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Substance P and Prostaglandin E_2 Release After Shock Wave Application to the Rabbit Femur

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The biologic action of extracorporeal shock wave application on the musculoskeletal system is poorly understood. To prove the hypothesis that alterations of tissue concentrations of substance P and prostaglandin E_2 are involved in the biologic action of shock waves, extracorporeal shock waves with energy flux density of 0.9 mJ/mm2 (1500 pulses at 1/second) were applied in vivo to the distal femur of rabbits. The concentrations of substance P and prostaglandin E_2 eluted from the periosteum of the femur were measured. Compared with the untreated contralateral hindlimbs, substance P release from the periosteum from the femur was increased 6 hours and 24 hours after extracorporeal shock wave application, but was decreased 6 weeks after extracorporeal shock wave application. By contrast, extracorporeal shock wave application did not result in altered prostaglandin E_2 release from the periosteum from the femur. Remarkably, there was a close relationship between the time course of substance P release found here, and the well-known clinical time course of initial pain occurrence and subsequent pain relief after extracorporeal shock wave application to tendon diseases. Accordingly, substance P might be involved in the biologic action of extracorporeal shock wave application on tissue of the musculoskeletal system. This is the first study providing insights into the molecular mechanisms of extracorporeal shock wave application to the musculoskeletal system.

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List of abbreviations used

PG	prostaglandin
PGE_2	prostaglandin E_2

Extracorporeal shock wave application is currently the standard therapy for lithotripsy of re-

nal stones.[7] Extracorporeal shock waves are mechanical pressure pulses resulting in peak pressures of as much as 100 mPa lasting for nanoseconds.[20] Extracorporeal shock wave application has been extended to the treatment of various diseases of the musculoskeletal system, such as calcifying tendinitis of the shoulder,[19,22,34] radial humeral epicondylitis,[16,28] and plantar fasciitis.[23,29] Patients experiencing these tendon diseases usually report local pain during and shortly after extracorporeal shock wave application.[19,21] Pain relief has been reported to occur between several months[19] and several years[23] after extracorporeal shock wave application, depending on the study design.

A weak correlation on radiographs has been found between pain relief and the disappearance of the calcified deposits on radiographs after extracorporeal shock wave therapy for calcifying tendinitis of the shoulder.[19,34] This indicates that mechanical disintegration of calcified deposits cannot be the exclusive mechanism explaining the clinically successful treatment of calcifying tendinitis of the shoulder with extracorporeal shock wave application. In general, the biologic mechanisms of extracorporeal shock waves on tissue of the musculoskeletal system are poorly understood. A frequently cited hypothesis is Melzack's concept of hyperstimulation analgesia.[25,29,30] According to this concept, a moderate sensory stimulus can relieve pain when it is administered directly to the site, and a relatively short stimulus (seconds to minutes) may relieve chronic recalcitrant pain for a long period.[25]

In this regard it is of interest that shock wave application directly to nerves in vitro has been shown to result in induction of action potentials.[33] If this also applies to unmyelinated primary afferent fibers, extracorporeal shock wave application might result in release of substance P. Substance P is a polypeptide of the tachykinin family formed by 11 amino acids.[6] In the peripheral nervous system, substance P is concentrated in C-fibers and a subpopulation of slowly conducting A-δ nerve fibers,[12,14] which are found in skin, mucous membranes, viscera, and vessels throughout the body.[5] In the central nervous system, substance P exerts complex synaptic effects, presynaptic, and postsynaptic, and usually acts as an activating or facilitating factor.[5] Substance P is released at central and peripheral terminals of sensory nociceptive neurons after stimulation.[24] There are several known peripheral actions of substance P such as induction of neurogenic inflammation,[18] extravasation of plasma,[40] or stimulation of the proliferation of various cell types (osteoblasts).[11] Furthermore, it was shown that extracorporeal shock wave application to the musculoskeletal system can result in tissue damage and inflammation.[8,30] Therefore, extracorporeal shock wave application to the musculoskeletal system also might result in the release of inflammatory mediators such as PGs. Prostaglandins are a complex group of oxygenated fatty acids that have been detected in virtually all mammalian tissues.[17] It is known that PGE_2 concentrations in tissue are increased in numerous inflammatory conditions.[10] In addition, PGE_2 is involved in the modulation of inflammation.[17]

