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Shockwave Treatment Enhances Proliferation and Improves WoundHealing via Purinergic Signaling-Induced Erk1/2 Pathway

107- Translationale Gesundheitsforschung – Brücken bauen von Grundlagenwissenschaft zu angewandter Forschung

Abstract

In clinical settings, shockwave treatment (SWT) has proven to accelerate poor woundhealing of burn wounds or diabetic ulcers although the underlying principles of this beneficial effect remain to be fully elucidated. Therefore the aim of this study was to identify the underlying signaling pathways involved in the proliferative and wound-healing effect of SWT.
Primary human adipose tissue-derived stem cells, mouse mesenchymal stem cells, and a human Jurkat T cell line were subjected to SWT *in vitro* and ATP release was measured. Proliferation after SWT was determined and immunoblotting was performed to evaluate mitogen activated protein kinase pathway activation. In addition, an *in vivo* rodent ischemic excision wound-healing model was used to assess the dependency of SWT-induced woundhealing on ERK1/2 signaling.
SWT dose-dependently released ATP in all three cell types and significantly increased the number of proliferating cells. Hydrolysis of released ATP using the scavenging molecule apyrase diminished the proliferative effect of SWT. Shockwaves significantly activated ERK1/2 signaling, which was prevented by the P2 receptor antagonist suramin as well as by ATP depletion. Our *in vivo* study confirmed that SWT induced wound healing in an ERK1/2 dependent manner.
We conclude that *in vitro* SWT releases cellular ATP, activating downstream ERK1/2 signaling via predominantly P2Y purinergic receptors, ultimately causing the proliferative effects of SWT. Our *in vivo* data endorse the ERK1/2 signaling pathway being essential in the SWT wound healing effect. Thus, this signaling cascade is one of the underlying principles of the beneficial effects of SWT and will aid to emphasize the application of SWT as a routine wound healing treatment.

Keywords:

Shockwave therapy; wound healing; proliferation; mechanotransduction; Erk1/2, purinergic signaling

1. Background

Shockwaves – which are acoustic waves – have been used as the golden standard therapy for the treatment of kidney stones in the field of urology already since the early 1980s. Soon after the first successful disintegration of kidney stones, various favorable effects of shockwaves on the treated patients were reported. Here, the first observation was the bone densification of the iliac crest of the patients, which occurred after shockwave treatment of kidney stones.

Over the years, the beneficial potential of shockwave therapy has also been used in fields apart from urology. Nowadays, shockwave treatment is recommended for patients suffering from achilles tendinopathy, radial epicondylopathy ("tennis elbow"), plantar fasciitis or delayed bone healing. Moreover, shockwave therapy is also used to treat impaired soft tissue wound healing indications such as diabetic foot ulcers and non-healing, chronic wounds (d'Agostino, Craig, Tibalt, & Respizzi, 2015; Mittermayr et al., 2012).

This promising, non-invasive, cost-efficient and low side-effect therapy has been shown to be highly efficient in the clinic – e.g. with a healing rate of up to 85% in treated patients suffering from delayed-/non-union long bone fractures (Schaden, Fischer, & Sailler, 2001). Shockwave therapy also induced wound healing of acute as well as chronic soft tissue wounds, with success rates of up to 75% (Schaden et al., 2007).

Nevertheless, the general mechanistic principles underlying the beneficial effect of shockwave therapy are still not completely understood. The importance of mechano-transductory signaling pathways – cascades that are activated intracellularly to translate a mechanical stimuli applied on the outside of the cell into an intracellular signal – has already been reported by various groups so far, using *in vitro* and *in vivo* approaches. Pathways involving extracellular signal regulated kinase (Erk1/2) and p38 mitogen activated protein kinase (MAPK) have been shown to be essential in the shockwave induced effect on bone cells (Chen et al., 2004; Wang et al., 2004).

