LONG-TERM FOLLOW UP OF THE EFFECTS OF EXTRACORPOREAL SHOCKWAVE THERAPY (ESWT) ON MICROCIRCULATION IN A DENERVATED MUSCLE FLAP*

ŁUKASZ KROKOWICZ1, MARIUSZ MIELNICZUK2, MICHAŁ DREWS1, MARIA SIEMIONOW3

Department of General, Gastroenterological and Endocrine Surgery, K. Marcinkowski Medical University in Poznań1
Kierownik: prof. dr hab. M. Drews
Department of Anesthesiology and Intensive Care, University of Nicolaus Copernicus, Collegium Medicum of Ludwik Rydygier in Bydgoszcz2
Kierownik: prof. dr hab. K. Kusza
Department of Plastic and Reconstructive Surgery, Cleveland Clinic, Cleveland, Ohio, United States of America3
Kierownik: dr J. Zins

Extracorporeal Shock Wave Therapy (ESWT) is a golden standard for treatment of kidney and urinary calculi. It is also widely used in a number of orthopedic pathologies and other fields of medicine. Although clinical success the exact mechanism of shock wave technology is not well established. The cremaster muscle model used in our experiment is structurally and functionally similar to other skeletal muscles (striated muscle).

The aim of the study was to evaluate influence of ESWT treatment on microcirculation and leukocyte-endothelial interactions after longer time period post ESWT application.

Material and Methods: In experiment we used 34 Lewis rats weighting 125-160 grams. Animals were divided into 4 groups – Group 1 (n=10) control, without ESWT application, group 2 (n=8), in which measurements were performed 3 days after application of 500 impulses of ESWT; group 3 (n=8) in which measurements were performed 7 days after application of 500 impulses of ESWT; group 4 (n=8), in which measurements were performed 21 days after application of 500 impulses of ESWT.

Results. The experiment showed a decrease in functional capillaries activity, we also observed the reduction in leukocyte rolling over the endothelium and an increase in flow velocity in V1 venules.

Conclusions. ESWT therapy after 3, 7 and 21 days decreases inflammatory process in the muscle, the other of its effect is weakened. This confirms that the treatment had a positive effect if ESWT is applied repeatedly, because only in this case a wave maintains its beneficial effects.

Key words: microcirculation, endothelium, Extracorporeal Shockwave Therapy, rat, cremaster muscle, red blood cell velocity, functional capillary density, leukocytes, leukocyte-endothelial interactions

Extracorporeal Shock Wave (ESW) is an acoustic wave generated by releasing a high-voltage discharge in an aqueous environment, which by means of a semicircular reflector may be transferred to a specific tissue (1).

ESWT has been used in medicine since 1980, when the first patients suffering from urolithiasis were treated with the use of an extracorporeal wave. Until 99, over 2 million patients worldwide have had their kidney stones broken down with the use of lithotripsy (ESWT of higher wave intensity) (2). Currently, ESWT is used in many branches of medicine. Scientific reports mention experimen-

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nal use of the abovementioned waves in reduction of myocardial scarring and in improvement of cardiac muscle function post ischaemia. Nishida and colleagues have demonstrated that ESWT may reduce cardiac muscle dysfunction post its artificially induced ischaemia (3). Uwatoku and colleagues have shown that ESWT might facilitate the remodelling of left ventricle post experimentally induced infarction (4). Similar observations have been made by Zimpfer, who has found that the use of ESW in animals with surgically induced cardiac muscle ischaemia results in an improved left ventricular contractibility and an induction of angiogenesis in ischaemic myocardium (5). The promising results of experiments in animals have prompted Fukimoto and colleagues to use ESWT in patients post myocardial infarction with no percutaneous coronaryography or bypass contraindications. The ESWT produced an improvement in patient wellbeing as well as a reduction in the taken drug doses. No adverse effects of applied treatment have been observed (6). Owing tot the reports of ESWT in the treatment of inflammation in orthopaedics, ESWT has found use in the treatment of extensive burns and difficult to heal wounds. This treatment facilitates healing, lowers the inflammation in the skin and subcutaneous tissue, as well as reduces the size of the formed scar (7, 8, 9). Studies on animal models have demonstrated the application of ESWT in reducing the region of tissue necrosis and extending the time of survival of skin folds in medical experiments (10-14).