Therefore, the authors hypothesize that extracorporeal shock wave application to the musculoskeletal system might result in altered release of substance P and PGE_2 from the treated tissue. Because this cannot be tested in humans without surgical intervention, the authors decided to prove this hypothesis in an animal model. High-energy shock waves with energy flux density of 0.9 mJ/mm^2 were applied in vivo to the distal femur of rabbits. The release of substance P and PGE_2 from preparations primarily of the periosteum covering the femur surface then was measured using established enzyme immunoassays.[9,15,32]

MATERIALS AND METHODS

Experiments were done on 20 female mature rabbits (race, Chinchilla bastard), approximately 1 year old with a body weight between 4 and 5 kg. The animals were obtained from Charles River Inc, Kissleg, Germany. During the treatment period, animals were maintained on a daily cycle of 12 hours of light alternating with 12 hours of darkness, at 21° C room temperature, and with ad libitum access to standard

food pellets and water. All research and animal care procedures were approved by the local district government animal use study committee.

With respect to extracorporeal shock wave application and survival time, animals were assigned randomly to three groups. Group A comprised four animals, whereas Groups B and C comprised eight animals each. For each animal, either the left or the right hindlimb was selected randomly for extracorporeal shock wave application, with the untreated hindlimb as an internal control. Extracorporeal shock wave application was done with the rabbits under deep intravenous anesthesia. Anesthesia was initialized by intravenous injection of a combination of xylazine hydrochloride (1.5 mg/kg body weight) and ketamine (6 mg/kg body weight), and was maintained by intravenously administering 2.4 mg/kg body weight per hour xylazine hydrochloride and 10 mg/kg body weight per hour ketamine by means of a perfusion pump. During anesthesia, animals were supplied with oxygen by mask. Administration of intravenous drugs was discontinued immediately after shock wave application. After shaving both hindlimbs, animals were positioned for extracorporeal shock wave application as described in detail previously.[8] Application of the extracorporeal shock waves to the distal femur of the selected hindlimb was done once using an electrohydraulic shock wave source (XL1, Dornier MedTech, Wessling, Germany). The shock wave device was coupled to the selected distal femur by means of a water bath. Shock wave focusing to the distal femur was controlled by two laser pointers adjusted in two planes.[8] The shock waves were applied as 1500 shock wave pulses of 1 Hz frequency for 25 minutes. Energy flux density was 0.9 mJ/mm^2 and was measured by a polyvinyl-fluoride hydrophone before the application of extracorporeal shock waves.[20]

Either 6 hours (Group A), 24 hours (Group B), or 6 weeks (Group C) after extracorporeal shock wave application, the animals were sacrificed. Immediately thereafter, both femurs were disarticulated at the hips and knees and all soft tissues adherent either to the periosteum or to the bone were removed carefully by a sharp scalpel. Great care was taken to avoid any periosteal or bone damage during this preparation. For measuring release of substance P and PGE_2, each prepared femur was passed successively through a series of five custom-made glass eluation tubes containing synthetic interstitial fluid as eluation medium (saline solution treated with a gas mixture made of 95% O_2 and 5% CO_2; pH 7.4; 37° C).[3] Eluation tubes were placed in a water bath at 37° C and were shaken continuously. The first eluation lasted for 30 minutes to achieve equilibration, whereas the subsequent eluations lasted for 5 minutes each. The experiment was completed by placing each prepared femur for 5 minutes in a sixth glass eluation tube containing the inflammatory mediators bradykinin, serotonin, and histamine (all 10^{-5} mol/L) in synthetic interstitial fluid. To place the specimens in the eluation tubes, it was necessary to cut off the distal half of the major trochanter. Appropriate placement of the specimens in the eluation media prevented contamination by substances released from the femur bone marrow such as substance P, which is known to be contained in the bone marrow.[35]

Substance P and PGE_2 release were determined in the successive eluation media (six measurements per specimen) using enzyme immunoassays as described previously.[9,15,32] Substance P assay kits were purchased from Cayman Chemical Company (Ann Arbor, MI). Prostaglandin E_2 antibody was provided by Dr. K. Brune (Department of Pharmacology, University of Erlangen, Germany).[4] Each eluation medium was divided into two equal portions and mixed with commercial enzyme immunoassay buffer (fivefold concentrated, ratio eluation medium : buffer 5:1) immediately after eluation. One of each of these portions was used for measuring substance P content immediately at the conclusion of the experiment. The other portion was deep-frozen at −20° C, and was used for measuring PGE_2 on the following day. Enzyme immunoassay plates were evaluated photometrically using a microplate reader (Dynatech, Guernsey, Channel Islands, United Kingdom). Because of simultaneous extracorporeal shock wave treatment of all animals, each group was evaluated by a separate charge of the enzyme immunoassay kits used. Accordingly, the absolute data could not be compared among the groups.