To establish the broader clinical application of shockwave therapy as a treatment tool for impaired soft tissue wound healing, it is essential to unravel the underlying mechanistic principles in detail. Only these data could lead to the application of shockwave treatment as a standardized therapy.

2. Aim of the study

The aim of this study was to decipher the underlying mechanisms that are involved in the beneficial effect of shockwave therapy on soft tissue wound healing. To elucidate crucial intracellular signaling cascades, a thorough *in vitro* study was conducted, in which the effect of shockwaves on different cell types was analyzed, to identify general mechanisms leading to the cell proliferative effect of shockwave treatment. Erk1/2 and p38 MAPK signaling pathways have already been described to play a role in SWT-induced bone regeneration (Chen et al., 2004). ATP release upon SWT was shown to be involved in increased osteogenic differentiation after SWT (Sun et al., 2013). Therefore, the influence of released ATP and MAPK signaling on the general proliferative effect of SWT was analyzed. Furthermore, an *in vivo* wound-healing study should ultimately prove the findings of the *in*

vitro part of the study. This should give information on the underlying mechanistic principles on the beneficial effects of shockwave treatment on cell proliferation and soft tissue wound healing.

3. Results

In all the three different cell types we subjected to *in vitro* shockwave treatment – mouse mesenchymal progenitor cells, human Jurkat T-cells and primary human adipose tissue-derived stem cells – similar effects were observed (Weihs et al., 2014):

- *In vitro* shockwave treatment dose-dependently increased cellular ATP release, Erk1/2 signaling pathway activation as well as cell proliferation
- Purinergic receptor activation (predominantly P2Y receptors) are essential for SWT enhanced Erk1/2 signaling pathway activation
- Cell proliferation
- Ultimately, the cell proliferative effect of *in vitro* shockwave treatment depends on signal transduction via active purinergic and Erk1/2 signaling