Despite the success in the clinical application of ESWT, the precise mechanism of its action on the microcirculatory haemodynamics is not known. Numerous hypotheses and results of scientific research suggest that ESWT has an inhibitory effect on tissue inflammation by reducing the secretion of pro-inflammatory cytokines and free radicals. In addition, the waves are thought to dilate the blood vessels though releasing nitric oxide (NO). Numerous hypotheses state that ESWT leads to neovascularisation that increases the blood supply to tissues. It is also believed that ESWT can modulate the inflammatory response of tissues by its direct effect on the expression of adhesion molecules, secretion of interleukins and activation of growth factors. Studies on ESWT use have indicated its numerous clinical applications, and demonstrated that their use leads to the release of angiogenesis and neovascularisation factors (15).

The endothelium lines the blood vessels. It consists of a single layer of closely adhering mononuclear cells. Together with the basement membrane on which the endothelial cells lie, it forms the internal membrane constituting the internal layer of blood vessels. In adults, the basement membrane together with endothelial cells takes up 1000 m². The cells making up the endothelium are physiologically active, take part in the “active” transport of chemical substances and secrete a number of biologically active substances. Owing to this, the endothelium actively participates in numerous inflammatory processes taking place in the body. Depending on the produced substances, the endothelium may have different biological activity (pro- and anti-coagulating, fibrinolytic, vasoconstrictive and vasodilating, neovasculating and pro-angiogenic, pro-inflammatory, oedema-inducing and atherogenic). The activation of endothelial cells occurs under the influence of numerous stimuli. The strongest one is the progression of inflammation, as a result of which the inflammatory response cells accumulate and cross outside the vessel lumen. The above process occurs in the following stages:

- leukocyte rolling when, as a result of their moving to the blood vessel wall (marginalisation), an interaction takes place between the selectins on the leukocyte surface (L) and on endothelial surface (E and P) and their receptors. There are formed labile bonds between the leukocytes and the endothelial surface, which are not strong enough to counteract the blood flow and cause the leukocyte rolling. At the same time, as a result of the activity of pro-inflammatory cytokines (mainly IL-1 and TNF-α) the proteins called integrins become activated and there starts the process of
  - close adhesion, in which the strong bonds formed between the proteins on the surface of leukocytes (integrins α and β) and the immunoglobulins being their receptors (ICAM-1 and VCAM-1) lead to their adhesion to the endothelial wall, where as a result of the activity of chemokines (mainly IL-8) the leukocytes are transferred outside the vascular wall and there starts the process of
  - leukocyte extravasation, i.e. leukocyte migration through the endothelial barrier to
The process of extravasation of the immune system cells is dependent on the activity of various substances, the main ones of which are cytokines (IL-1, TNF-α, IL-8), selectins (L, P, E), integrins and immunoglobulins (ICAM i VCAM) (16-19).

In the study, there was used a model of rat cremaster muscle – a well studied model of microcirculation haemodynamics and interactions between leukocytes and the vascular endothelium in various settings translating into clinical situations such as perioperative injury, anaemia, reperfusion or drug action.

The aim of the study was the evaluation of the effects of ESWT on the microcirculation haemodynamics, the numbers of active capillaries and the interactions between the capillary walls and leukocytes over a longer time (3, 7 and 21 days) from ESWT application.

MATERIAL AND METHODS

24 Lewis rats weighing 125-160 g were used in the experiment, as per the protocol approved by the Animal Research Committee Cleveland Clinic. The animals used in the experiment were kept in an accredited animal house of Cleveland Clinic, in which they were treated humanely, in compliance with the guidelines of the United States Department of Health. The rats were kept in cages at room temperature in the day-night cycle. They were given standard feed and water.

Experimental groups

Group 1 – control group (n = 10). In this group, after the isolation of cremaster muscle and placing it on the tissue bath, the microcirculation haemodynamics measurement was made. The animals were not subject to ESWT.

