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Extracorporeal shock wave treatment protects skin flaps against ischemia–reperfusion injury

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ABSTRACT

Advances in the treatment of ischemia–reperfusion injury have created an opportunity for plastic surgeons to apply these treatments to flaps and implanted tissues. Using an extended inferior epigastric artery skin flap as a flap ischemia–reperfusion injury (IRI) model, we examined the capability of extracorporeal shock wave treatment (ESWT) to protect tissue against IRI in a rat flap model. Twenty-four rats were used and randomly divided into three groups ($n = 8$ for each group). Group I was the sham group and did not undergo ischemic insult; rather, the flap was raised and immediately sutured back (non-ischemic control group). Group II (ischemia control) and Group III (ESWT) underwent 3 h of ischemic insult. During reperfusion Group III was treated with ESWT and Group II was left untreated. Histological evaluation was made to investigate treatment induced tissue alterations. Survival areas were assessed at 5 d postoperatively. Skin flap survival and perfusion improved significantly in the ischemic animals following ESWT ($p < 0.001$, respectively). The tissue protecting effect of ESWT resulted in flap survival areas and perfusion data equal to non-ischemic, sham operated flaps. In line with the observation of better flap perfusion, tissue from ESWT-treated animals (Group III) revealed a significantly increased frequency of CD31-positive vessels compared to both the ischemic (Group II; $p = 0.003$) and the non-ischemic, sham operated control (Group I; $p < 0.005$) and an enhanced expression of pro-angiogenic genes. This was accompanied by a mild suppression of pro-inflammatory genes. Our study suggests that ESWT improves flap survival in IRI by promoting angiogenesis and inhibiting tissue inflammation. The study identifies ESWT as a low-cost and easy to use technique for surgical techniques that aim at reducing ischemia–reperfusion-induced tissue injury

Introduction

Microsurgical procedures, such as free flaps or replantation surgery, involve a mandatory period of ischemia during surgery. An extended ischemic period harbours the risk of unfavourable results as restoration of blood flow after a certain period of no-flow ischemia results in tissue damage referred to as ischemia–reperfusion injury (IRI). To minimize IRI unspecific local or systemic physical stressors, pharmacological agents and/or growth factors were tested in the past.[1–4] However, these approaches had two major flaws. The presumably beneficial substances had to be administered invasively in a time consuming and/or expensive procedure and the results achieved with these protocols in experimental studies were not necessarily reproducible in clinical practice. To address these issues, efforts have been made to further refine and simplify alternative non-invasive methods to increase tissue viability. Shock waves are high-energy acoustic waves generated by high voltage explosion and vaporization under water.[5] Since its successful introduction in 1980 for fragmentation of kidneys stone, ESW has been adapted for many other clinical indications such as musculoskeletal disorders (non-union of long bone fractures, calcifying tendonitis). In experimental and clinical studies extracorporeal shock-waves (ESW) induced bone and wound healing, cell differentiation and neo-vascularization.[5–8] Recent data suggest that skin flaps might profit from extracorporeal shock-wave treatment (ESWT),[6,7,9,10] a simple, time-saving and inexpensive procedure that is easy to implement in day-to-day

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practice. Improved tissue perfusion and a significant rise of growth factors i.e. vascular endothelial growth factor (VEGF) as well as endothelial nitric oxide synthase and proliferating cell nuclear antigen are thought to explain the enhanced skin flap survival following ESWT.[6,7,9,10] However, all these studies implemented standardised pedicled flaps which by definition do not fulfil all requirements of a flap model for ischemia–reperfusion injury (IRI).[6,7,9–13] Pedicled flaps remain connected to their original blood supply by their pedicle, do not undergo global ischemia and reproducibly show signs of ischemia in the distal part of the flap only as a consequence of their extremely large size. In truly free flap- or replantation surgery the tissue undergoes an obligatory time of global ischemia until vascular anastomoses are finished and the period of flap-reperfusion starts. Simulated free flaps in combination with total ischemia for an extended period of time, as performed in this study are better suited as an IRI-model. To the best of our knowledge no attempt has been made to date to define whether ESWT can prevent or attenuate IRI in free or simulated free skin flaps. The purpose of this study was to determine the influence of ESWT on ischemia and ischemia–reperfusion injury as a reasonable next step in the evaluation of ESWT as an adjunct to improve flap survival.

