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ORIGINAL ARTICLE

Could radial extracorporeal shock wave therapy have an effect on wound healing in clinical practice by creating genotoxic damage? An *in vitro* study in mouse fibroblasts

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ABSTRACT

Objectives: This study aims to evaluate wound healing effects of *in vitro* radial extracorporeal shock wave (rESW) application on mouse fibroblasts and whether the cytotoxic effect of extracorporeal shock wave (ESW) was due to a possible genotoxic effect.

Patients and methods: After creating an *in vitro* wound healing model in L929 mouse fibroblast culture, fibroblasts were stimulated with a frequency of 3 Hz, and 100, 250, 500, 1,000 and 1,500 pulses shock waves were applied. Energy flux densities ranging from 0.01 to 0.23 mJ/mm^2 (14.3 MPa) at a constant pressure level of 0.5 and 1 bar were applied. Wound healing, cell viability, and genotoxicity were evaluated at 24 and 48 h.

Results: All shot numbers for both pressures significantly reduced cell viability ($p<0.05$). For both 0.5 and 1 bar pressures, in both intervals, the rate of wound healing decreased, regardless of the number of shots ($p<0.05$). *In vitro* genotoxic damage was detected at both 0.5 and 1 bar pressures, in both time intervals, regardless of the number of shots. The genotoxic damage increased from 24 to 48 h.

Conclusion: The study results suggest that, when ESWT is applied in this *in vitro* experimental setup, cell viability decreases and wound healing is delayed under all conditions. Furthermore, genotoxic damage can be prevented by using shots below 1,000 pulses. Therefore, while investigating the therapeutic effect of ESW therapy *in vitro*, the upper limit for the number of shots should be 1,000 pulses.

Keywords: Cell viability, extracorporeal shock wave therapy, genotoxicity, *in vitro*, wound healing.

Extracorporeal shock wave therapy (ESWT) is a non-invasive treatment method using sound waves to stimulate healing. The number of studies in the literature has increased, since the date of the use of shock wave therapy in the field of urology for lithotripsy and its effects have been better understood.[1] Currently, ESWT is actively used in orthopedics and traumatology clinics in the treatment of many different musculoskeletal pathologies. In the literature, the applications mainly focus on bone, tendon, and soft tissue. The first applications on bone tissue started with nonunion and delayed healing case series,[2-5] followed by applications on osteochondrosis dissecans,[6] osteonecrosis,[7] bone marrow edema.[8,9] Applications on tendon tissue are often performed on plantar fasciitis.[10,11] Achilles tendinopathy,[4,12] calcifying tendinitis[13] and lateral epicondylitis.[14,15] On the other hand, soft tissue applications focused on chronic wounds,[16] scar tissues[17] and hypertrophic wounds.[18]

The high-amplitude sound waves (shock waves) used in ESWT is the result of a transient pressure

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change resulting from a sudden increase in ambient pressure in three-dimensional space. These shock waves have a peak pressure of about 1,000 times higher in terms of their physical properties than ultrasound waves. It is known that the effect of shock waves on the tissue occurs in four phases. In the physical phase, extracellular cavitation, ionized molecules, and membrane permeability increase due to the direct effect of shock waves. In the physicochemical phase, interaction occurs with spreadable radicals and biomolecules. In the chemical phase, intracellular reactions and molecular changes occur and, finally, the biological phase occurs.[19]

Two types of focused (f) and radial (r) extracorporeal shock waves (ESW) are used in the clinic. In focused ESW (fESW), shock waves from a large shockwave source are directed to a single targeted point, whereas in radial ESW (rESW), shock waves from the source propagate as expanding waves in three-dimensional space and reach the targeted point and surrounding regions (Figure 1).[20]

Wound healing refers to a complex process in which inflammatory reactions, proliferation and remodeling occur. In the proliferative phase of wound healing, collagen synthesis increases with rapid fibroblast proliferation in response to chemotactic factors synthesized during the inflammatory phase. In addition, capillaries formed by angiogenesis contribute to the formation of granulation tissue. In the remodeling phase, collagen synthesis is maintained by fibroblasts and wound healing is completed.[21] Several studies have shown that ESWT increases the growth rate of fibroblast via messenger ribonucleic acid (mRNA) expression level and increases collagen type I and type III synthesis in fibroblasts in a dose-dependent manner. These increase the interest in ESW application in the treatment of wound healing problems.[16,17,22-24]

There are theories related to the cellular explanation of how shock waves increase wound healing; however, there is no consensus in the literature. In a limited number of studies, it has been shown that ESW application may have a cytotoxic effect and it has been suggested that this is caused by both the mechanical cavitation effect and triggering the pro-inflammatory response and catabolic process in cells. As a result of this process, tissue healing mechanisms have been proposed to be activated.[25]

In the present study, we hypothesized that ESW application had genotoxic effects on cells. We, therefore, aimed to evaluate wound healing effects of *in vitro* rESW application on mouse fibroblasts and whether the cytotoxic effect of ESW was due to a possible genotoxic effect. To the best of our knowledge, this is the first study in the literature to examine possible genotoxic effects of *in vitro* rESW application on mouse fibroblasts.