Group 2 – (ESWT500.3d n = 8). In this group, the studies were made 3 days after the application of 500 ESWT impulses with energy density of 0.10 mJ/mm².

Group 3 – (ESWT500.7d n = 8). In this group, the studies were made 7 days after the application of 500 ESWT impulses with energy density of 0.10 mJ/mm².

Group 4 – (ESWT500.21d n = 8). In this group, the experiment was performed 21 days after the application of 500 ESWT impulses with energy density of 0.10 mJ/mm².

Anaesthetised rats (with the use of anaesthetics: acepromazine, ketamine and xylazine) were placed in a supine position. The gel used in ultrasound examinations was used as a medium between the device emitting ESW (EvoTron®, SanuWave, Alpharetta, GA) and the scrotum. According to the protocol, the animals received 500 impulses with the energy density of 0.10 mJ/mm². The device applicator was focused on the right testicle and was moved with circular movements to ensure complete coverage of the cremaster muscle with the ESW (fig. 1).

The surgical procedures were performed under the Zeiss OPMi6 surgical microscope providing 40 x zoom (Carl Zeiss OPMi 6-SD, Carl Zeiss, Goettingen, Germany).

The cremaster muscle was isolated according to the technique developed by Siemionow and colleagues (20-28). The skin was cut above the inguinal ligament in the right iliac fossa. Upon dissecting the right testicle with the spermatic cord, the cremaster muscle was isolated together with its bundle up to the external iliac vessels.

The cremaster muscle was next cut on its anterior wall, the testicle was dissected along with the spermatic cord, which produced a circular muscle flap with visible main vessels. After dissecting the genitofemoral nerve, it was resected at the 1-cm section to minimise the influence of the sympathetic nervous system on microcirculation.

The animal was next placed in a supine position on the tissue batch of Plexiglass. For survival observation, the muscle was stretched...
with the use of silk sutures of 7/0 thickness. The muscle was kept moistened with the Ringer’s solution and covered with isolation foil to prevent drying. The animal body temperature was maintained at 35-37°C with the use of heating lamp (fig. 2).

Survival observation of microcirculation

The rat secured in the “cell bath” of Plexiglass was placed under the microscope (Nikon Optiphoto-2, Japan), fitted with a Doppler optical instrument for measuring the blood cell flow rate (Texas A&M, College Station, TX), 19-inch monitor (Sony Trinitron, Japan) and a colour digital camera (Carl Zeiss Axiocam MR, Carl Zeiss, Goettingen, Germany). The survival microcirculation image was displayed on the monitor and recorded on the computer hard drive (Hewlett Packard HPL1940T and HP xw8400 Workstation, Hewlett Packard, Palo Alto, CA). The final magnification on the monitor was 1800 x (fig. 3).

The measurements of microcirculation haemodynamics of the cremaster muscle were made in each of the groups after spreading the cremaster muscle on the tissue bath and after a 15-minute break necessary for microcirculation stabilisation, depending on the group: after 3 (group 2), 7 (group 3) and 21 days (group 4) from the application of 500 ESWT impulses of energy density of 0.10 mJ/mm².

The following haemodynamic parameters were evaluated:
1. Vessel diameter – with the use of a digital system for measuring the vessel diameter (Carl Zeiss Axiocam MR and Carl Zeiss AxioVision Rel.4.6, Carl Zeiss, Goettingen, Germany) there was measured the diameter of the main (primary) muscle arteriole A1, its secondary (A2) and tertiary (A3) branches, and the muscle main vein (V1).
2. The blood cell flow rate (PPE) was measured in millimetres per second (mm/s) in the same vessels in which the diameter was measured with the use of the optical Doppler Velocimeter device (Texas A&M, College Station, TX).
3. Functional capillary perfusion (CPW) was measured in the proximal, median and distal section of the muscle, around the selected extracapillary veins. In each of the three sites, the numbers of functionally active capillaries was measured in 9 adjacent fields, which gave a total of 27 fields in a given muscle.
4. The observation of interactions between the leukocytes and the extracapillary vein endothelium. The measurements were performed in extracapillary veins which were selected in the proximal, median and distal part of the muscle. In 2 minutes, with the use of a manual counter, the numbers of leukocytes rolling in the vessel lumen were...
counted, in over 20 seconds – the numbers of leukocytes adhering to the vessel wall and the numbers of leukocytes transmigrating across the vascular wall.

