binding protein, differential expression of several proteins was also identified in serum of shock wave–treated rats that may also play a role in wound healing by extracorporeal shock wave therapy. Reduced vascularity during wound maturation was shown to be mediated by endothelial apoptosis, and albumin has an antiapoptotic activity for endothelium.[27] Lower albumin expression and higher total antioxidant level expression in the chronic wound fluid content were correlated with delayed wound healing.[25,28] These results indicated that modulation of albumin expression could increase wound healing. Immunoglobulin light chain lambda and immunoglobulin kappa have been shown to increase the percentage of viable polymorphonuclear leucocytes by inhibiting apoptosis.[29] Patients with inflammatory diseases have also been shown to have

increased levels of immunoglobulin light chain.[30,31] We observed upregulated immunoglobulin light chain lambda and downregulated immunoglobulin light chain kappa in the serum of extracorporeal shock wave–treated rats, which suggests that immunoglobulin light chains, at least in part, are involved in extracorporeal shock wave–enhanced diabetic wound healing.

In the serum of extracorporeal shock wave–treated rats, we detected downregulated T-kininogen 1, serotransferrin, serine protease inhibitor A3N, apolipoprotein A-IV precursor, and immunoglobulin kappa chain C region. Kininogen is the protein precursor of kinin, which is generated by the action of kallikreins (serine proteases) on the kininogens.[32] Kinins are responsible for many effects in leukocytes, such as the release of inflammatory mediators and reactive oxygen species.[33] The kinins exert their biological effects through the activation of G protein–coupled receptors with seven transmembrane domains, designated B1 and B2 receptors.[34] Recently, it was postulated that neutrophils may control vascular permeability by generating kinin.[35] Therefore, the downregulated T-kininogen in extracorporeal shock wave–treated rats may indicate that extracorporeal shock wave therapy could modulate T-kininogen expression through suppression of reactive oxygen species and inflammatory effect and thus enhance wound healing. Serotransferrin plays an important role in the host immune response, and an increased concentration was found to be associated with inflammation and oxidative stress.[36,37] Thus, the suppressed serotransferrin level in the extracorporeal shock wave therapy group may imply that shock wave–enhanced diabetic wound healing is correlated with serotransferrin expression through an anti-inflammatory effect. Serine protease inhibitor A3 is an inhibitor of several proteases, such as elastase, cathepsin G, and chymase derived from mast cells and neutrophils.[38] Apolipoprotein A-IV is a component of lipoprotein particles similar to apolipoprotein E.[39] Concentrations of inflammatory protein have been shown to be associated with apolipoprotein A-IV, which suggests a link between inflammation and apolipoprotein A-IV synthesis or catabolism.[40,41] The downregulation of apolipoprotein A-IV precursor and serine protease inhibitor A3N found in serum of extracorporeal shock wave–treated rats should support the fact that they both are important factors in enhancing wound healing after extracorporeal shock wave therapy through suppression of the inflammatory response.

This proteomic study uncovered that the differential plasma protein expressions that enhance wound healing in extracorporeal shock wave therapy–treated diabetic rats are involved in angiogenesis and anti-inflammatory effects. Our results provide the basis for further elucidation of the biomechanism of these proteins and the related signaling pathway of wound healing enhancement by extracorporeal shock wave therapy. We are also interested in investigating whether the local wounding tissues have the consistent protein expression observed in the serum. We believe the elucidation of the biomechanisms of haptoglobin and vitamin D-binding protein will be helpful for extracorporeal shock wave therapy application in wound healing.

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ACKNOWLEDGMENT

This study was supported in part by grants from Chang Gung Memorial Hospital (CMRPG8A1081).

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