Materials and methods

Animals

Twenty-four male Lewis rats, each weighing between 250 and 300 g, were housed under pathogen-free conditions and had *ad libitum* access to filtered water and standard rat chow. All animals were maintained in accordance to the guidelines of the German Animal Welfare Act. The experimental protocol was approved by a review committee of the state of Baden-Wurttemberg, Germany. Animals were randomly divided into three groups of 8 animals each (Table 1).

Epigastric skin flap model

In all experiments anaesthesia was induced and maintained by intraperitoneal injection of 100 mg/kg ketamine hydrochloride (Ketamine 100 mg/mL Gräub, aniMedica GmbH, Senden-Bösensell, Germany) and 5 mg/kg xylazine (Xylazin 2%, Riemser Arzneimittel, Greifswald, Germany). The operative procedure was identical for all groups and was performed by one surgeon (SH) on all animals. All operative procedures were performed under aseptic conditions. With the animals in a state of deep anaesthesia a modified extended epigastric island flap was raised (6 cm × 10 cm) as previously described.[14–17] The flap remained connected solely to the left superficial epigastric blood vessels including the underlying panniculus carnosus.

Study design

To differentiate tissue alterations induced by ischemia from those induced by the surgical procedure itself one group of animals underwent sham operations (Group I, non-ischemic control). The flap was raised and immediately sutured back without a period of ischemia as previously described.[14,15] In Group II (ischemia control) and Group III (ESWT-treatment group) flap ischemia was maintained for 3 h using a Yasargil clip (65-g compression) on the pedicle (left superficial epigastric vessels) after flap harvest. Following ischemia the flap was sutured back to its native configuration and placed on to a silicon sheet to prevent neovascularisation from the wound bed. Group II was left untreated. In Group III ESWT was applied immediately after wound closure. Ultrasound transmission gel was used as a contact medium between the ESWT apparatus and the skin. Shock wave was applied in a dose of 500 impulses at 0.15 mJ/mm^2 (dermaPACE®, Sanuwave Inc., Alpharetta, GA, USA) whilst the applicator was randomly moved to cover full size of the flap. Procedure and dosage of ESWT was based on previous studies.[7,9,12,13,18]

Table 1
Overview of study design and experimental animal groups.

Group ID	*n*	Description
I	8	Control group, no adjuvant therapy
II	8	Control group, induced ischemia for 3 h, no adjuvant therapy
III	8	ESWT, immediately postoperative after induced ischemia for 3 h

Evaluation

All animals were observed daily and five days after flap harvesting, full-thickness necrosis was fully developed in each rat. Animals were re-anaesthetised and follow-up evaluations were performed. All evaluations were performed independently by one investigator who was blinded to the different groups.

Estimation of survival area in flaps

Standardised digital pictures of the flaps were taken and transferred to the computer. The mean area of flap necrosis, defined by surgical borders (expressed as a percentage of the total flap area) was calculated for each animal using ImageJ-Software (NIH, USA).

Indocyanine green laser-fluoroscopy (ICG)

Indocyanine green-fluorescence was induced and recorded using a laser-fluorescence imaging device (IC-View®, Pulsion Medical Systems AG, Munich, Germany), comprising a near-infrared (NIR) laser light source (0.16 W, wavelength λ 780 nm) and a NIR-filtered digital camcorder as previously described.[12,13] The perfusion index (PI) was recorded in relation to surrounding skin with normal blood flow and blood supply as an increase of grey value over time.

Microvessel density

Skin specimens were divided into proximal and distal halves respective to the flap design. One third of the surviving proximal half was shock frozen in liquid nitrogen and stored at −80 °C afterwards. Quantification of flap microvessel density was assessed using a monoclonal anti-CD31 antibody as previously described.[19] To exclude false positive counting of CD31-co-stained leukocytes or platelets, only plain cross-sections of vessels were counted, which allowed visual identification of endothelial cells and vessel morphology. To exclude any potential observer-dependent bias in relation to tissue area selection the complete two-step procedure was done twice and the investigator was blinded to the groups of the animals.

