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● *Original Contribution*

EFFECT OF SHOCK WAVE TREATMENT ON PLATELET-RICH PLASMA ADDED TO OSTEOBLAST CULTURES

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Abstract—The aim of this study was to verify the effects on osteoblast cultures of adding a platelet-rich plasma (PRP) concentrate pretreated with 500 shock wave (SW) at an energy flow density of 0.17 mJ/mm^2, emitted by an electromagnetic generator Minilith SL1 (STORZ, Germany), reproducing the conditions of our previous study in which we apply SW directly on osteoblasts. Real-time PCR showed that in osteoblast cultures with added PRP pretreated with SW, there was an increased expression at 48 h of insulin-like growth factor binding protein 3 (IGFBP-3) and runt-related transcription factor 2 (RUNX2) and at 72 h, of collagen type I, osteocalcin, insulin-like growth factor 1 (IGF-1) as well as IGFBP-3. Western blotting confirmed the increased protein synthesis of IGFBP-3. This experience suggests that extracorporeal shock wave treatment (ESWT) should stimulate osteogenesis also by indirect platelets-mediated network. It therefore seems possible that combining the two methods, ESWT and bioengineering procedures to infiltrate PRP and growth factors, could be a successful approach. (E-mail: angelanotarnicola@yahoo.it) © 2011 World Federation for Ultrasound in Medicine & Biology.

Key Words: **Shockwaves, Platelet-rich plasma, Osteogenesis.**

INTRODUCTION

The insulin-like growth factor (IGF) and insulin-like growth factor binding protein (IGFBP) system is an important regulator of bone homeostasis throughout life (Ohlsson et al. 1998). Insulin-like growth factor 1 (IGF-1) and insulin-like growth factor 2 (IGF-2) are the most abundant growth factors secreted by skeletal cells and are considered autocrine regulators of osteoblast cell function (Canalis et al. 1989; Frolik et al. 1988; Mohan et al. 1988). IGF-1 may be important during early osteoblast differentiation as well as very late on, during the mineralization phase. IGF-1 can modulate osteoblast differentiation *via* runt-related transcription factor 2 (RUNX2) (Niu and Rosen 2005), which is a well-known crucial transcription factor for osteoblast development, because it regulates collagen type I and osteocalcin (OC) gene transcription (Komori 2002; Wada et al. 2006).

Another member of the IGF regulatory system is a family of IGFBPs that can regulate the biological activities of IGF. IGFBPs are capable of IGF-dependent and IGF independent actions (Lee et al. 1988; Binkert et al. 1989; Wood et al. 1988; LaTour et al. 1990; Kiefer et al. 1991; Shimasaki et al. 1991). IGFBPs act as shuttle proteins for the IGF to increase their half-life. Moreover, the IGFBPs play a role in delivering IGF to the target tissues, where they are capable of modulating IGF biological responses (Yakar et al. 1999). IGFBP-3 and IGFBP-5 have also been shown to modulate cell growth independently of the IGF (Miyakoshi et al. 2001; Hong et al. 2002), and are a major component of the circulating IGF complex (Jones and Clemmons 1995; Gazzerro and Canalis 2006). *In vitro*, IGFBP-3 can inhibit or stimulate IGF activity, the latter by upregulating IGF-1 delivery to cell surface receptors (Longobardi et al. 2003). IGFBP-4 and IGFBP-5 are potent inhibitor of osteoblast differentiation, bone growth and mineralization through their

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ability to neutralize IGF-1 action by high-affinity growth factor binding and presumptive sequestration away from the IGF receptor (Richman et al. 1999; Mukherjee and Rotwein 2008).

Collagen type I is the most abundant protein of the bone extracellular matrix and its expression is an important differentiation marker of osteoblasts (Kern et al. 2001).

Osteocalcin is a vitamin K–dependent noncollagenous bone matrix protein. It is synthesized by osteoblasts and is a well-known marker of viability, differentiation and osteogenic ability in these cells (Camarda et al. 1987). The tissue-specific expression of OC and its transcriptional control are mainly regulated by runt-related transcription factor 2 (RUNX2) (Ducy and Karsenty 1995; Ducy et al. 1997) and can be modified by growth factors (Javed et al. 2000; Alliston et al. 2001; Jeon and Sayre 2003).

