

Cardiac shock wave therapy: assessment of safety and new insights into mechanisms of tissue regeneration

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Abstract

Although low-energy extracorporeal cardiac shock wave (ECSW) therapy represents an attractive non-invasive treatment option for ischaemic heart disease, the precise mechanisms of its action and influence on the cardiac tissue remain obscure. The goal of this study was to evaluate the effects of SW application on cardiac function and structure. Four-month-old Fisher 344 rats were subjected to ECSW therapy. Echocardiographic measurements of cardiac function were performed at baseline and at 1 and 3 months after treatment. Signs of inflammation, apoptosis and fibrosis were evaluated by immunohistochemistry in the control and treated hearts. ECSW application did not provoke arrhythmia or increase the troponin-I level. At all time points, the left ventricular ejection fraction and fractional shortening remained stable. Histological analysis revealed neither differences in the extracellular matrix collagen content nor the presence of fibrosis; similarly, there were no signs of inflammation. Moreover, a population of cardiac cells that responded eagerly to ECSW application in the adult heart was identified; c-kit-positive, Ki67-positive, orthochromatic cells, corresponding to cardiac primitive cells, were 2.65-fold more numerous in the treated myocardium. In conclusion, non-invasive ECSW therapy is a safe and effective way of activating cardiac stem cells and myocardial regeneration. Because many factors influence cellular turnover in the ischaemic myocardium during the course of ischaemic heart disease, cardiac remodelling, and heart failure progression, studies to identify the optimal treatment time are warranted.

Keywords: ischaemic heart disease • low-energy shock waves • extracorporeal cardiac shock wave therapy • heart failure • cardiac primitive cells • cardiac regeneration

Introduction

According to the current understanding of cardiac biology, cardiomyocytes, smooth muscle and endothelial cells are continuously renewed to preserve heart function throughout life [1]. Myocardial ischaemia and infarction are the results of coronary vessel diameter reduction or occlusion that affect all cardiac cells, which leads to the impairment of their function or death by apoptosis or necrosis. This process, in turn, can lead to heart failure.

Over 14 million people in Europe suffer from heart failure. This number is forecast to increase to 30 million by the year 2020 [2]. Contrary to public belief, heart failure is far more common than most tumours, including breast, cervical and intestine cancers. If new treatment options are not implemented soon, society will have to cope with the burden of increasing hospitalizations and escalating economic costs, two factors acceptable neither for cardiologists nor for national health services.

With the lowering of SW energy, new indications for SW use have come to light. Although destructive at about 10 times higher energy flux density, SWs are regenerative at ≤ 0.1 mJ/mm² [3]. Their application has been tested in animal models of ischaemic heart disease [4–6] and in patients with angina pectoris, where ECSW therapy alleviated symptoms and improved myocardial perfusion [7–10]. While the precise mechanisms of SW action and the influence of SWs on cardiac tissue remain obscure, ECSW

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1 therapy seems to offer great promise for patients with ischaemic
2 heart disease and represents an attractive non-invasive treatment
3 option. However, SW can cause non-selective tissue damage and
4 as such may induce an injury-and-repair process in the
5 myocardium, even at the low energy used in ECSW treatment. This
6 process can be expected to result in muscle cell loss, interstitial
7 fibrosis and vascular sclerosis [11]. Consequently, a reasonable
8 demand for safety and mechanism studies has been forcefully
9 expressed by practicing cardiologists.

10 The clinical benefits derived from ECSW therapy have been
11 attributed to the improvement of myocardial blood flow and
12 enhanced angiogenesis [6]; however, in light of the relevant cardiac
13 biology, it is inconceivable that the effects of SW should be limited
14 to the induction of angiogenesis and endothelial progenitor cell
15 activation. The shear stress on cell membranes provoked by SWs
16 results in cell membrane permeabilization and in the activation of
17 membrane receptors and intracellular signal transduction pathways
18 [12]. This action could affect all of the cardiac cells responsible for
19 cardiac regeneration, including cardiac stem cells and primitive cells
20 that represent the progenitors and precursors of cardiac cell line-
21 ages (cardiomyocytes, smooth muscle and endothelial cells).

22 The aim of the study was to evaluate the safety of ECSW appli-
23 cation on the adult heart and its effects on cardiac tissue function
24 and morphology. Moreover, the histological analysis of treated
25 hearts allowed us to identify a population of cardiac cells that
26 responds eagerly to SW application in the adult heart.