Semi-quantitative real time-PCR (sqRT-PCR)

We analysed the differential expression of selected genes of interest. Skin biopsies were taken from the surviving flap area, snap frozen in liquid nitrogen and stored at −80 °C. Skin flap tissue from the sham operated Group I was used as the normal, control for subsequent experiments. After homogenisation of tissue samples with Precellys-Keramik-kit 1.4 mm (cat. no. 91-PCS-CKM14,

Table 2
List of selected housekeeping and candidate genes.

Gene name	Gene symbol	Primer assay cat. no.
Beta-2 microglobulin, *housekeeping gene*	B2m	QT00176295
Tumour necrosis factor (TNF superfamily, member 2)	TNF	QT00178717
Vascular endothelial growth factor A	VEGF-A	QT00198954
Vascular cell adhesion molecule 1	VCAM-1	QT00178500
Prostaglandin E receptor 2 (subtype EP2)	PTGER2	QT00183526
Macrophage inflammatory protein	CCL4	QT00187075

Peqlab, Erlangen, Germany) total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA). Before starting reverse transcription of 1 μg RNA, genomic DNA was eliminated using QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA). Real time PCR was performed with the QuantiFast SYBR Green PCR Kit (Qiagen) using the 7300 Real-Time PCR System (Applied Biosystems, Carlsbad, CA) according to the manufacturer's protocol. The reaction mixture (25 μl) was made of 2.5 μl of cDNA (12.5 ng/reaction), 12.5 μl of 2× QuantiFast SYBR Green PCR Master Mix, 2.5 μl of certain QuantiTect Primer and 7.5 μl RNase free water (Qiagen). Details of the sqRt-PCR program were as follows: 37 cycles, PCR initial activation step (95 °C, 5 min), denaturation (95 °C, 10 s), combined annealing and extension (60 °C, 30 s). Each run was followed by melting curve analyses to affirm the specificity of the reaction products. Finally gene expression results were normalised to beta-2-microglobulin (housekeeping gene).[20] Table 2 provides the list of genes of interest including the housekeeping gene accompanied by their source catalogue numbers.

Statistical analysis

The statistical analysis was performed by Statistical Package for Social Sciences (SPSS) 16.0 software (SPSS Inc., Chicago, IL) and conducted by the Department of Biostatistics at the University of Heidelberg. Experimental results were expressed as mean ± SD percent survival. Independent-samples *t*-test was used to identify any significance amongst the three groups and in order to determine *p*-values. Significances of differences were Bonferroni-adjusted for three comparisons. A value of $p < 0.017$ was considered statistically significant. Results were represented as bar charts with the height of the columns showing the mean value of the determined results.

7300 System SDS Software (Applied Biosystems, Carlsbad, CA) was used to analyse quantitative data. To determine relative changes in the expression of selected genes (Table 2), results were normalised to beta-2-microglobulin acting as housekeeping gene. Expression levels of reference genes could be calculated using following arithmetic formula:

$$\Delta\Delta CT = \Delta CT(\text{examined groups}) - \Delta CT(\text{control group})$$

$$\text{Ratio} = 2E - \Delta\Delta CT$$

Differences in expression of tested genes compared to the control group (Group I) were considered to be statistically significant when $p < 0.05$ using independent-samples *t*-test and differentially expressed for a fold change greater than 1.5 or less than 0.5.

Results

Skin flap survival (planimetric measurement)

Data on the mean skin flap survival area in the respective experimental groups are provided in percent of the total skin flap area ± standard deviation. The sham operated animals (Group I) harboured the largest average skin flap survival area (80.9 ± 4.2%) followed by the animals that received ESWT following ischemia (Group III, 70.9 ± 11.3%). The smallest average skin flap survival area was observed in the ischemic animals without treatment (Group II, 33.3 ± 10.7%).

In comparison, sham operation without ischemia (Group I) resulted in a significantly larger average skin flap survival area than operation plus ischemia only (Group II, $p < 0.001$). ESWT treatment after ischemia (Group III) significantly enlarged the mean flap survival area compared to ischemia without treatment (Group II, $p < 0.001$).