During extracorporeal shock wave treatment (ESWT) *in vivo*, the various cell lines present in the focal field undergo stimulation and their reciprocal interactions are thought to simultaneously induce the final therapeutic effect. Up to now, experimental studies have been focused on the effects of ESWT on single cell lines including the osteoblasts (Hausdorf et al. 2010; Murata et al. 2007; Sugioka et al. 2010). The osteoblasts are mononucleate cells that are responsible for bone formation. They are sophisticated fibroblasts that express all genes that fibroblasts express, with the addition of the genes for bone sialoprotein and OC (Hakki et al. 2010). It has been found that ESWT directly stimulated the proliferation and differentiation of osteoblasts and this led to a stimulation of osteogenesis process (Tamma et al. 2009). Only recently have some authors began to study the network of interactions among cells after shock wave (SW) treatment, demonstrating a chemotactic effect of the endothelial cells on the monocytes-macrophages and stem cells, inducing an antiinflammatory and neoangiogenic action (Aicher et al. 2006; Mariotto et al. 2005). Moreover, in a previous work, we had shown that SW stimulation of osteoblasts can reduce the receptor activator NF kappa B ligand and osteoprotegerin ratio (RANKL/OPG), causing an anti-osteoclastogenic effect (Tamma et al. 2009). SW is also able to cause bone healing, inducing growth factor synthesis (Hausdorf et al. 2010; Chen et al. 2004).To better clarify the effect of ESWT in bone, we studied indirectly the effect of ESWT on platelets by their effect on osteoblast culture.

There is currently strong clinical interest in ascertaining how SWs modulate the platelets, because the release of growth factors has important effects on the surrounding cells. In fact, in recent years, bioengineering has applied the growth factors as vascular endothelial growth factor (VEGF), FGF-2, PDGF and IGF-1, produced by the platelets in association with other treatments such as surgery to strengthen the effects and improve the tissue reparative response (Smith and Roukis 2009). However, the effects associated with SW therapy have not yet been explored.

Firstly, the effects of ESW on the platelets need to be considered; in fact, to our knowledge no previous work has examined these effects. This is probably a result of the difficulty in studying cells lacking a nucleus and with limited survival (Harrison and Cramer 1993).

We set up an *in vitro* study to assess the effects of ESW on platelet-rich plasma (PRP) concentrate added to osteoblast cultures. The end-point of the study was to observe whether the SW could induce the platelets to activate the osteoblasts, so as to assess not only the possible clinical effects *in vivo*, where osteoblasts and platelets are present together, but also to provide a rationale for the association of SW therapy and the administration of growth factors.

In this study, we reproduced the same protocol applied in our last paper, in which we verified SW effects on osteoblasts (Tamma et al. 2009). Preventively, for that previous study, we tested several different ESW energy levels and dosages and we found that a higher number of impulses did not change the results, whereas higher energy levels caused necrotic effects. We monitored osteoblasts until 96 h after SW and we found the SW effects were already evident at 24 h. In this work we did not extend the experiment time over 72 h because our previous experience demonstrated that SW induced precocious effects on cells in the early hours. In that study, we demonstrated a direct, early upregulating effect of ESWT on RUNX2. Moreover, the promoting action of IGF-1 on RUNX2 expression has been described in the literature; this prompted us to study the effect of ESW pretreated PRP on the expression of RUNX2 in osteoblasts. We considered that the proliferation and differentiation of osteoblasts is induced by the insulin-like growth factor binding protein-3 (IGFBP-3) and inhibited by IGF-4 and IGFBP-5 (Ohlsson et al. 1998). We also analyzed index of differentiation for osteoblast activity as the bone matrix proteins collagen type I and OC (Niu and Rosen 2005).

MATERIALS AND METHODS

The study was approved by the local university's Ethics Committee and by the local institutional Animal Care and Use Committee.

Murine calvaria osteoblasts (OBs)

In 5–6-day-old mice (c57bl/6j), the frontal and parietal bones were removed in sterile conditions and the periosteum was detached using scissors. The calvaria

fragments were digested in 0.5 mg/ml *Clostridium* histolyticum neutral collagenase (Sigma Chemical Co., St. Louis, MO, USA) in phosphate-buffered saline (PBS) at 37°C for 60 min. After digestion, calvaria fragments were washed vigorously three times with α-minimal essential medium (α-MEM), then transferred to a 12.5-cm^2 cell culture flask and cultured in α-MEM supplemented with 10% fetal bovine serum (FBS, Gibco, Uxbridge, UK), 100 IU/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL amphotericin B and 50 IU/mL Mycostatin (Gibco, Uxbridge, UK) at 37°C, in a water-saturated atmosphere containing 5% CO_2. The medium was changed every three days. Under these conditions the osteoblasts in the fragments proliferated and migrated to the culture surface, reaching confluence within two weeks. Cells were then trypsinized and transferred to appropriate dishes for characterization and experiments.