30 Materials and methods

31 Study protocol

32 Four-month-old Fisher 344 male rats were divided randomly into control
33 ($n = 10$) and ECSW-treated ($n = 10$) groups. The latter was subjected to
34 echocardiography-guided ECSW therapy. An electrocardiography tracing
35 was recorded during the treatment to identify signs of arrhythmia. The car-
36 diac troponin-I (cTnI) concentration was measured in plasma obtained
37 from control and treated animals at 1 hr after the first ECSW application.
38 Echocardiographic measurements of the left ventricular ejection fraction
39 and fractional shortening were performed at weeks 5 and 13 in the control
40 and treated animals. Five animals in each group were sacrificed at week
41 5 and at week 13, and animal hearts were excised and processed for his-
42 tological study. The protocol was approved by the local ethics committee,
43 and all experiments were supervised by the Laboratory Animal Care
44 Committee of the Second University of Naples.

45 ECSW treatment

46 Animals in the control and treated groups were anaesthetized with 1.5%
47 inhaled isoflurane and became unresponsive to a moderate pain stimulus
48 while still normally breathing spontaneously. This anaesthetic regimen
49 does not affect the left ventricular dimensions or ejection fraction in
50 healthy animals [13]. Treatment was performed using a Duolith Vet compact
51 shock wave unit that electromagnetically generates focused SWs (Storz
52 Medical AG, Tägerwilen, Switzerland). A total of 100 shots were delivered
53 at a total energy flux density of 0.25 mJ/mm^2 during every treatment ses-
54 sion, with three sessions in 1 week. A specially designed hand-piece
55 enabled the mechanical adjustment of the therapeutically effective depth of
SW penetration in rats.

56 Echocardiography

All measurements were performed with a 25-MHz linear transducer coupled
to a high-resolution Micro-Ultrasound System (Vevo 770, VisualSonics,
Inc., Toronto, Canada). Serial M-mode echocardiographic images were
taken in the short axis view at the level of the papillary muscles. Images
were stored digitally for offline analysis. The end-diastolic and end-systolic
left ventricular diameter and posterior wall thickness were measured, and
fractional shortening and ejection fraction were calculated.

57 Cardiac troponin-I measurement

A high-sensitivity ELISA kit (Life Diagnostics, Inc., West Chester, PA, USA)
was used for the determination of cTnI in rat plasma. Samples were incu-
bated simultaneously with two different affinity-purified antibodies, includ-
ing an antibody for solid phase immobilization and a horseradish peroxidase
(HP)-conjugated antibody. Incubation was performed in microtitre wells on
a plate shaker for 1 hr at room temperature. After washing, a solution of
tetramethylbenzidine was added for 20 min. The reaction was stopped by
the addition of HCl, and the absorbance was measured at 450 nm with a
microplate reader (ELx800, BioTek, Winooski, VT, USA). A standard curve
was constructed by measuring the mean absorbance of the serially diluted
cTnI standard. All samples were assayed in triplicate and the actual concen-
tration of cTnI ($\mu\text{g/l}$) was determined from the standard curve.

58 Tissue staining

Rats were anaesthetized with ketamine (100 mg/kg, i.p.). The abdominal
aorta was cannulated with a polyethylene catheter filled with 0.2M PBS
(pH 7.4) and 100 IU/ml heparin. In rapid succession, the heart was
arrested in diastole by injection of 100 mM cadmium chloride through the
aortic catheter, the thorax was opened, and the heart was perfusion-fixed
with 10% (v/v) neutral buffered formalin. The hearts were dissected and
post-fixed for 90 min. at room temperature. Cardiac tissue was embedded
in paraffin, sliced into serial 4- μm -thick sections and placed on poly-L-
lysine-coated glass slides.

Slides were deparaffinized, rehydrated and stained with haematoxylin
and eosin, Masson's trichrome stain or Picrosirius red stain, in accordance
with standard practices [14]. Alternatively, sections were immunostained
with primary antibodies against Ki67 (1:600, rabbit polyclonal; Novocastra
Laboratories Ltd., Newcastle upon Tyne, UK), c-kit (1:10, rabbit polyclonal)
and Flk-1 (1:25, mouse monoclonal, both from Santa Cruz Biotechnology,
Santa Cruz, CA, USA), and detected with indirect immunoperoxidase
technique by employing the IHC Select Immunoperoxidase Secondary
Detection System (DET-HP1000; Millipore, Temecula, CA, USA). To identify
mast cells in tissue samples, deparaffinized sections were stained with 0.2%
toluidine blue for 5 min., washed, quickly dehydrated and cover-slipped for
observation. Negative controls (*i.e.* isotype-matched non-specific antibodies)
were included for each staining. Microscopic analysis was performed with
a Leica DMLB microscope (Leica Microsystems, Wetzlar, Germany).