PATIENTS AND METHODS

This study was approved by the Başkent University, Faculty of Medicine University, Institutional Review Board (No: 94603339-604.01.02/1985).

Cell culture

The L929 mouse fibroblasts (CCL-1, ATCC, Rockville, USA) were cultured in RPMI 1640 medium (Biochrome AG, Berlin, Germany) supplemented with heat inactivated 10% fetal bovine serum (FBS; Biochrom AG, Berlin, Germany), and antibiotic mix (10,000 U/mL penicillin and 10,000 µg/mL streptomycin, Biological Industries, Israel) in a 95% humidified incubator (Heraeus Deutschland GmbH & Co. KG, Hanau, Germany). Cells were subcultured every 24 h and cells at the 5 to 10 passage level were used in the study.

rESW treatment

The shock-wave generator (Masterpuls® MP100; Storz Medical, Switzerland) used in orthopedics and traumatology clinic used for the *in vitro* experiments. Shock wave application was performed using a 15-mm diameter radial shock wave transmitter (Model No: R15, Storz Medical), which has a tissue penetration of ≥30 mm and has proven to have a therapeutic effect. The shock wave applications were performed as previously described with minor modifications (Figure 2).[16] Briefly, cells were seeded in a 9 cm^2 sterile Petri dishes. After cells were reached 95% confluency,

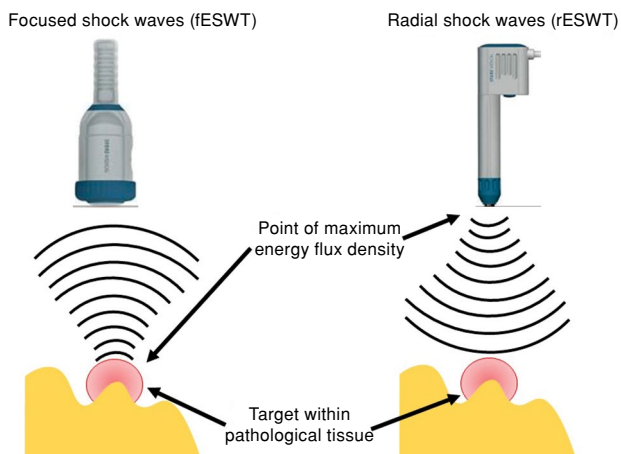

FIGURE 1. Mechanism of action of focused and radial extracorporeal shock wave technology.