Osteoblast characterization

Murine osteoblasts were characterized according to the well-established parameters of alkaline phosphatase activity, the production of cAMP in response to parathyroid hormone (PTH)10^{-8} M (Sigma Chemical Co.) and synthesis of OC in response to 1,25-dihydroxyvitamin D3 10^{-8} M (Sigma Chemical Co.).

PRP concentrate

The PRP was prepared at the Immunohematology Department of Bari University Hospital by apheresis of venous blood from three healthy volunteers (aged 25 to 35 y) who gave informed consent to participate in this study. We chose human blood because it would have been difficult to collect the same amount of PRP from rodents. The platelets count was 250,000/μL (range of normal concentration 150,000 to 450,000/μL) and after concentration the count became 1420,000/mL. The PRP was diluted in α-MEM to obtain a normal concentration/mL and aliquoted in 1.8-mL test tubes. Tubes containing the PRP were subjected to SWT produced by an electromagnetic generator SL1 (STORZ, German) at an energy flow density of 0.17 mJ/mm^2 for 500 pulses. A common ultrasound gel was used as a contact medium between the tube containing the PRP and the cylinder. The environment conditions were a temperature of 25°C and an air pressure of 101 kPa. Other tubes of PRP did not undergo any treatment. Moreover α-MEM–containing tubes were subjected to ESWT and used as control. Subsequently, the same volume of untreated PRP, the ESW pretreated PRP and the ESW-treated α-MEM were added to the osteoblast cultures extracted and purified from the calvaria of newborn mice. Comparison was made between osteoblast cultures added with ESW-pretreated PRP and the culture added with the untreated PRP *versus* control represented from ESW-treated α-MEM culture.

The OB activity was assessed by real-time polymerase chain reaction (PCR) and Western blotting, measuring both the genes expression and protein synthesis involved in the differentiation process of the pre-osteoblasts and mature osteoblast activities. In literature it is supported that collagen type I and (RUNX2) have important roles on bone metabolism and the modulation of different mechanical and biological effects have been studied by PCR (Hakki et al. 2010; Zhu et al. 2009).

We analyzed IGF-1 and IGFBP-3 by PCR, because IGF-1 is the principal IGF involved in bone growth and IGFBP-3 is the primary IGFBP that modulates the bioactivity of IGFs in bone tissue (Ohlsson et al. 1998) Therefore, we supposed that they should show a genetic modulation after SW. For the other IGFBPs, we consider that they received an important regulation at level of protein synthesis by IGFs (Lemmey et al. 1997) and we analyzed them by Western blot.

RNA extraction and reverse transcriptase reaction

Osteoblast culture flasks were lysed and stored at –80°C at 24, 48 and 72 h after their stimulation. After that RNA extraction was completed using spin columns (RNAeasy Mini Kit, Qiagen, Hilden, Germany) according to the manufacturer's instructions.

RNA (1 μg) was reverse transcribed to complementary DNA (cDNA) with the AccuScript High Fidelity 1st Strand cDNA Synthesis Kit (Stratagene, La Jolla, CA, USA). Ten microliters of initial mix (1 μg RNA, 1 mM dNTPs, 50 pmol Oligo[dT], DEPC H_2O) was incubated at 65°C for 5 min and in ice for 1 min, and then 10X RT buffer, 25 mM $MgCl_2$, 0.1 M DTT and 1 μL (40 U) of RNaseOUT were added. After 2 min of incubation at 42°C, 1 μL (50 U) of SuperScript II RT was added and the incubation at 42°C was resumed for 50 min and then at 70°C for 15 min. Rnase-H 1 μL (2 U) was added and a further 20 min of incubation at 37°C was done to complete the reaction.

The samples were stored at –80°C until real-time PCR was done.

Real-time PCR

cDNA was amplified with the iTaq SYBR Green supermix with the ROX kit (Bio-Rad Laboratories, Hercules, CA, USA) and the PCR amplification was performed using the Chromo4 Real-Time PCR Detection System (Bio-Rad Laboratories).

The –80°C stored cDNA samples, obtained from the lyses of the osteoblast flasks at 24, 48 and 72 h from their stimulation, were analyzed by real time PCR the day after the reverse transcriptase process.

By real-time PCR, the expression of mRNA for IGF-1, IGFBP-3, RUNX2, OC and type I collagen was evaluated, as well as glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as housekeeping gene. The primers sequences, all at 60°C annealing temperature (Operon Biotechnologies GmbH, Cologne, Germany), are reported in Table 1.

The amplification process included three steps:

(i) Incubation at 95°C for 3 min;
(ii) PCR cycling (40 cycles):
  (iia) incubation at 95°C for 15 s,
  (iib) annealing and extension at 60°C for 30 s (collect and analyze data); and
(iii) after the last cycle, melting curves (Tm) analysis was performed in the 55°–95°C interval by 0.5°C increments in the temperature.