Of note, the mean skin flap survival area after ischemia plus ESWT treatment (Group III) almost equalled that observed after sham operation without ischemia (Group I). Even though the surviving skin flap was still slightly larger in the sham operated animals (Group I), the difference in size did not reach statistical significance (Fig. 1a).

Indocyanine green laser-fluoroscopy

Perfusion data are provided as mean perfusion index (PI) of the whole flap area in percent ± standard deviation in relation to surrounding skin with normal blood flow and blood supply. A PI of less than 25% of the reference skin is considered as a sign of developing flap necrosis.[21] A PI of 25% and more predicts skin flap survival. The higher the PI value is the better for reliable tissue survival. The mean PI for the sham-operated animals (Group I) was 80.0 ± 4.4%. The animals with ischemia only (Group II) revealed a mean PI of 34.2 ± 7.7%, and the animals with ischemia plus ESWT had a mean PI of 80.8 ± 8.7%. Both, the sham operated flaps not subjected to ischemia (Group I) as well as the flaps subjected to ischemia plus ESWT treatment (Group III) revealed significantly higher mean PI values when compared to the ischemia control group (Group II, $p < 0.001$). Of note, even though subjected to a period of ischemia the mean PI values of the ESWT treatment group (Group III) were almost identical to the mean PI values of the sham operated group without ischemia, predicting a similar skin flap survival rate in these two groups. These data substantiate the planimetric measurements of the mean skin flap survival area provided above (Fig. 1b).

Microvessel density

The endothelial marker anti-CD31 detected significantly more newly formed blood-vessels per high-power field (HPF; the visual field in ×400 magnification) in the ischemic flaps that received ESWT (Group III) compared to both the ischemic flaps without treatment (Group II) (36.3 ± 11.0 vs. 19.0 ± 6.0; $p = 0.003$) and the sham operated flaps without ischemia (Group I) (20.5 ± 7.2; $p = 0.005$). Of note, there was no significant difference in microvessel density between the ischemic flaps without treatment (Group II) and flaps without ischemia (Group I) (19.0 ± 6.0 vs. 20.5 ± 7.2; $p = 0.657$) (Figs. 1c and 2).

Gene expression (sqRT-PCR)

Fold increase in gene expression was expressed as mean ± standard deviation of the mean. Gene expression of all individual genes was compared between all three experimental groups, respectively. Differences in gene expression were considered to be statistically significant when $p < 0.05$. Transcript levels were expressed relative to levels in untreated specimens from the sham operated group (Group I, baseline = 1). Ischemic stress resulted in an overexpression of the pro-angiogenic factors vascular endothelial growth factor A (*VEGF-A*), vascular cell adhesion molecule (*VCAM-1*) and prostaglandin E receptor 2 (*PTGER2*) in both experimental groups that underwent three hours of ischemia (Group II and Group III)