Statistical analysis was done by calculating the mean and standard deviation of the values of substance P concentration and PGE_2 concentration for each group of animals and eluation medium. Effects of the application of extracorporeal shock waves on substance P concentration and PGE_2 concentration in the eluation medium were analyzed by two-way repeated measures analysis of variance (ANOVA). The results represent matched values with respect to successive eluations of the same specimen (first matching), and with respect to treated versus untreated specimens of the same animal (second

matching). However, two-way repeated measures ANOVA only considers one type of matching. In consequence, statistical analysis was done twice, considering the first matching during the first run, and the second matching during the second run. In either run, statistical significance was established at $p < 0.05$. Calculations were done using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA).

RESULTS

Figure 1 shows the results for the substance P concentration in the eluation medium as a function of the number of the successive eluation tubes. Figure 2 shows the corresponding data for PGE_2. In Table 1, the results of the statistical analysis of these data are summarized.

Considering the first matching described above (matching with respect to successive eluations of the same specimen), for all groups of animals a statistically significant difference was found for substance P concentration in the eluation medium between the treated and the untreated specimens. Furthermore, this variable showed a statistically significant difference between the successive eluations, on the treated and the untreated femurs for all groups. By contrast, a statistically significant difference was not found for the PGE_2 concentration in the eluation medium between the treated and the untreated specimens in any group. Except for Group A, this variable showed a statistically significant difference between the successive eluations, on the treated and the untreated femurs (upper part of Table 1).

Considering the second matching described above (matching with respect to treated versus untreated specimens of the same animal), for all groups of animals a statistically significant difference was found for substance P concentration in the eluation medium between the treated and the untreated specimens. Further-