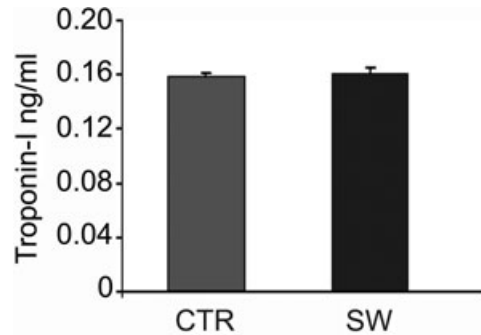


Fig. 1 Application of ECSW does not induce myocardial necrosis. High-sensitivity ELISA was used to determine the actual concentration of cTnI in plasma collected from rodents at baseline and at 1 hr after the application of 100 shots of SW at 0.25 mJ/mm². The cTnI levels remained stable after the treatment ($n = 4$, $P = 0.45$).

Pictures were taken in bright field with a digital camera (Leica DC200) connected to the microscope.

Apoptosis evaluation

Apoptotic cells were detected *in situ* by labelling and detecting DNA strand breaks in the TUNEL assay. Paraffin-embedded tissue sections were deparaffinized and pre-treated with proteinase K (20 µg/ml). Sections were incubated with terminal deoxynucleotidyl transferase in the presence of digoxigenin-labelled and -unlabelled nucleotides, which marked the free 3'OH DNA termini *in situ*. This step was followed by incubation with an anti-digoxigenin antibody conjugated to a peroxidase reporter molecule (all reagents were provided by Millipore, Billerica, MA, USA). Microscopic analysis was performed with a Leica DMLB microscope, and pictures were taken in bright field with a digital camera connected to the microscope.

Statistical analysis

All numerical data are presented as mean ± S.E.M. Statistical differences between groups were evaluated with Student's two-tailed unpaired *t*-test (paired *t*-test was used for the cTnI concentration comparison). A *P*-value of < 0.05 was considered significant.

Results

ESCW application did not provoke arrhythmia or myocardial necrosis

Shock waves generate a physical force that may influence the membrane action potential. This process in turn may interfere with contractile stimuli generation in the nodal cells and conduction in the Purkinje fibres and cardiomyocytes. Therefore, we secured a direct electrocardiographic tracing during the ECSW application. Even if

Table 1 Echocardiographic characteristics of control and SW-treated rats

	3 Weeks		13 Weeks	
	Control ($n = 5$)	SW-treated ($n = 5$)	Control ($n = 5$)	SW-treated ($n = 5$)
EF (%)	70 ± 3	71 ± 5	59 ± 4	61 ± 7
FS (%)	47 ± 5	48 ± 6	41 ± 2	40 ± 6

EF, ejection fraction; FS, fractional shortening. Mean ± S.E.M. No significant differences were seen between the groups at any time point.

not R wave-triggered (due to the high physiological frequency of heartbeat in rats), the ECSW application did not induce arrhythmia.

The laboratory measurement of cTnI concentration is a highly specific marker of acute myocardial infarction and myocardial injury associated with rhabdomyolysis, pulmonary embolism, myocarditis and acute pericarditis [15], all of which are possible complications of ECSW application. High-sensitivity ELISA revealed stable TnI levels of 0.155 ± 0.0005 µg/l and 0.158 ± 0.002 µg/l ($n = 4$, $P = 0.05$) at baseline (before ECSW treatment) and at 1 hr after 100 shots at 0.25 mJ/mm², respectively (Fig. 1).

ECSW therapy did not induce functional or morphological damage to the heart

Echocardiographic evaluation of the control and treated rats at weeks 5 and 13 revealed preserved cardiac left ventricular ejection fraction and fractional shortening after treatment at both time points (Table 1).