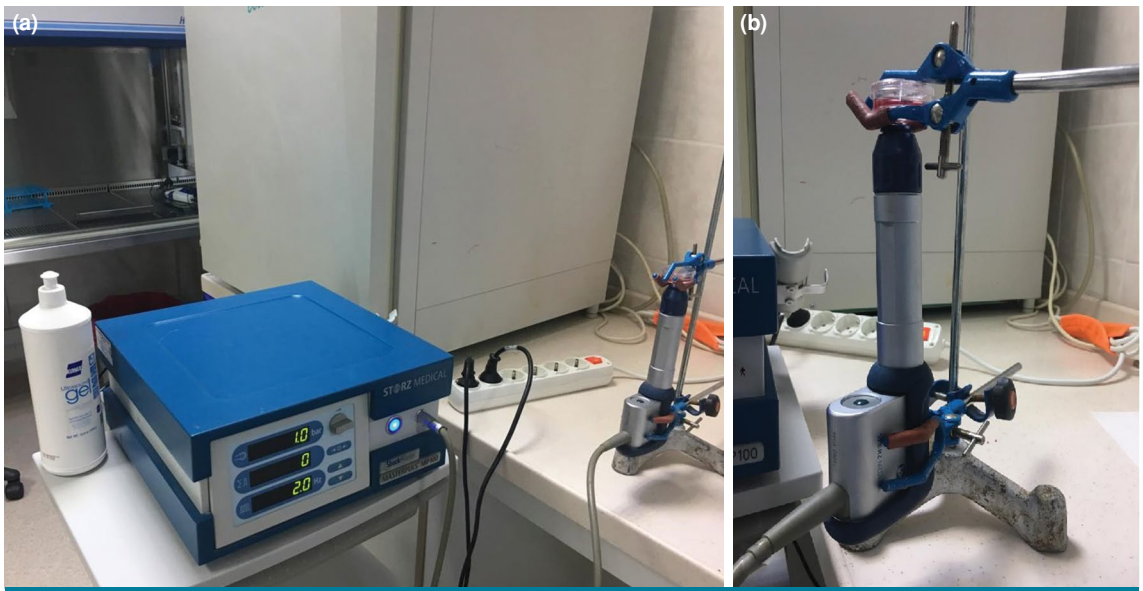

FIGURE 2. Experimental setup in which shock wave is applied to cell cultures. **(a)** Experimental setup. **(b)** Shock wave application to cell cultures.

they were stimulated with a frequency of 3 Hz and 100, 250, 500, 1,000, and 1,500 pulses of shock waves. Energy flux densities ranging from 0.01 to 0.23 mJ/mm^2 (14.3 MPa) at a constant pressure level of 0.5 and 1 bar were applied. This experimental protocol was determined by examining the number and frequency of shock wave pulses used in previous studies to make a detailed evaluation in cell culture.[2,16,26-28] Additionally, separate cell cultures are used for each pulse shock and pressure level. Energy transfer from the radial (r)ESWT transmitter to the Petri dish was maximized by coating the surface of the Petri dishes with coupling agent. The rESWT application was performed at 24-h intervals, until the *in vitro* wound sites were closed (for two days). The cell cultures to be used in the study were, then, plated and an *in vitro* wound model was created, and the rESWT was not applied to the cell cultures in the control group. Evaluation of *in vitro* wound healing in the control group was performed blindly by a researcher familiar with this method, but not involved in the study to avoid bias.

In vitro scratch assay (wound healing)

The L929 cells were seeded in a Petri dish, and scratch wounds were made using a sterile 0.1 to 10 µL pipette tip in fibroblast cell culture and, then, the closure time and percentage of the scratch wounds were evaluated as previously described.[29] Scratch wounds area was photographed using an inverted microscope (Olympus IX73, Japan) at 24-h intervals and analyzed by ImageJ version 1.53k14 (University of Wisconsin, Madison, WI, USA) software. For quantification, the area in the wound edges was measured at least of four areas for each application, and the mean values were calculated (d). The percentage of wound healing (WH) was calculated as follows:

$$WH\% = [(d_{\text{original wound}} - d_{\text{healing}}) / d_{\text{original wound}}] \times 100$$

Under *in vitro* conditions, scratch wounds that do not have any rESW treatment close within 48 h (Figure 3). Therefore, we determined the follow-up period as 48 h in our study.

Determination of cell viability

Cytotoxic effects of rESWT application were determined using 3-[4.5-thiazol-2-yl]-2.5-diphenylte trazolium bromide (Sigma-Aldrich Inc., St Louis, MO, USA). Briefly, following the application in specified time and conditions, 5 µL of 5 mg/mL MTT were added to total of 1×104 cells (final volume 0.59 mg/mL) and the cells were incubated at 37°C for 4 h. At the end of the incubation, 100 µL 10% of sodium dodecyl sulfate solution was added to cells and a final incubation step was carried out at 37°C for 16 h. Optical density of the chromogenic product was determined at 540 nm in a spectrophotometer (Epoch™; BioTek Instruments Inc., VT, USA) and viability of cells was calculated using spectrophotometer outcomes.

Assay for genotoxicity (Comet assay)

Genotoxic effects of rESWT application were determined by alkaline single-cell gel electrophoresis