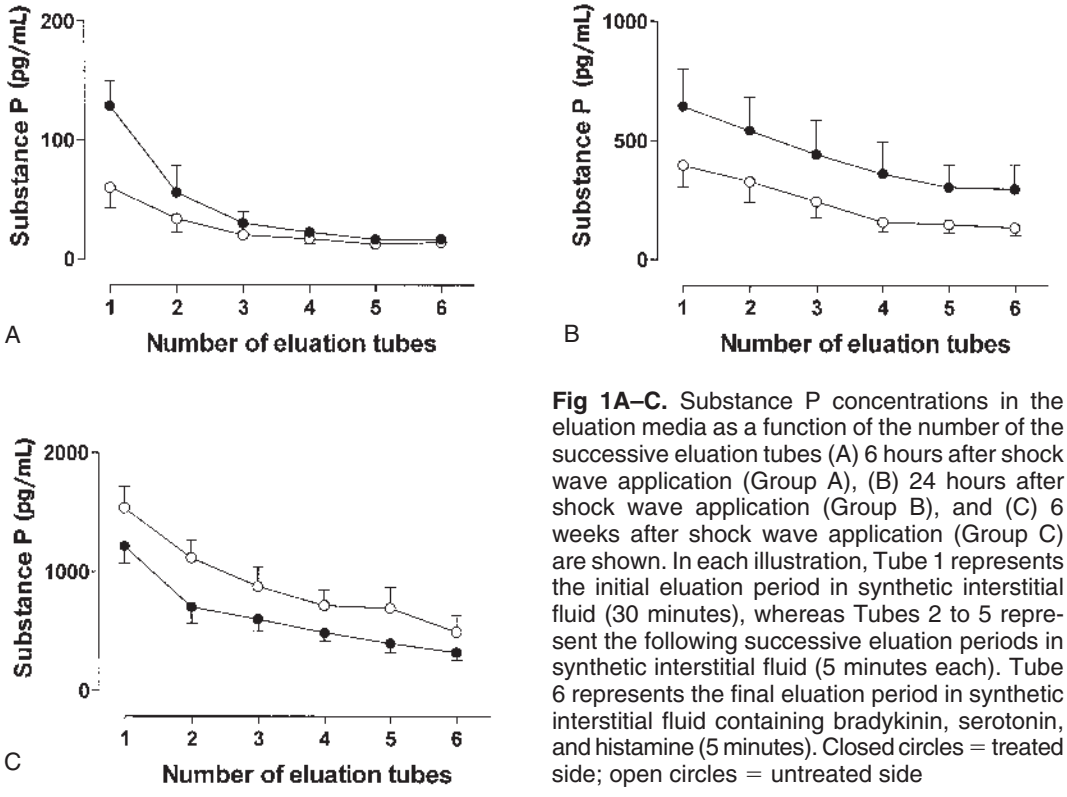

Fig 1A–C. Substance P concentrations in the eluation media as a function of the number of the successive eluation tubes (A) 6 hours after shock wave application (Group A), (B) 24 hours after shock wave application (Group B), and (C) 6 weeks after shock wave application (Group C) are shown. In each illustration, Tube 1 represents the initial eluation period in synthetic interstitial fluid (30 minutes), whereas Tubes 2 to 5 represent the following successive eluation periods in synthetic interstitial fluid (5 minutes each). Tube 6 represents the final eluation period in synthetic interstitial fluid containing bradykinin, serotonin, and histamine (5 minutes). Closed circles = treated side; open circles = untreated side

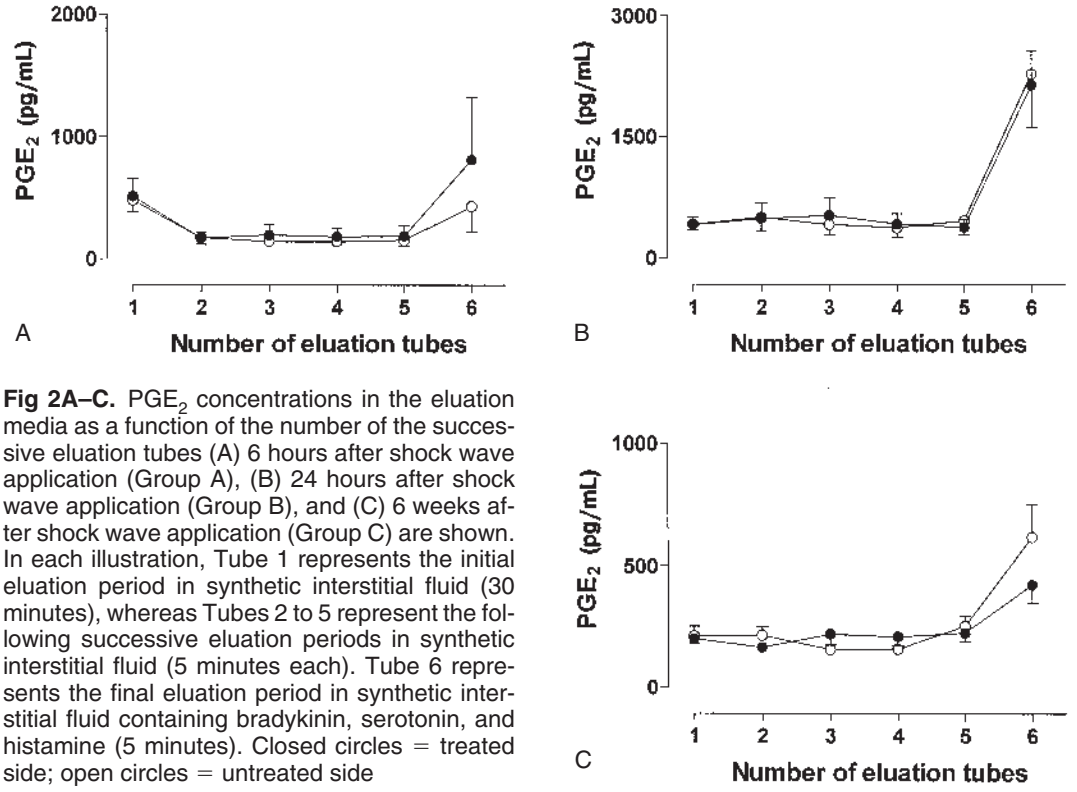

Fig 2A–C. PGE_2 concentrations in the eluation media as a function of the number of the successive eluation tubes (A) 6 hours after shock wave application (Group A), (B) 24 hours after shock wave application (Group B), and (C) 6 weeks after shock wave application (Group C) are shown. In each illustration, Tube 1 represents the initial eluation period in synthetic interstitial fluid (30 minutes), whereas Tubes 2 to 5 represent the following successive eluation periods in synthetic interstitial fluid (5 minutes each). Tube 6 represents the final eluation period in synthetic interstitial fluid containing bradykinin, serotonin, and histamine (5 minutes). Closed circles = treated side; open circles = untreated side