Collagen plays a vital role in maintaining structural integrity and in determining tissue function. In the heart, both decreases in [16] and excess accumulations of collagen [17] can be detrimental. We used Masson's trichrome and Picrosirius red stains (Fig. 2C–F) to visualize the fine collagen fibres in the myocardium of control and ECSW-treated animals. Neither control nor ECSW-treated animals manifested cardiac fibrosis throughout the study period. The myocardial collagen content was similar in control and treated animals up to 3 months after ECSW treatment. In terms of possible endothelial damage, there was no thickening of the blood vessel intima. Local subepicardial vasculogenesis was observed in treated rats in regions corresponding to the transthoracic SW application zone; however, inflammatory interstitial infiltration was not observed in the cardiac tissue of treated animals (Fig. 2A and B).

Several mechanisms of SW action, such as direct mechanical stress or paracrine action of the factors secreted from damaged or activated cells, may lead to cellular apoptosis. In the heart, increased myocyte apoptosis is associated with the progression of cardiac failure of both ischaemic and non-ischaemic origin [18]. After ECSW therapy in rats, the percentage of apoptotic cells (Fig. 3)

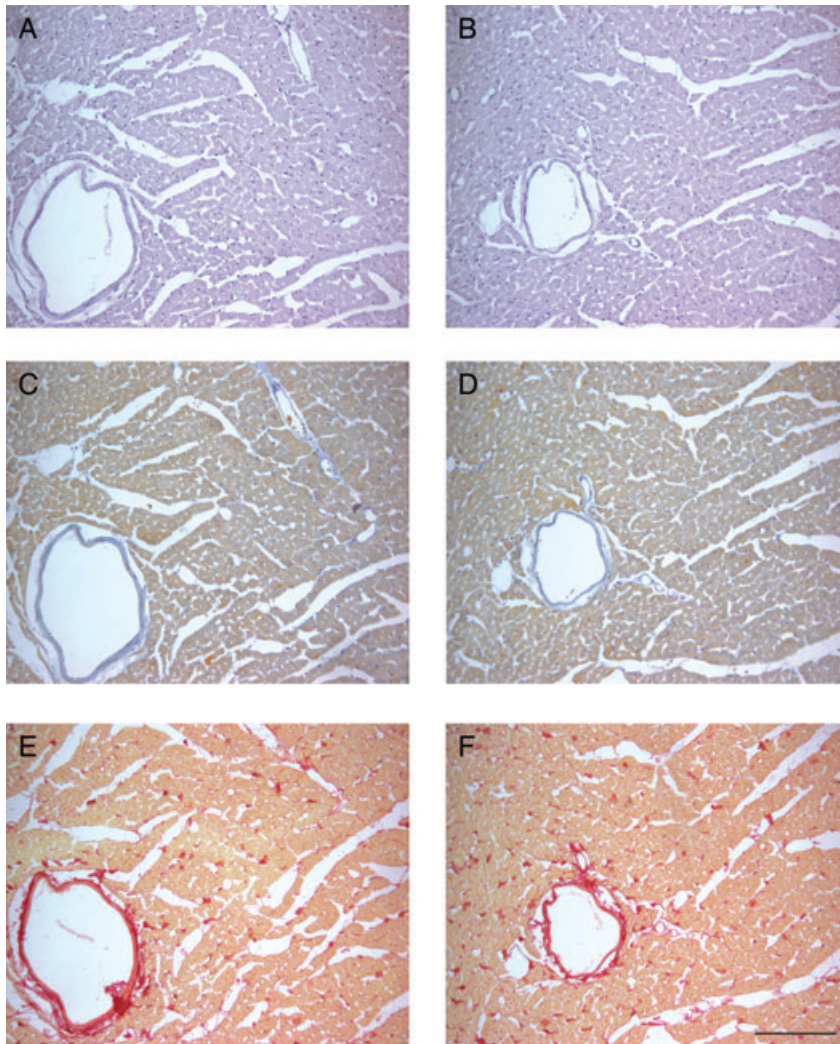


Fig. 2 ECSW application does not induce structural damage to the heart. Histochemistry and immunohistochemistry methods were used to evaluate the morphology of cardiac tissue in control and treated animals at 3 months after ECSW application. Haematoxylin and eosin staining revealed no signs of inflammatory infiltration in control (A) or SW-treated hearts (B). Masson's trichrome (collagen in blue; C, D) and Picrosirius stainings (collagen in red; E, F) revealed no signs of fibrosis in control (C, E) or SW-treated hearts (D, F). Scale bar: 100 μ m.

was similar in control and treated rats at 5 weeks ($0.33 \pm 0.019\%$ and $0.37 \pm 0.037\%$, respectively; $n = 4$, $P = 0.35$) and at 13 weeks ($0.35 \pm 0.024\%$ and $0.39 \pm 0.023\%$, respectively; $n = 4$, $P = 0.28$). Remarkably, all apoptotic cells were non-myocytes.