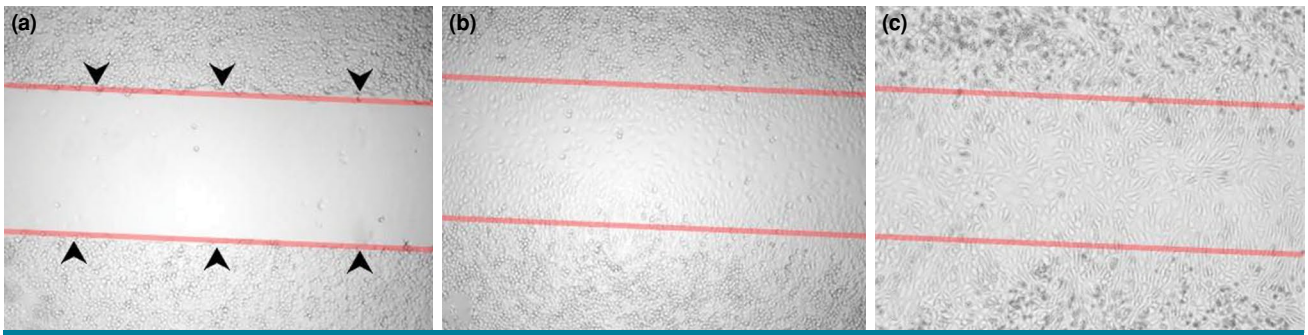

FIGURE 3. Representative images of single-cell gel electrophoresis assay. **(a)** *In vitro* wound model created in fibroblast culture with pipette tip: Cell-free area between both red lines marked with black arrows. **(b)** Partial invasion of the cell-free area (*in vitro* wound) between both red lines by fibroblasts at 24 h. **(c)** Complete invasion of the cell-free area (*in vitro* wound) between both red lines by fibroblasts at 48 h.

method (SCGE; comet assay) as previously described.[30] Briefly, trypsinized cells were resuspended in phosphate-buffered saline (PBS) and mixed with 1% (w/v) low melting point agarose (LMPA; Sigma-Aldrich Corp., MO, USA). After this step, the cells were spread out on to the pre-coated slides with 0.5% (w/v) normal melting point agarose (NMPA; Sigma-Aldrich Corp., MO, USA). For the solidification of agarose slides placed with coverslips were put on ice packs. After the solidification of agarose coverslips were removed and third layer of LMPA was added on to the slides. After the incubation in lysis solution (10 mM Tris, 100 mM ethylenediaminetetraacetic acid (EDTA) disodium salt and 2.5 M sodium chloride; pH 10) for 2 h at 4°C (dark), slides were incubated in an electrophoresis buffer (1 mM EDTA disodium salt, 300 mM sodium hydroxide; pH >13) for 20 min in the dark in an opaque electrophoresis tank. Following the electrophoresis which was carried out at 300 mA for 30 min, slides were neutralized (0.4 M Tris; pH 7.5) and stained with ethidium bromide (2 μg/mL). One hundred nuclei were blindly scored using a fluorescence microscope (Nikon, Eclipse 600, Japan) and this process was repeated three times for each treatment. Thus, a total of 300 nuclei were scored. By the visual scoring, nuclei were categorized regarding to shape and comet tail. Undamaged, i.e., intact nuclei have globular shape were scored as 0, extremely damaged nuclei were scored as 4+ (Figure 4). Each counted nucleus was multiplied by its score, and total scores were expressed as arbitrary units (AU).

Statistical analysis

Statistical analysis was performed using the IBM SPSS for Windows version 25.0 software (IBM Corp., Armonk, NY, USA). Descriptive data were expressed in mean ± standard deviation (SD), median (min-max) or number and frequency. Normality assumption was tested using the Kolmogorov-Smirnov normality test. After evaluating whether the data were suitable for the parametric test, t-test was used for parametric data and Wilcoxon signed-rank test was used for non-parametric data. Analysis of variance was used in factorial order for comparisons of the means. If the prerequisites of parametric tests (analysis of variance in factorial order) were not met, the data were analyzed with the general linear model. A two-tailed p value of <0.05 was considered statistically significant.

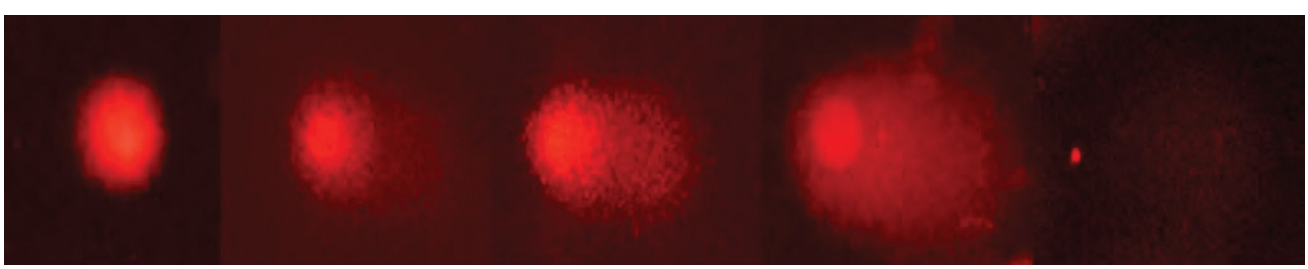

FIGURE 4. Representative images of single-cell gel electrophoresis assay.