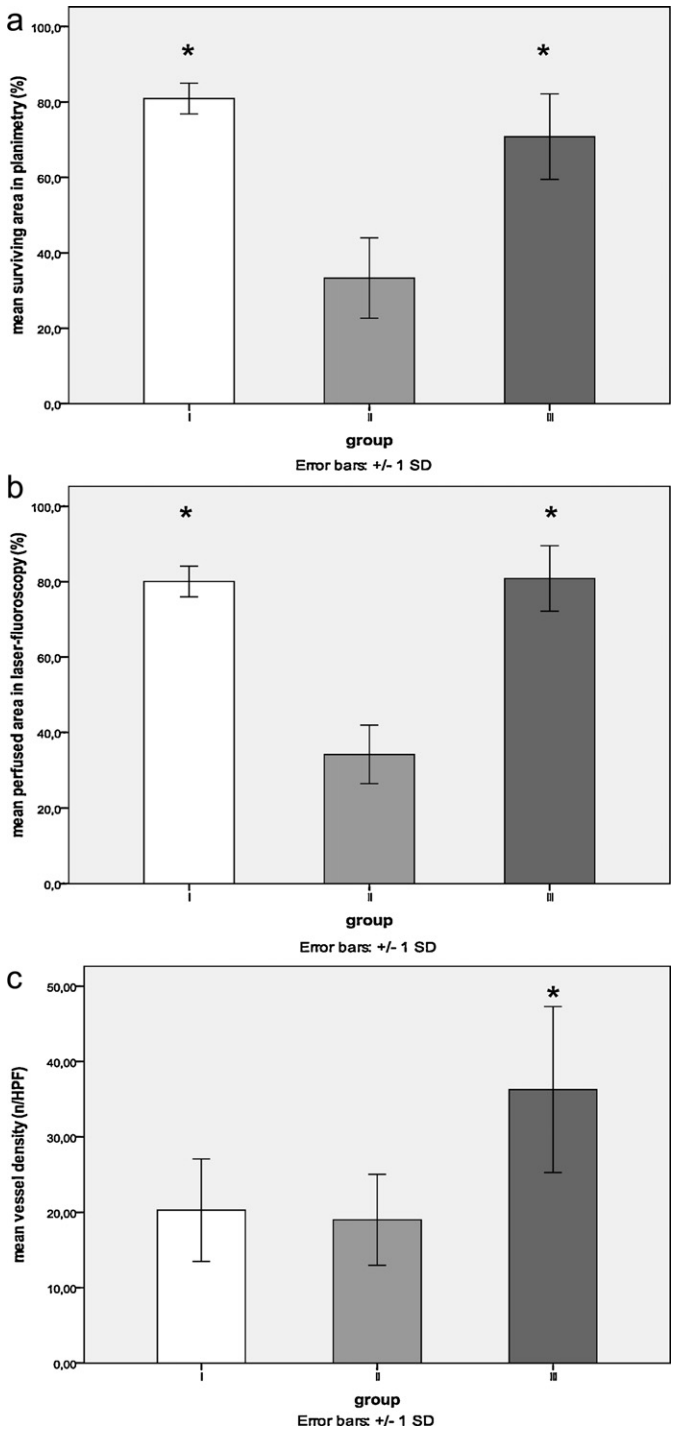

Fig. 1. (a) Bar charts show mean surviving skin flap area in percent five days postoperatively. Both, sham surgery without ischemia (I) and ESWT immediately after ischemia (III) significantly enhance flap survival compared to the control group with ischemia (II) (*$p < 0.001$). (b) ESWT improves flap perfusion. Sham surgery (I) and ESWT (III) showed a significantly higher survival rate when compared to the control group with ischemia (II) (*$p < 0.001$). (c) Microvessel density was assessed by enumerating the number of CD31-positive vessels in all experimental groups on day 5 postoperatively per HPF (magnification ×400). Graphs highlight statistically significant increase in vessel density for ESWT group compared to controls with/without ischemia (*$p = 0.003$ versus control group with ischemia (II); *$p = 0.005$ versus sham surgery group without ischemia (I)). *Y*-axis represents the mean number (n) of plain cross-sections of CD31-marked microvessels/HPF within the individual experimental groups.

relative to levels of the sham operated group (Group I). Between the ischemic flaps the increase in gene expression was significantly higher for all three genes in the ischemic flap plus ESWT with $p = 0.031$ for *VEGF-A*, $p < 0.001$ for *VCAM-1* and $p = 0.014$ for *PTGER2*, respectively. Of note, *PTGER2* and *VCAM-1* revealed the highest normalised expression levels of all investigated genes in both experimental groups with ischemia (Group II and Group III).

Compared to ischemia alone (Group II), ESWT treatment after ischemia (Group III) was accompanied by a statistically significant downregulation of the chemokine **CC**-Motif **L**igand **4** (*CCL4*), also known as **s**mall **i**nducible **c**ytokine **A4** (*SCYA4*) ($p < 0.001$). Interestingly enough, together with *CCL4* the multifunctional proinflammatory cytokine tumour necrosis factor alpha (*TNF-α*) was also found to be significantly down-regulated in the ischemic flaps that received ESWT (Group III, $p < 0.001$).