ECSW treatment resulted in the activation of c-kit-positive cardiac cells

C-kit (also known as CD117)-positive cardiac cells represent a population of cardiac stem cells and progenitors/precursors of cardiac cell lineages in the developing [19] and adult heart [20]. In contrast to control hearts, immunohistochemical analysis of the rat myocardium at 3 months after ECSW treatment revealed the presence of c-kit-positive cell clusters (Fig. 4A). ECSW treatment increased the number of c-kit-positive cells 2.65-fold compared to control hearts (279.1 ± 52.86 vs. 105.4 ± 15.25 c-kit-positive cells/100 mm^2 ; $n = 4$, $P < 0.05$).

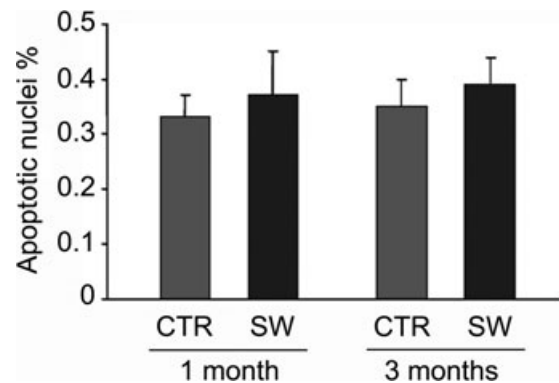


Fig. 3 ECSW therapy did not provoke apoptosis of the cardiac cells. The percentage of apoptotic cells, as evaluated by the TUNEL assay, was similar at 5 weeks ($n = 4$, $P = 0.35$) and 13 weeks ($n = 4$, $P = 0.28$) in control and treated rats.