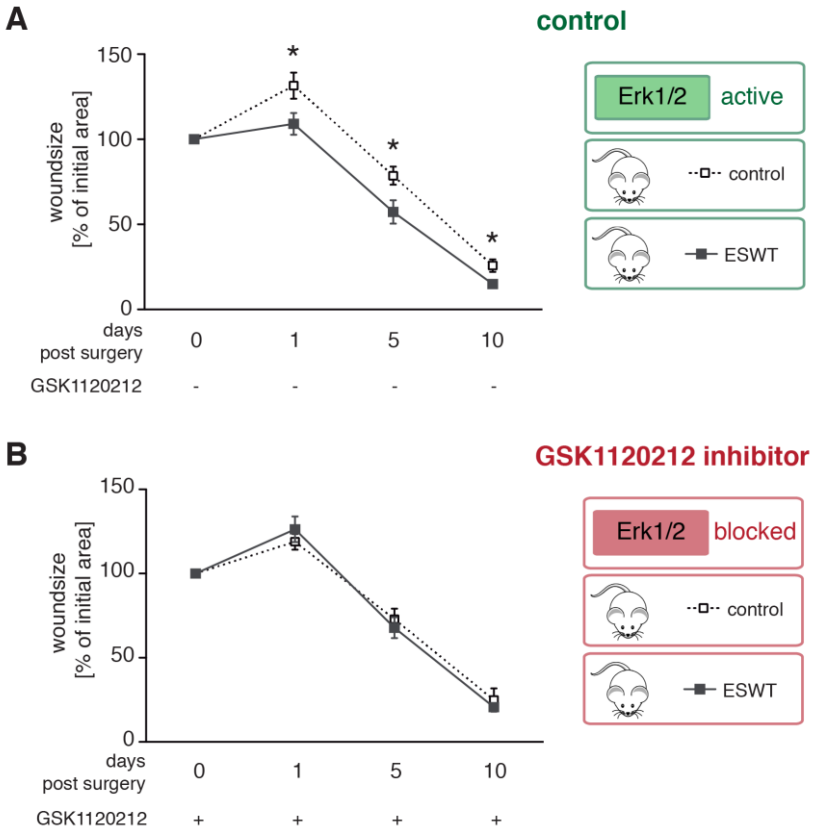

Figure 1: *In vivo* shockwave treatment enhanced wound healing in an epigastric skin flap model is dependent on active Erk1/2 signaling. Ischemic rendered wounds of Sprague-Dawley rats were shockwave treated using 0.13 mJ/mm^2 and 100 pulses. (A) Compared to untreated controls, wounds of animals with active Erk1/2 signaling show significantly smaller wounds at indicated timepoints after surgery when wounds were subjected to shockwave treatment. (B) Shockwave treatment did change the wound healing rate in animals where Erk1/2 signaling was blocked by GSK1120212 inhibitor (n=9; mean +/- S.E. (error bars); * p=0.05; two-tailed unpaired Student's t test or Mann-Whitney U test (GSK1120212 day 5) for pairwise comparison at different time points). Modified from (Weihs et al. 2014)

After the identification of the signaling cascade which is responsible for the shockwave treatment induced proliferative effect *in vitro* – ATP release, activation of purinergic receptors, activation of

Erk1/2 signaling – an *in vivo* wound-healing study was performed to identify the role of this cascade in the wound-healing effect of shockwave treatment.

For this purpose, a rodent ischemic excision wound-healing model was conducted (Mittermayr et al., 2011), where wound healing in animals was compared after shockwave therapy. To identify the role of Erk1/2 in SWT-enhanced wound healing, also animals which received the Mek1/2 inhibitor GSK1120212 (thereby blocking Erk1/2 activation) were included in the study. Ischemic wounds were generated, and immediately after surgery, shockwave treatment using 100 pulses at 0.19 mJ/mm^2 (dermagold100, MTS) was performed.

As depicted in Figure 1A, shockwave treatment in animals with intact Erk1/2 signaling resulted in significant smaller wound sizes in the shockwave group compared to untreated controls, analyzed 1, 5 and 10 days after surgery and shockwave treatment. In animals which received the inhibitor to block Erk1/2 activation, shockwave therapy did not affect wound healing compared to untreated controls (Figure 1B). Analysis of wound size as well as histological analysis confirmed the crucial role of Erk1/2 signaling in shockwave treatment induced wound healing *in vivo*.

4. Conclusion

The findings of this study demonstrate that *in vitro* as well as *in vivo* shockwave treatment affects intracellular signaling processes in treated cells and tissue. We could describe a relevant signaling cascade which is activated upon shockwave treatment, ultimately leading to increased cell proliferation *in vitro* and enhanced wound healing *in vivo* (Figure 2). Initially, shockwave treatment induced cellular ATP release, followed by activation of predominantly P2Y purinergic receptors. Subsequently, intracellular Erk1/2 pathways are activated, leading to an increase in cell proliferation *in vitro* as well as improved wound healing *in vivo*.

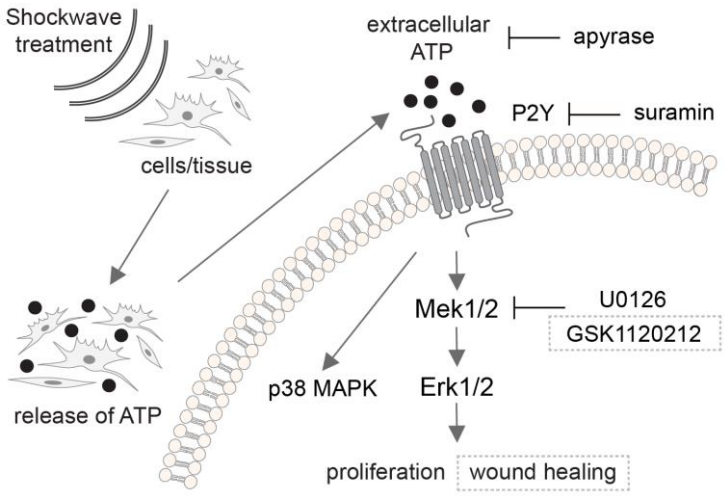

Figure 2: Schematic illustration of shockwave treatment triggered cellular mechanisms. Shockwave application on cells or tissue induces cellular release of ATP, which couples to purinergic P2 receptors, followed by the activation of intracellular MAPK signaling pathways. Ultimately, shockwave treatment enhances cell proliferation and improves wound healing. Modified from (Weihs et al. 2014)