TABLE 1. Statistical Analysis of the Substances P and PGE_2 Data

		Shock Wave Effects ESWA Versus NonESWA		Eluation Effects Successive Eluations	
Group	**Measures**	*F*	*p*	*F*	*p*
Matching done with respect to successive eluations of the same specimen					
A	Substance P	20.6	< 0.001	9.66	< 0.001
B	Substance P	5.98	0.019	17.0	< 0.001
C	Substance P	11.5	0.002	17.0	< 0.001
A	PGE_2	1.39	0.254	1.94	0.137
B	PGE_2	0.01	0.930	14.0	< 0.001
C	PGE_2	0.79	0.379	12.9	< 0.001
Matching done with respect to treated versus untreated specimens of the same animal					
A	Substance P	17.2	< 0.001	10.2	< 0.001
B	Substance P	32.7	< 0.001	1.73	0.150
C	Substance P	24.9	< 0.001	9.34	< 0.001
A	PGE_2	2.13	0.162	1.71	0.184
B	PGE_2	0.04	0.846	8.39	< 0.001
C	PGE_2	1.69	0.200	8.03	< 0.001

The table shows the test values (F values) and the significance levels (p values) of the effects of extracorporeal shock wave application and successive eluations of the same specimen on differences of either substance P and PGE_2 concentration in the eluation medium in ANOVA models with repeated measure design (data are shown in Fig 1 and Fig 2). ESWA = extracorporeal shock wave application

more, except for Group B this variable showed a statistically significant difference between the successive eluations, on the treated and the untreated femurs. By contrast, a statistically significant difference was not found for the PGE_2 concentration in the eluation medium between the treated and the untreated specimens in any group. Except for Group A, this variable showed a statistically significant difference between the successive eluations, on the treated and the untreated femurs (lower part of Table 1).

Accordingly, the results of the current study can be summarized as follows: (1) extracorporeal shock wave application resulted in an increase of substance P release from the treated femur specimens 6 hours and 24 hours after shock wave application when compared with the specimens of the corresponding untreated hindlimbs. By contrast, extracorporeal shock wave application resulted in a decrease of substance P release from the treated femur specimens 6 weeks after shock wave application; (2) the more eluation steps that were done, the less substance P concentration was obtained in the respective eluation tubes; (3) extracorporeal shock wave application did not result in altered PGE_2 release from the treated femur specimens either 6 hours, 24 hours, or 6 weeks after shock wave application; (4) successive eluation steps did not influence PGE_2 concentrations in the respective eluation tubes. However, eluation with synthetic interstitial fluid containing bradykinin, serotonin, and histamine led to higher PGE_2 concentrations in the eluation medium than eluation with synthetic interstitial fluid only.

DISCUSSION

The sensitivity and specificity of the enzyme immunoassays used in the current study have been described.[1] The interindividual variability of the results found in the current study was comparable with the interindividual variabilities of data obtained by similar enzyme immunoassay analyses reported in the literature.[1,9,15] The current authors observed a decrease of substance P concentration in the eluation medium with ongoing eluation time. This might indicate eluation of interstitial substance P without additional liberation from nerve endings during eluation. By contrast, at the end of the experiments the authors found constant levels of PGE_2 concentration in the eluation medium with ongoing eluation time, and an increase of PGE_2 concentration after treatment with inflammatory mediators. This probably reflected continuous liberation of PGE_2 from cellular membranes.

From basic experimental studies it is known that the effects of extracorporeal shock wave application will appear at the interfaces of tissues with different acoustic impedances.[8] The various kinds of soft tissues have comparable low acoustic impedances, whereas the acoustic density of bone has been found to be five times higher.[39] It therefore is reasonable to expect the effects of extracorporeal shock wave application to the distal femur in the rabbit to be greatest on the periosteum covering the cortical bone surface. Periosteum is known to contain substance P immunoreactive nerve fibers,[2] which also were found in bone marrow, synovial membrane, and soft tissues adjacent to bone.[2] However, the special handling of the specimens investigated in the current study as outlined above guaranteed that substance P release was almost exclusively from the periosteum.