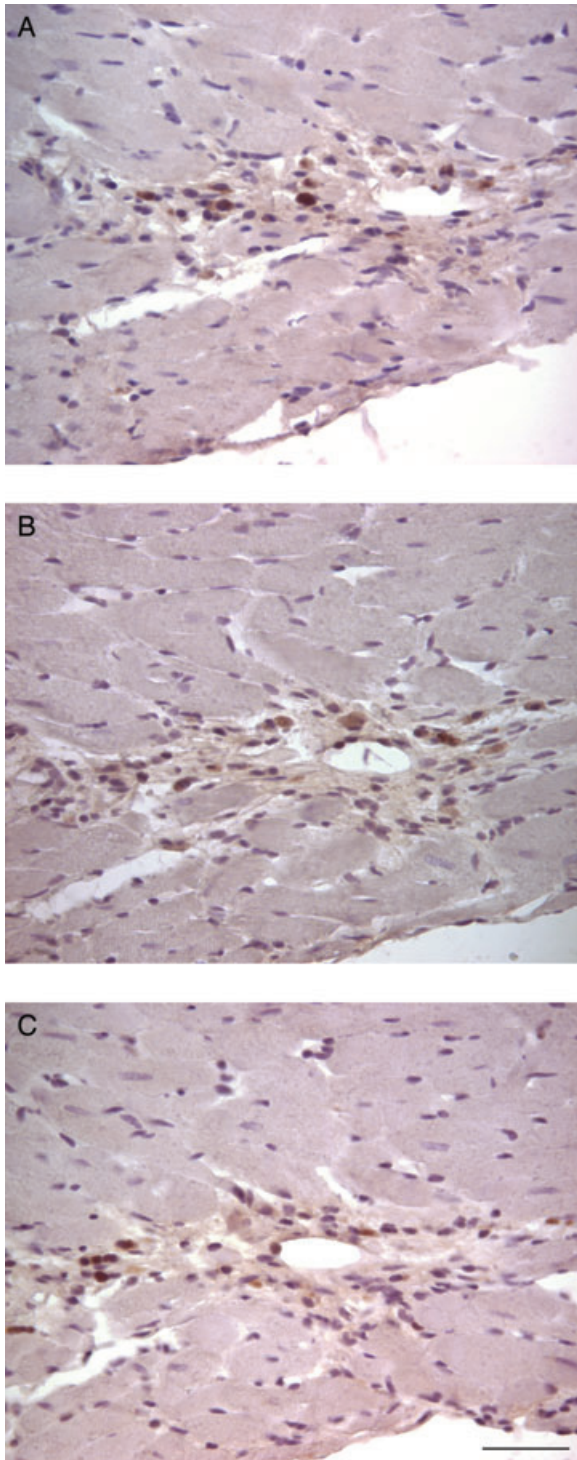


Fig. 4 ECSW application increases the number of cardiac primitive cells. Clusters of c-kit-positive, Ki67-positive and Flk-1-positive cells were observed in the myocardium of ECSW-treated animals only. Representative images of the serial sections of cardiac tissue stained by immunohistochemistry for c-kit (A), Flk-1 (B) and Ki67 (C) are shown. Scale bar: 50 μ m.

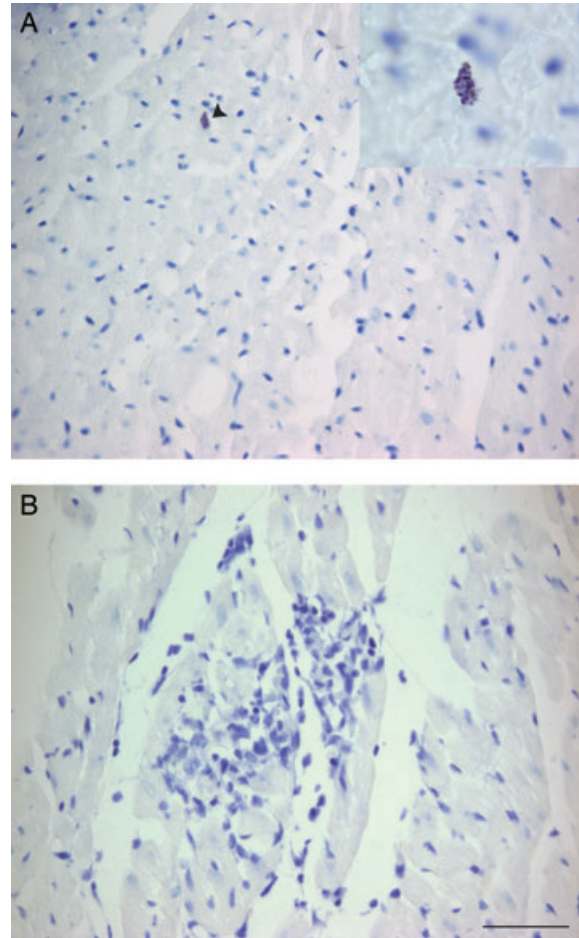


Fig. 5 Toluidine blue staining excludes the presence of mastocytes in the activated cell clusters. Representative images with a mastocyte (A) demonstrating typical metachromatic granules in the cytoplasm and a cluster of orthochromatic c-kit-positive cells (B). Scale bar: 100 μ m.

Because a sub-population of c-kit-positive cells represents endothelial progenitor cells [21], we identified cells expressing the vascular endothelial growth factor receptor (VEGFR) Flk-1 in the clusters of cells detected after ECSW treatment (Fig. 4B). The vascular density increased from $380.75 \pm 13.49/\text{mm}^2$ in the control to $428.00 \pm 14.71/\text{mm}^2$ in the SW-treated hearts ($n = 4$, $P < 0.05$). Moreover, Ki67 staining, which marks cells in the G₁, S, G₂, and M phases of the cell cycle, revealed that the fraction of actively cycling c-kit-positive cells was 1.56-fold higher in treated animals compared to control ($55.70 \pm 1.34\%$ vs. $35.74 \pm 2.93\%$; $n = 4$, $P < 0.05$) at 3 months after SW application (Fig. 4C).

The possibility that the observed c-kit-positive cells were cardiac mastocytes was ruled out by a toluidine blue staining. The clusters of c-kit-positive cells stained blue, while mastocytes were extremely rare among myocardial cells and displayed unique morphological features, including the presence of fine metachromatic granules in the cytoplasm (Fig. 5).

Discussion

The rapid progression from orthopaedics, where low-energy SWs are used to treat non-union fractures and degenerative diseases of the connective tissue, to cardiology; from bench, at which the exact mechanisms of SW action on cardiac tissue have not been satisfactorily elucidated, to bedside; from animal studies, which have been limited mostly to the observation of functional outcomes, despite the availability of tissue samples for further histological analysis, to clinical trials, all testify of the urgent need for new treatment options for heart failure patients.