Fig. 3 illustrates the fold changes in gene expression of all individual genes. Fold changes in gene expression are compared between the sham-operated animals without ischemia (Group I) and both the animals with ischemia only (Group II) and the animals with ischemia plus ESWT (Group III), respectively.

Discussion

The present study demonstrates that ESWT protects skin flaps from ischemia–reperfusion injury (IRI). The data illustrate that ESWT is capable to significantly enhance skin flap survival in the aftermath of ischemia to an extent that almost equals surgical results without ischemia. Morphologically, this effect is accompanied by a significant increase in microvessels in flap tissue that received ESWT. Biologically, this effect seems associated with an up-regulation of vasoactive and a simultaneous down-regulation of proinflammatory genes and proteins.

The mechanisms underlying ischemia–reperfusion injury (IRI) are complex. The literature attributes potentially important roles in IRI to the local sequestration and activation of leukocytes and to the secretion of inflammatory and pro-angiogenic cytokines/chemokines.[3] Recently, experimental data have shown that ESWT promotes tissue revascularisation and wound healing by its pro-angiogenic and anti-inflammatory effects.[6,10,22–27] Hence, ESWT seems to have significant effects on two of the major factors influencing IRI, inflammation and angiogenesis.

TNF-α is a multifunctional proinflammatory cytokine, which is known to induce amongst other genes *CCL4*, a molecule belonging to the family of CC-chemokines that are important in recruiting and targeting monocytes to inflamed tissue.[28,29]

Following ESWT we detected a significant, concomitant down-regulation of both *TNF-α* and *CCL4*, which is nicely explained by the interdependence of their gene expression. Our data are substantiated by findings of others. Suppression of *TNF-α* was demonstrated in critically perfused random skin flaps on day 1 and 7 after ESWT.[24] Interestingly enough, significant over-expression of *TNF-α* is associated with the induction and maintenance of IRI.[30–32] In line with these results, our data indicate that subdued or reduced *TNF-α*-expression in ischemic tissue is required to protect the tissue from *TNF-α*-induced IRI and that ESWT succeeds in *TNF-α*-down-regulation. Whilst down-regulation of *TNF-α* and *CCL4* support the supposed anti-inflammatory effect associated with ESWT, up-regulation of *Ptger2*, *VCAM-1* and *VEGF-α*, which was detected after ESWT in our series may seem at first sight contradictory as these genes can have proinflammatory function. However, these genes concomitantly stimulate angiogenesis, which constitutes the second major factor influencing IRI.[3] In fact, a shift in the balance of pro-angiogenic chemokines/cytokines and proinflammatory mediators has been suggested in a murine skin isograft model.[26] This shift was significant for an enhanced and prevailing early angiogenic response to ESWT.[26] From these