This yields a model of action of shockwave treatment-induced signaling mechanisms which are involved in proliferation and wound healing at a molecular level. This data helps to decipher the working principles and underlying mechanisms of shockwave treatment, which could lead to an overall improvement of clinical treatment efficacy. Thereby this non-invasive, cost efficient, low risk bearing therapy which is performed in an outpatient setting, could drastically improve wound-healing therapy.

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References:

Chen, Y.-J., Kuo, Y.-R., Yang, K. D., Wang, C.-J., Sheen-Chen, S.-M., Huang, H.-C., et al. (2004). Activation of extracellular signal-regulated kinase (ERK) and p38 kinase in shock wave-promoted bone formation of segmental defect in rats. *Bone*, *34*(3), 466–477.

d'Agostino, M. C., Craig, K., Tibalt, E., & Respizzi, S. (2015). Shock wave as biological therapeutic tool: From mechanical stimulation to recovery and healing, through mechanotransduction. *International Journal of Surgery (London, England)*, *24*(Pt B), 147–153.

Mittermayr, R., Antonic, V., Hartinger, J., Kaufmann, H., Redl, H., Téot, L., et al. (2012). Extracorporeal shock wave therapy (ESWT) for wound healing: Technology, mechanisms, and clinical efficacy. *Wound Repair and Regeneration*, *20*, 456–465.

Mittermayr, R., Hartinger, J., Antonic, V., Meinl, A., Pfeifer, S., Stojadinovic, A., et al. (2011). Extracorporeal shock wave therapy (ESWT) minimizes ischemic tissue necrosis irrespective of application time and promotes tissue revascularization by stimulating angiogenesis. *Annals of Surgery*, *253*(5), 1024–1032.

Schaden, W., Fischer, A., & Sailler, A. (2001). Extracorporeal shock wave therapy of nonunion or delayed osseous union. *Clinical Orthopaedics and Related Research*, (387), 90–94.

Schaden, W., Thiele, R., Kölpl, C., Pusch, M., Nissan, A., Attinger, C. E., et al. (2007). Shock wave therapy for acute and chronic soft tissue wounds: a feasibility study. *The Journal of Surgical Research*, *143*(1), 1–12.

Sun, D., Junger, W. G., Yuan, C., Zhang, W., Bao, Y., Qin, D., et al. (2013). Shockwaves induce osteogenic differentiation of human mesenchymal stem cells through ATP release and activation of P2X7 receptors. *Stem Cells (Dayton, Ohio)*, *31*(6), 1170–1180.

Wang, F.-S., Yang, K. D., Wang, C.-J., Huang, H.-C., Chio, C.-C., Hsu, T.-Y., & Ou, C.-Y. (2004). Shockwave stimulates oxygen radical-mediated osteogenesis of the mesenchymal cells from human umbilical cord blood. *Journal of Bone and Mineral Research : the Official Journal of the American Society for Bone and Mineral Research*, *19*(6), 973–982.

Weihs, A. M., Fuchs, C., Teuschl, A. H., Hartinger, J., Slezak, P., Mittermayr, R., et al. (2014). Shock Wave Treatment Enhances Cell Proliferation and Improves Wound Healing by ATP Release-coupled Extracellular Signal-regulated Kinase (ERK) Activation. *The Journal of Biological Chemistry*, *289*(39), 27090–27104.