The concept of cardiac regeneration first emerged when stem cells were described in the human heart. These cells express the stem cell factor receptor c-kit, are multipotent, and generate myocardial, endothelial and smooth muscle lineage cells [22]. Two hypotheses have been suggested for the origin of stem cells (*e.g.* that circulating stem cells home to the heart from other tissues, or that stem cells are developmental residents in the heart), although the true origin remains to be established. Both hypotheses give rise to different regenerative approaches, including bone marrow/peripheral blood-derived cell transplantation and local activation of cardiac intrinsic regenerative properties by growth factor injection.

Although a precise mechanism of action remains unknown, cardiac SW therapy seems to influence both the peripheral and resident cardiac stem cell pools. A recent experimental study demonstrated that the pre-treatment of ischaemic tissue with low-energy SW facilitates the recruitment of circulating progenitor cells into chronic ischaemic tissue [23]. In addition to VEGFR up-regulation [24], stromal cell-derived factor-1 (SDF-1) up-regulation was suggested as the predominant mechanism for the enhanced recruitment of endothelial progenitor cells after SW treatment. If SDF-1 becomes up-regulated by SW treatment, other cytokines and growth factors might be up-regulated in the ischaemic target tissue, which would enhance the activation of resident stem cells. Indeed, low-energy SW treatment of c-kit-positive adult human cardiac primitive cells *in vitro* enhances the proliferation of cells and the expression of markers of cardiomyocyte, endothelial and smooth muscle cell lineages [25]. Similarly, evidence for cardiac stem cell activation was observed *in vivo* in this study: in contrast to control hearts, the number of c-kit-positive cycling cells increased in the treated myocardium. This novel finding forms a foundation for the use of ECSW for the activation of myocardial regeneration.

Ischaemic heart remodelling and heart failure progression are closely related to the increased concentration of factors such as oxidants, nitric oxide, angiotensin II and catecholamine, which can trigger the apoptosis of cardiomyocytes [26]. A rapid enhancement of endothelial nitric oxide synthase activity and an increase in nitric oxide production have been observed *in vitro* as a consequence of SW application [27]. This effect presumably represents a local and transitory anti-inflammatory action of SWs, but may contribute to the apoptosis of cardiac cells. Similarly, SWs may influence the cell membrane potential and interfere with contrac-

tile stimuli generation and propagation in the conduction system cells and cardiomyocytes.

Given the lack of safety and mechanism studies, it remains uncertain whether the benefits of ECSW application outweigh its risks. Accordingly, we evaluated the effects of low-energy SW application on cardiac tissue function and structure. ECSW treatment did not provoke arrhythmia or increase the cTnI level. Histological analysis revealed neither differences in the extracellular matrix collagen content nor the presence of fibrosis. Moreover, the SWs did not contribute to the apoptosis of cardiac cells in treated hearts *in vivo*, and there were no signs of inflammation at 3 months after ECSW treatment.

Fibrosis is a hallmark of the cardiac remodelling that leads to increased myocardial stiffness and cardiac dysfunction. Because this condition may represent an earlier inflammation of the cardiac tissue or cardiac cell loss [28], its absence at the 3-month time point indicates that no relevant damage was inflicted in the cardiac tissue following ECSW application. This result is particularly evident in our setting, because the cTnI increase in the rodent heart is directly and constantly correlated with histological damage to the cardiac tissue. A normal cTnI concentration precludes cardiac tissue damage and *vice versa* [29]. The absence of histological indices of cardiac lesions in our study, independent of the measurement time of the cTnI concentration, indicates that there was no myocardial tissue damage at any time point. Accordingly, the heart function, as evaluated by the echocardiographic measurement of left ventricular ejection fraction and fractional shortening, remained stable at all time points in the SW-treated rats.

In conclusion, non-invasive ECSW therapy is a safe and effective way to activate cardiac stem cells and myocardial regeneration. With regards to its application in ischaemic heart disease, the optimal treatment time should be established. It may be that ischaemia-induced cardiac remodelling provokes changes that limit the responsiveness of cardiac primitive cells to SW treatment, while cardiac tissue at the early stages of coronary artery disease may represent the best soil for plant growth: namely, a responsive environment in which the cardiac stem cells activated by ECSW therapy can bloom and thereby regenerate the ischaemic heart.

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Conflict of interest

The authors confirm that there are no conflicts of interest.

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