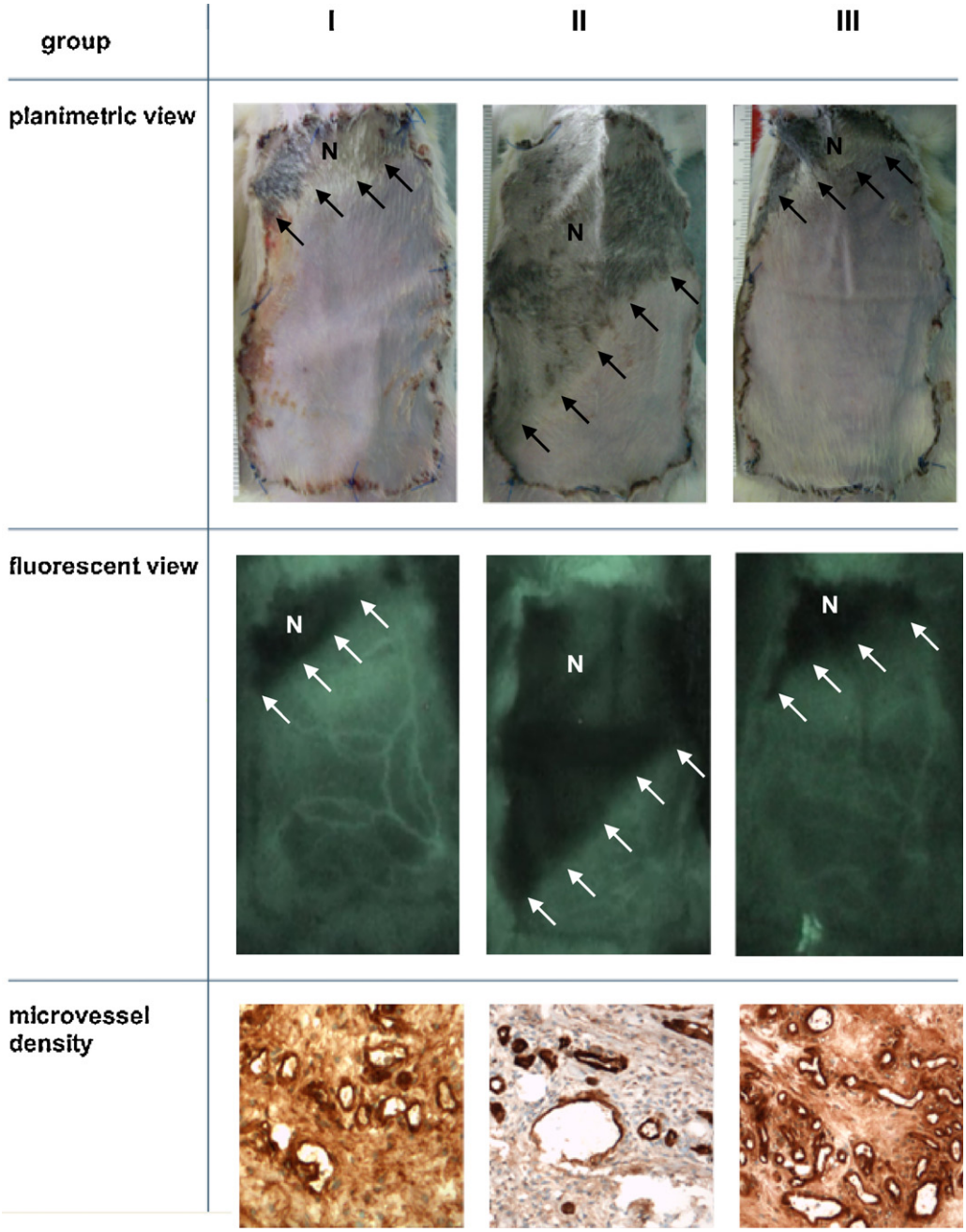

Fig. 2. Representative samples of all three groups at day 5 postoperatively. Planimetric view in upper row shows survival of the flap tissue. Fluorescent view indicates skin flap perfusion in relation to the surrounding skin with unchanged blood flow. Microvessel density demonstrated by histological sections with CD31-immunostaining. *N* = necrosis, unperfused area; arrows mark the individual boarders between vital/perfused and necrotic/unperfused skin flap area (*N*).

and other data it has become evident that in wound healing of ischemic tissue, inflammation and angiogenesis interact closely and that the temporal dominance of one of these processes over the other might have significant impact on the healing process itself.[26,33,34] In line with these observations we demonstrate that improved tissue protection following ESWT went along with a significant up-regulation of genes associated with pro-angiogenic function, i.e. *VEGF-α*, *PTGER2* and *VCAM-1*. Our data confirm recent publications that report an increased expression of *VEGF-α*, a multifunctional cytokine that is able to stimulate neovascularisation and angiogenesis during an enhanced skin flap survival after ESWT.[9–11,19,23] Increased *VEGF-α* tissue expression also leads to vasodilatation, which may help better perfuse the damaged tissue and increased vascular permeability, which may provide a provisional scaffold for migrating endothelial cells. The vascular adhesion molecule-1, *VCAM-1*, facilitates *VEGF-α* mediated vascular permeability.[35] This might explain why a concomitant up-regulation of both *VEGF-α* and *VCAM-1* by ESWT as demonstrated in our study is so beneficial for tissue protection from IRI. Interestingly enough, improvement in tissue perfusion by ESWT has been associated with a *VEGF-α*-mediated vasodilatation of pre-existing vessels during an early stage and with neovascularisation at late stages.[10] In support of these data, we observed a significant increase in vessel density in those flaps that received ESWT immediately after prolonged ischemia. It has been shown that the expression of the EP2 subtype of the Prostaglandin E Receptor 2 (PTGER-2) is a critical element for a hypoxia inducible factor (HIF)-1 alpha independent induction of the vascular endothelial growth