It is not known whether the same temporal pattern of alterations of substance P release observed in the distal rabbit femur in the current study also occurs after extracorporeal shock wave application in anatomic sites in humans afflicted by the typical pain of the aforementioned tendon diseases. However, an initial increase and subsequent long-lasting decrease of substance P release from the site of treatment might help to explain the initial local pain during and shortly after extracorporeal shock wave application to tendon diseases and the subsequent, long-lasting pain relief. This can be deduced from a recent study regarding the potential biologic mechanisms of the substance capsaicin, for which a powerful analgesic effect is known.[38] Capsaicin (the irritant in red hot chili peppers) belongs to the family

of vanilloids. The substance has a direct and selective stimulatory action on most polymodal C-fibers and probably A-δ fibers, and causes the release of substance P by depolarization.[13] After excitation, capsaicin results in the depletion of substance P in the nerve fibers. This eventually is followed by a long-lasting degeneration of nerve fibers. In humans, capsaicin can be applied directly on the skin. Because capsaicin primarily causes the release of substance P, topically applied capsaicin initially causes a state of hyperalgesia. After prolonged administration of capsaicin, depletion of substance P occurs. Capsaicin then produces its antinociceptive effect.[26]

Furthermore, it is important to consider that substance P may mediate neurogenic inflammation.[18] Signs of inflammation within the paratenon of the Achilles tendon in rabbits have been described as early as 1 day after high-energy extracorporeal shock wave application.[30] Therefore, the current authors hypothesized there was an altered concentration of PGE_2 in the femurs of rabbits after extracorporeal shock wave application. However, PGE_2 concentration in the eluation medium was unchanged 6 hours, 24 hours, and 6 weeks after extracorporeal shock wave application. Assuming that there was no increase of PGE_2 concentration in the periosteum between 24 hours and 6 weeks after the application of extracorporeal shock waves, this indicates that there only was very mild inflammation in the periosteum. However, this must be examined in additional studies.

Intradermal injection of capsaicin in humans produces degeneration and subsequent reinnervation of epidermal nerve fibers.[36] Therefore, an initial increase and subsequent long-lasting decrease of substance P concentration in the respective anatomic sites after extracorporeal shock wave application also could be attributable to damage to nerves innervating the region that was treated. In this regard, it is important to consider that high-energy extracorporeal shock wave application has been shown to result in vacuolic swelling of axons[27] and other alterations of nerve fibers.[37] Furthermore, transection of the mental nerve of rats recently was shown to result in a week-long disappearance of substance P fibers in the skin of the lower lip.[31] Obviously, this also would result in initial local pain followed by subsequent, long-lasting pain relief. As shown in Figure 1, there was a convergence of the curves describing the extent of substance P release from treated and untreated femurs 6 hours after the application of extracorporeal shock waves. This could not be observed 24 hours or 6 weeks after extracorporeal shock wave application. Based on these findings one might hypothesize that 6 hours after extracorporeal shock wave application there was an increased interstitial concentration of substance P attributable to the excitatory action of the application of extracorporeal shock waves, without increased basal secretion of substance P. Furthermore, 24 hours after extracorporeal shock wave application there was an increased basal secretion of substance P attributable to a slight (neurogenic) inflammation and an increased sensitivity of the surviving nerve endings. Finally, 6 weeks after extracorporeal shock wave application there was a decreased basal secretion of substance P attributable to degeneration of a part of the nerve endings. However, detailed knowledge about possible damage to nerves caused by the application of extracorporeal shock waves to the musculoskeletal system still has to be established. This currently is being investigated in the authors' laboratories.

The authors found an initial increase and subsequent decrease of substance P concentration in the periosteum covering the cortical femur surface after high-energy extracorporeal shock wave application to the distal femur in the rabbit. Assuming that the same temporal pattern of alterations of substance P concentration also occurs after extracorporeal shock wave application in those anatomic sites in humans that are afflicted by the typical pain of tendon diseases, the results presented in the current study might help to explain the initial local pain during and shortly after extracorporeal shock wave application for tendon diseases and the subsequent, long-lasting pain relief. In this regard, the findings of the current study may be

seen as the first insight into the molecular mechanisms of extracorporeal shock wave application to the musculoskeletal system.

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