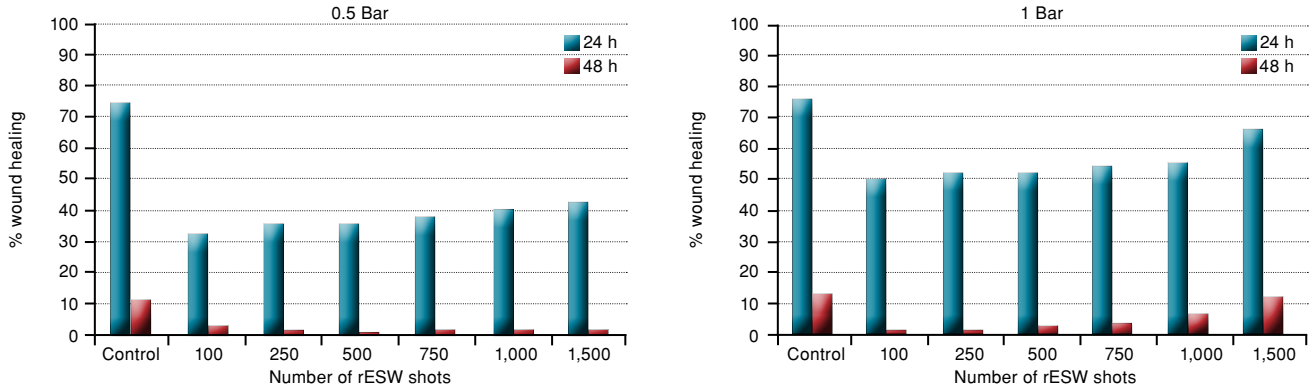

FIGURE 5. Representative images of wound closure rate of scratch assay. Scratch areas were captured at 24-h intervals and evaluated. In both graphs, the y-axis shows the percentage patency of the wound.
rESWT: Radial extracorporeal shock wave therapy.

RESULTS

Effects of rESW on wound healing (*In vitro* scratch assay)

When the control group and the 0.5 bar pressure group were compared, the wound healing rate at the 24 and 48 h decreased, regardless of the number of shots. However, when the control group and the 1 bar pressure group were compared, the wound healing rate decreased at 24 and 48 h, similarly to the 0.5 bar applied group, regardless of the number of shots (Figure 5). The p values obtained as a result of statistical comparison of both groups with the control group are given in Table I.

Effects of rESW on cell viability

According to the general linear model analysis, when 100 shots were applied in the 0.5 and 1 bar pressure group, there was no significant difference in viability between the control group and the experimental groups (p=0.122). When ≥250 shots were exceeded, the viability decreased significantly after the 24 h compared to the control group, regardless of the pressure (Table II). The lowest viability values were reached in 1,500 shots at each pressure level (Figure 6).

Effects of rESW on genotoxic damage (Comet assay)

In vitro genotoxic damage was assessed at both 0.5 and 1 bar pressures, in both time intervals,

TABLE I
Comparison of wound healing rates of 0.5 bar and 1 bar pressure groups with the control group

	Control *vs.*	0-24 h	0-24 h	0-48 h	0-48 h	24-48 h	24-48 h
		p	Mean±SD	*p*	Mean±SD	*p*	Mean±SD
0.5 bar	100	0.005*	5259601.50±3225384.97	0.001*	7532564.88±1260227.48	0.001*	2272963.38±2480397.17
	250	0.012**	4702930.25±2379211.37	0.012**	7168327.25±1168250.99	0.012**	2465397.00±2064067.82
	500	0.001*	4723806.88±2233347.21	0.001*	7253320.25±1372915.97	0.015*	2529513.38±2234489.71
	750	0.012**	4763945.50±3430106.49	0.012**	7409069.88±1811918.77	0.012**	2645124.38±2564325.46
	1000	0.012**	3978795.50±3423041.93	0.012**	6387547.88±1126229.31	0.012**	2408752.38±2715997.14
	1500	0.012**	4166815.00±2562602.56	0.012**	7002917.63±893627.73	0.012**	2836102.62±2694849.72
1 bar	100	0.017**	3604867.00±3484374.23	0.012**	7000993.75±911799.22	0.012**	3396126.75±3206028.09
	250	0.001*	3901035.25±1613607.30	0.001*	7645078.00±1178166.47	0.003*	3744042.75±2388005.43
	500	0.017**	3124100.63±2640394.76	0.012**	6311927.63±628624.39	0.012**	3187827.00±2746801.96
	750	0.036**	3093055.00±3112098.01	0.012**	6449591.63±889393.50	0.012**	3356536.63±3017819.26
	1000	0.012**	3134051.7516±69308.60	0.012**	6507387.63±1056586.79	0.012**	3373335.88±794965.93
	1500	0.025**	2410278.13±2523595.19	0.012**	6133526.00±1660161.12	0.012**	3723247.88±1319097.14

SD: Standard deviation; * Paired t-test; ** Wilcoxon signed-rank test.