Statistical analysis. A statistical analysis of mean values of the following parameters was performed in all the study groups: vessel diameter, RBC flow rate, number of capillaries with functional flow, the counts of rolling leukocytes, adhering to the vessel wall and transmigrating across its wall. In the analysis of variable values of all the study groups and at different time points, ANOVA (analysis of variance) was used. For each of the studied parameters, the response to the causative factor was examined in relation to the study group, measurement time and interactions between the study group and the measurement time. When the p value for interactions within the group was statistically significant, each of the study groups was compared with the control group. The level of statistical significance was set at 0.05, and in each group the comparison with the control group was made with the use of Bonferroni correction. Due to the small number of animals in each of the study groups, the results with the p value lower than 0.05 were deemed as potentially statistically significant and described on charts. All the statistical analyses were performed with the use of SAS (version 9, Cary, NC). The majority of statistical studies were performed during the stay in the United States, due to which the charts contain English explanations, explained in the legends.

RESULTS

Vessel diameter. In group 2, there was observed a drop in the mean diameter of the main vein by 18.5% as compared with the control group (216 µm vs 265 µm) (p<0.05). In the same group there was also observed a drop in the primary arteriole diameter by 22% as compared with the control group (71 µm vs 91 µm) (p < 0.05) and a decrease in the tertiary arteriole diameter by 20% as compared with the control group (44 µm vs 55 µm) (p<0.05) (fig. 4).

In group 3 there were observed no statistically significant changes in the vessel diameter measured during the observation of haemodynamics in the cremaster muscle.

In group 4 there was observed an increase in the secondary arteriole diameter by 12% as compared with the control group (79 µm vs 70 µm) (p<0.05) (fig. 4).

In group 4 there also occurred a drop in the primary vein diameter by 20% as compared with the control group (213 µm vs 265 µm) (p<0.05) (fig. 4).

Fig. 4 Comparison of the mean values of arteriole and vein diameter

(Vein, Diameter, Mean, Median)
The remaining parameters in the groups in which the observation was performed after 3, 7 or 21 days from the application of 500 ESWT impulses were not statistically significant (fig. 4).

The measurement of the RBC flow rate (PPE). In all the groups the mean PPE value in the vein was statistically significantly higher: by 27% in group 2, by 41% in group 3 and by 48% in group 4 as compared with the control group (9 mm/s vs 10.9 mm/s vs 11.4 mm/s vs 7.1 mm/s) (p<0.05) (fig. 5).

In groups 2 and 4, in secondary arterioles, the mean PPE value dropped by 28% and by 10% (7.05 mm/s vs 8.7 mm/s vs 9.8 mm/s) as compared with the control group, and the in the tertiary ones the mean PPE value in groups 2 and 4 dropped by 17.5% and by 19% as compared with the control group (7.12 mm/s vs 7 mm/s vs 8.6 mm/s) (p < 0.05) (fig. 5).

In group 4 there was measured the statistically significant PPE increase in the primary arteriole by 14% as compared with the control group (13.2 mm/s vs 11.6 mm/s) (p<0.05) (fig. 5).

The remaining parameters in the groups in which the observation was performed after 3, 7 or 21 days from the application of 500 ESWT impulses were not statistically significant (fig. 5).

Functional Capillary Perfusion (CPW). During the measurements in all groups there was observed a statistically significant drop in capillary perfusion in the studied muscle, in group 2 the drop was by 26%, in group 3 16%, and in group 4 26% as compared with the control group (8.6 vs 9.7 vs 8.6 vs 11.5) (p<0.05) (fig. 6).

Interactions between leukocytes and the vascular endothelium in extracapillary veins
Leukocytes rolling on the endothelium: As a result of haemodynamic measurements there were found statistically significantly reduction in the counts of leukocytes rolling on the endothelium in group 2 by 41%, in group 3 by 66% (5.43 vs 3.2 vs 9.3) (p<0.05). In group 4, the number of leukocytes rolling on the endothelium also decreased but was not statistically significant (fig. 6).