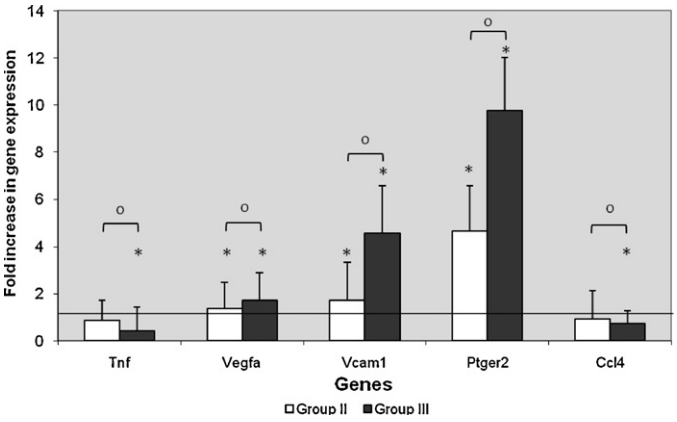

Fig. 3. Real-time polymerase chain reaction analysis of selected proangiogenic and proinflammatory genes. Differences in expression of tested genes compared to the control group and between both experimental groups considered to be statistically significant when $p < 0.05$. Fold increase in gene expression is depicted here relative to the expression values in untreated specimens (group I, baseline = 1). *significant p-values between group I vs. II or group I vs. III. ○significant p-values between group II vs. III.

factor (VEGF).[36] Thus, *PTGER-2* overexpression may have mediated in part the enhanced *VEGF-α*-expression in ischemic flaps that received ESWT and thereby contributed to tissue protection from IRI. Based on the literature and our own results we hypothesise that during an early stage following ischemia ESWT protects the flap tissue from the effects of IRI by an increased blood supply which is mediated by an ESWT-induced enhanced expression of proangioactive factors (i.e. *VEGF-A*, *PTGER2* and *VCAM-1*) and the suppression of proinflammatory factors (i.e. *TNF-α* and *CCL4*).

This additional blood supply provides the minimum requirement of oxygen and nutrition for the ischemic tissue to survive until new vessels are formed in a second, later stage of wound healing. As we detected a higher vessel density in the ischemic flaps treated with ESWT, our data suggest, that this later stage of wound healing already starts before day 5 after ischemia. Of note, ESWT of ischemic flaps yielded survival areas and perfusion indices almost equal to sham-operated flaps without periods of ischemia. These results underscore the power of ESWT to protect ischemic skin flaps from IRI. Our data encourage future studies targeting earlier and later observational time points in an effort to better understand the dynamics of ESWT-mediated molecular events in ischemic tissue. This insight might help identify potential molecular targets to further improve the ESWT-mediated benefit.

Conclusion

In conclusion, this study demonstrates for the first time that ESWT protects ischemic soft tissue from IRI. This tissue protecting effect makes this easy, quick, cost-effective and non-invasive application of ESWT an attractive tool in all surgical procedures that aim at reducing IRI. Based on our findings, we conclude that ESWT may have benefits for therapeutic flap or replantation surgery in clinical practice. ESWT could be easily administered on the area of interest, after the surgical procedure without any risk for the treated tissue and no necessity of wound management. Moreover it may be of great benefit in more complex clinical situations with a patient population whose vascular conditions are frequently insufficient due to diabetes or arterial occlusive disease. In acute clinical situations the postoperative ESWT may be used when intraoperative microvascular perfusion failure occurs with a high risk of loss of nutritional oxygen supply and consecutive damage to the free flap or replanted tissue.

Conflict of interest

All authors disclose any financial and personal relationships with other people, or organisations, that could inappropriately influence (bias) their work, all within 3 years of the beginning the work submitted.

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