TABLE II
Comparison of cell viability rates between control and experimental groups according to the number of shots and time intervals

	Control *vs.*	*p*	Mean difference
Cell viability (24 h to 48 h)	100 shots	0.122	0.049
	250 shots	0.024	0.072*
	500 shots	0.009	0.084*
	750 shots	0.002	0.102*
	1,000 shots	0.001	0.124*
	1,500 shots	0.001	0.154*

* Statistical significance at p<0.05.

regardless of the number of shots. Genotoxic damage was increased progressively from 24 to 48 h. The results are shown in Table III.

Genotoxic damage observed in 24 h at 0.5 bar and 100 (p=1.000), 250 (p=0.558), 500 (p=0.135), and 750 (p=0.099) shots were similar to the control group, while significant genotoxic damage was observed in the 1,000 and 1,500 shots. On the other hand, higher genotoxic damage rate was detected at the end of 48 h in both groups compared to the control group (Table IV).

When the pressure was increased to 1 bar, a significant genotoxic damage occurred even at 100 shots, unlike 0.5 bar pressure application in the 24-h evaluation. A significant genotoxic damage occurred in all groups below this pressure, regardless of the number of shots and the duration (Table IV).

In addition, the univariate analysis of variance revealed the genotoxic damage per shot count with 1.0 bar pressure which was significantly higher than the genotoxic damage per shot with 0.5 bar pressure (Table V).

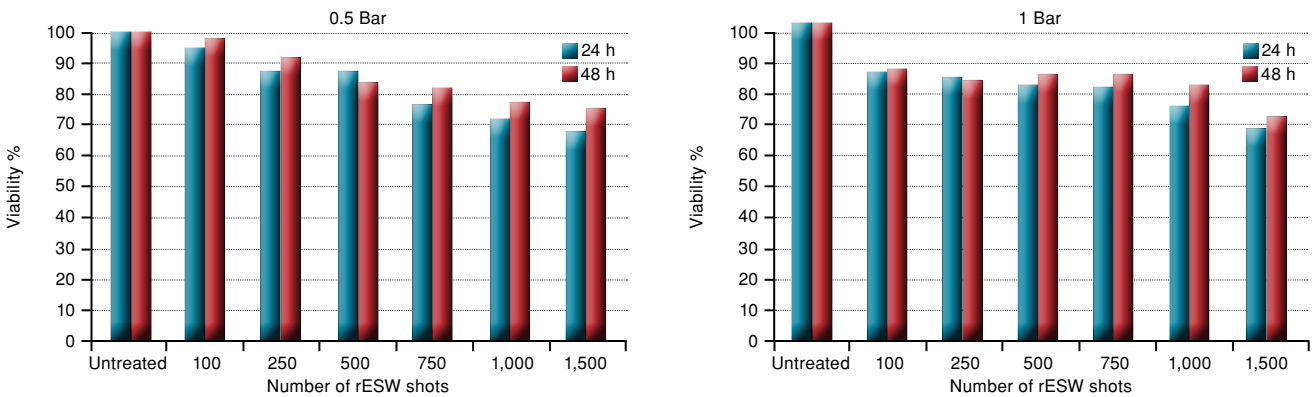

FIGURE 6. Effects of rESWT application on the *in vitro* wound healing of L929 cells for 24 and 48 h at 0.5 and 1 bar pressures.
rESWT: Radial extracorporeal shock wave therapy.

TABLE III
SCGE (comet) scores (mean±SEM)

	24 h		48 h	
	0.5 bar	1 bar	0.5 bar	1 bar
	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM
Control	7.50±2.158	7.50±2.158	4.167±0.926	4.167±0.926
100	7.50±2.158	15.833±1.747	10.333±0.926	25.667±2.437
250	9.17±2.158	20.167±1.747	13.833±0.926	25.5±2.437
500	12.17±2.158	21.167±1.747	15.833±0.926	25.5±2.437
750	12.67±2.158	22.833±1.747	17.5±0.926	26.667±2.437
1,000	14.17±2.158	24.167±1.747	23±0.926	31±2.437
1,500	15.83±2.158	25.667±1.747	24.167±0.926	32.333±2.437

SCGE: Single-cell gel electrophoresis; SEM: Standard error of the mean.