Leukocytes adhering to the endothelium. During the experiment it was found that the mean count of leukocytes adhering to the endothelium was significantly increased as compared with the control group in group 3 by 42% (4.4 vs 3.1) (p<0.05). In groups 2 and 4 there was not observed a statistically significant increase in the number of leukocytes adhering to the endothelium (fig. 6).

Leukocytes migrating across the vascular wall. In group 2 there was found a 78% drop
Etracorporeal shockwave therapy (ESWT) and microcirculation of denervated muscle flap

Fig. 6. Comparison of the mean values of functional capillary perfusion (CPW) and interactions between leukocytes and the endothelial wall (Capillaries, Rolling Leukocytes, Sticking Leukocytes, Transmigrating Leukocytes)

in the number of eukocytes migrating across the vascular wall as compared with the control group (0.47 vs 2.17) (p<0.05) (fig. 6).

In the remaining experimental groups there were observed no statistically significant differences in the number of leukocytes transmigrating across the vascular wall as compared with the control group (fig. 6).

DISCUSSION

Despite the increasingly wider use of ESW, its influence and effects on microcirculation after a longer period from its application is not known. In the previous experiment, in which there was observed the effect of ESW on the haemodynamics of small vessels of the cremaster muscle immediately and 24 h after its application, it has been demonstrated that ESWT has a beneficial effect on microcirculation in the studied muscle. The effect of its action starts immediately after the application and is maintained for 24 h (15), causing an increase in the blood flow though the muscle and also having anti-inflammatory effect. The aim of the present experiment was the examination of the ESWT action after 3, 7 and 21 days from the wave induction.

The use of the cremaster muscle model in the research studies enabled the performance of the in vivo experiment on a free muscle flap. This model, being for many years the object of research of Prof. Siemionow and her team, is a unique and universal model enabling the observation of changes occurring in microcirculation during the action of drugs, physical substances as well as pathological processes such as anaemia (29-33). The changes occurring in the cremaster muscle translate directly into the changes taking place in striated muscles in humans.

The study results indicated that the changes in the vascular diameter observed in microcirculation are short-term – after 3, 7 or 21 days, there was observed no increase in the vascular diameter, which had taken place in the previous experiment (15). On the contrary, after 3 days from exposure to ESWT there occurred a drop in their diameter, which was not maintained 7 days after ESWT application.

The same trend was observed during the evaluation of the RBC flow rate in arteries – after 3 and 21 days there was observed a drop in their flow, while after 7 days from the exposure to ESW, PPE did not change. This confirmed the speculations that the beneficial
effects caused by ESWT are short-term. The blood cell flow rate in the veins increased in all experimental groups. This could stem from an increase in the volume of the cremaster muscle and thus its contractibility, which may facilitate the venous return. It seems to be a result of the increase in the animal body weight between the ESWT application and the measurements performed after 3, 7 and 21 days, as a result of which the blood cell flow changes and the thickness of the studied muscle increases. The rats that are fed regularly gain on weight in the range of 5-7 g per day. In addition, as a result of ESWT application, there occurs a temporary stimulation of muscle vascularisation, which has been observed in the previous experiment, which may lead to its hypertrophy (15). The result of those changes is also the observed decrease in the number of active capillaries in the microcirculation during the haemodynamic measurements.

A positive and long-term result of action of the ESWT is its anti-inflammatory action. This is confirmed by the change in the balance in the leukocyte-endothelium system. In all groups, there was observed a persisting drop in the number of leukocytes rolling on the endothelium. These are leukocytes directly involved in the inflammatory response and the drop in their number indicates the inhibition of inflammation (18). During the experiment, there was no possibility of evaluating whether the same leukocytes were in contact with the endothelial surface, particularly since the mean survival of neutrophils is slightly over 5 days. The study results confirmed that the beneficial effect of ESWT may be of use in clinical situations in patients treated surgically due to non-healing wounds, ulcers or extensive burns. In such situations, in order to obtain the optimal therapeutic effect, it should be used cyclically.

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Adress correspondence: 60-355 Poznań, ul. Przybyszewskiego 49