TABLE V

Univariate analysis of variance to compare of the genotoxic damage caused by the 0.5 and 1 bar pressures

	Shots	24 h (p-values)			48 h (p-values)		
		Mean square	F	Significance	Mean square	F	Significance
0.5 Bar *vs.* 1 bar pressure	100	208.33	8.89	0.014*	705.33	21.07	0.001*
	250	363.00	19.14	0.001*	408.33	126.29	0.002**
	500	243.00	16.91	0.002*	280.33	23.69	0.001*
	750	310.08	15.04	0.003*	252.08	14.42	0.004*
	1,000	300.00	7.58	0.020*	192.00	7.74	0.019*
	1,500	290.08	9.54	0.011*	200.08	6.02	0.034*

* Paired t-test; ** Wilcoxon t-test.

TABLE IV

Time- and dose-dependent comparison of genotoxic damage between control and experimental groups

Control *vs.*		24 h		48 h	
		Mean±SE	*p*	Mean±SE	*p*
0.5 bar	100	0.001±3.051	1.000	-6.167±1.310*	0.001
	250	-1.667±3.051	0.588	-9.667±1.310*	0.001
	500	-4.667±3.051	0.135	-11.667±1.310*	0.001
	750	-5.167±3.051	0.099	-13.333±1.310*	0.001
	1,000	-6.667±3.051*	0.036	-18.833±1.310*	0.001
	1,500	-8.333±3.051*	0.001	-20.000±1.310*	0.001
1 bar	100	-8.333*±2.396	0.001	-21.500*±3.204	0.001
	250	-12.667*±2.396	0.001	-21.333*±3.204	0.001
	500	-13.667*±2.396	0.001	-21.333*±3.204	0.001
	750	-15.333*±2.396	0.001	-22.500*±3.204	0.001
	1,000	-16.667*±2.396	0.001	-26.833*±3.204	0.001
	1,500	-18.167*±2.396	0.001	-28.167*±3.204	0.001

SE: Standard error.

DISCUSSION

The positive effects of ESWT in many areas, particularly in the musculoskeletal system and chronic wounds, led to the investigation of the physical and biological effects of this method at the cellular and tissue level. Several studies in the literature have mainly evaluated the effects of fESW, and there are many questions to be answered about the molecular and cellular effects of rESW.[16,19,25,26,31]

In the current *in vitro* study, we showed rESW application reduced cell viability and delayed wound healing independently of the pressure level. The lowest viability values reached in the highest number of shots at each pressure level and time interval. In addition, in this study, for the first time in the literature,[32] we demonstrated the genotoxic effect of shock wave application on fibroblasts under *in vitro* conditions using the comet assay. An increase in comet assay scores was accepted as an increase in genotoxic damage. In cell culture, a gold-standard protocol for rESW application has not been established; therefore, we determined a wide range of shock wave numbers to better demonstrate the rESW effects.

The increasing clinical use of ESWT in recent years has aroused interest in its effects on wound healing and wound care. In their study, Aschermann et al.,[16] after applying 0, 375, 750, and 1,500 pulses of rESW, waited 18 to 24 h for the detachment and

re-attachment of the cells from the plate and, then, they formed the *in vitro* wound assay. The authors showed that fibroblast migration increased and, thus, *in vitro* wound healing increased in all groups after 48 h of follow-up. Contrary to their results, we showed *in vitro* wound healing (fibroblast migration) decreased in all groups, regardless of the number of shots. Although in our study, it was confirmed that fibroblasts did not separate from the base after each rESW application, the reason for this difference between the results may be that we first created the scratch assay and then performed rESW application. In addition, we believe that the reason for the decrease in fibroblast migration in our study is the decrease in viability and changes in the cytoskeleton due to the effect of cavitation. On the other hand, only fibroblasts are evaluated in the *in vitro* scratch assay. However, many cells such as neutrophils and macrophages play an active role in *in vivo* wound healing. Tepekoylu et al.[33] showed that shock wave therapy increased macrophage recruitment to the wound area by increasing the pivotal macrophage recruitment factors such as macrophage migration inhibitory factor and macrophage inflammatory protein 1 beta. The effect of shock wave therapy on enhancing *in vivo* wound healing may be due to the contribution of cytokines secreted from increased macrophages to the wound healing process.

In the literature, there are studies examining the effects of rESW application on cell viability. Hochstrasser et al.[25] examined the effect of rESW application in an *in vitro* study using human fetal foreskin fibroblasts (HFFF2). In their study, fibroblasts stimulated with a frequency of 1 Hz and 100, 200, 500, and 5,000 pulses shock waves were applied at a 2.5-bar pressure level. Cell viability was evaluated with the trypan blue exclusion assay. Viability decreased within the first 1 to 2 h after the application, regardless of the number of shock waves. In another study in which human dermal fibroblasts were used, 300, 1,000 and 2,000 pulses shock waves were applied and, 1 h after the application, fibroblast viability was evaluated by using MTT method.[26] It was reported that viability decreased independently of the energy level and the greatest decrease was observed in the highest number of shock waves. Studies in the literature have also emphasized that with the increase in the number of shock waves applied, the viability decreases, and the main determinant of the viability is the shock wave number.[19] In the current study, we showed that rESW application reduced cell viability compared to the control group independently of the pressure level when 250 or more shots were applied, and the lowest viability was achieved at the highest number of shots at both pressure levels. Therefore, it would be appropriate not to exceed 100 shots in rESW studies that are desired to be performed without changing cell viability. Our results seem to be compatible with previous studies.[16,19,25,26] The early cytotoxic effect seen immediately after rESW application suggests that rESW causes cell death by creating mechanical cell damage mediated by cavitation effect. The possible cause of cytotoxicity may be an increase in cell membrane permeability as a result of the direct effect of the shock wave in the first phase of ESW application, as well as acute disorganization of the cytoskeleton that cannot be repaired.

There are many studies in the literature investigating the effects of rESW application on different cell types. These studies usually evaluate cell viability, effects on cell proliferation, chemokine, and cytokine responses to shock wave application, activating and inhibiting cellular pathways, changes in gene expression, while there is no study on the genotoxic effect of shock wave on the cell in the literature.[34-36] In this study, we demonstrated the genotoxic effect of shock wave application on fibroblasts by the comet assay. According to our results, regardless of the number of shock waves applied, genotoxic effects were observed at the end of 48 h in both pressure groups. In the *in vitro* study of Ashermann et al.,[16] fibroblasts increased gene expression and proteins that regulate the cell cycle in response to the shock wave, thereby attempting to achieve genome stability. These findings are also consistent with our study. Genotoxic damage can be caused by direct physical effects, permeability changes, ionizing molecules and resulting soluble radicals created by ESW application on the cell. In addition, high-energy shock wave application may cause apoptosis or necrosis through genotoxic damage. According to the results obtained from this study, the severity of the genotoxic damage was directly affected by the pressure increase and 1,000 shots at 0.5 bar pressure were considered as the threshold value for genotoxic damage in *in vitro* conditions. Since the genotoxic effect of the application up to 1,000 shots at 0.5 bar pressure occurred significantly at the 48 h, it was thought that this application might stimulate apoptosis in the cells. On the other hand, genotoxic damage occurring in the first 24 h in all groups may be associated with necrosis. Therefore, we recommend staying below 1,000 shots for therapeutic effect studies and using shots above 1,000 at 0.5 bar or, regardless of the shot number, 1 bar pressure should

be used in experimental studies to induce genotoxic damage. Mechanisms of genotoxic damage caused by ESW application should be clarified in future studies.

More intriguingly, in an experimental study of rats with spinal cord damage, low-energy ESW application increased angiogenesis by increasing vascular endothelial growth factor expression in nerve cells, thus improving locomotor and sensory functions.[37] Finally, in an *in vivo* study, Haberal et al.[38] reported that shock wave therapy applied in rats with lumbar laminectomy reduced epidural fibrosis. The reason for the decrease in epidural fibrosis in this study may be the fibroblast death due to the genotoxic damage observed in the current study, and the possible effect of inflammation triggered by this cytotoxic effect on wound healing.

Nonetheless, this study has some limitations. Our research is based on *in vitro* experimentation and cannot be linked to *in vivo* conditions. *In vivo* wound healing is a complex process and not only through fibroblasts, and it depends on tissue architecture and composition of extracellular matrix. With chemokines and cytokines secreted from endothelial cells, cell-cell interactions and the barrier effect created by living tissues and changes in tissue integrity are both effective on *in vivo* results. In addition, different experimental setups can be used in *in vitro* rESW applications. Water bath setup, which is the method frequently used in the literature, was not used in this study, that may have affected the experimental results. Finally, comparing the results obtained from the *in vivo* study to be performed simultaneously with the *in vitro* study could provide a better examination of the mechanism of action.

In conclusion, the present study demonstrated the effect of rESW application on fibroblast viability, wound healing and fibroblast genotoxicity using MTT assay, *in vitro* scratch assay, and comet assay. According to these results, wound healing was delayed and cell viability decreased at all pressure levels, all shot numbers, and all time intervals in fibroblast cultures applied rESW in the *in vitro* setting. In addition, the severity of genotoxic damage increased proportionally with the increased pressure level. We believe that this study is valuable, as it is the first to reveal the genotoxic effects of rESW application *in vitro*. By using different energy and impulse regimes and *in vivo* studies, more useful data can be obtained and the effects of rESW application can be clearly demonstrated.

Declaration of conflicting interests

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

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