The fold change values were calculated with the Pfaffl method (Pfaffl 2001).

Preparation of cellular extracts

No sign of cellular suffering was revealed on optical microscope. Mice calvaria osteoblasts were lysed with radioimmunoprecipitation assay ice-cold buffer (20 mM TrisHCl, pH 7.4, 150 mM NaCl, 5mM EDTA, 1 % Nonidet-P40 (NP40), 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin and 8 μg/mL leupeptin) added with 1 mM sodium orthovanadate, for 10 min. Then extracts were centrifuged at 14,000 revolutions per minute (rpm) for 15 min at 4°C to separate the nucleus, while the supernatant was harvested for protein dosage. Protein extract concentrations were determined by the BCA Protein Assay Reagent Kit (Pierce Biotechnology, Inc. Rockford, IL, USA).

Western blot

About 30 μg of cell proteins were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) to determine variations in the expression of IGFBP-3 and IGFBP-5. Subsequently, proteins were transferred to nitrocellulose membranes (Hybond, Amersham Pharmacia, London, UK). The blots were blocked by incubation in 5% milk with Tris-buffered saline and tween (TBS-T) for 1 h at 37°C and probed overnight at 4°C with mouse anti-IGFBP3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-IGFBP-5 (Santa Cruz Biotechnology, CA, USA) and mouse anti-α-actin (Chemicon International Inc., Temecula, CA, USA).

After the primary antibody treatment, the membranes were washed four times for 5 min each at real time in PBS + 0.1% Tween-20 before the addition of secondary antibodies. PBS and 0.1% Tween-20–diluted secondary antibodies (anti-mouse and rabbit) were IRDye Labeled (680/800CW) (LI-COR Biosciences, Lincoln, NE, USA). The Li-cor Odyssey infrared imaging system (LI-COR Biosciences) was used for Immunoblotting analysis. The Western blot images were analyzed by imaging densitometry using Quantity One Software (Bio-Rad Laboratories) and compared with the actin. The data are expressed as optical density (OD) $\times$ mm^2.

Statistical analyses

Statistical analyses were performed by Student's t-test with the Statistical Package for the Social Sciences software (SPSS, Inc., Chicago, IL, USA), adapted to compare different average data for continuous variables. The results were considered statistically significant at $p < 0.05$.

RESULTS

IGF-1

Real-time PCR was carried on cDNA derived from RNA extracted from osteoblasts stimulated with untreated PRP, ESW-pretreated PRP and ESW-pretreated α-MEM as control at 24, 48 and 72 h from the stimulation. The results show an increased IGF-1

Table 1. Primers sequence from 5′ to 3′

Name	Sequence	Position	Tm (°C)	Product size (Bp)
Osteocalcin-S	CCATCTTTCTGCTCACTCTG	56	52.9	172
Osteocalcin-AS	TTTCCTCCCTGTTGTCTCC	227	52.9	
RUNX2-S	CGTCAGCATCCTATCAGTTC	2342	52.8	145
RUNX2-AS	CCGTCAGCGTCAACACCATC	2486	58.7	
IGFBP3-S	AATGGCCGCGGGTTCTGC	331	60.1	269
IGFBP3-AS	TTCTGGGTGTCTGTGCTTTGAG	599	58.2	
Collagen1-S	GGCTCCTGCTCCTCTTAG	23	52.5	194
Collagen1-AS	ACAGTCCAGTTCTTCATTGC	216	53	
IGF1-S	TTGCCAGAAGAGGGAGAG	8	51.8	134
IGF1-AS	CAGTGACAGTGAGATTTAGC	141	50.8	
GAPDH-S	TCAACGGCACAGTCAAGG	207	55.6	126
GAPDH-AS	ACTCCACGACATACTCAGC	332	52.6	

A = adenine; C = cytosine; G = guanine; T = thymine; S = sense; AS = antisense.

expression at 72 h from ESW-PRP treatment compared with ESW-treated α-MEM controls. In untreated PRP osteoblasts, we did not find any variation in IGF-1 expression (Fig. 1).

RUNX2

We evaluated the influence of the pretreated PRP on the gene markers of osteoblastic differentiation by real-time PCR to assess the expression of RUNX2, a crucial transcription factor for osteoblast development. The experimental results demonstrated that RUNX2 mRNA increased at 48 h from stimulation with ESW-PRP and returned approximately at the control level at 72 h (Fig. 2), in agreement with our previous study in which we verified that the gene expression of RUNX2 dies after 48 h from a cellular stimulation (Tamma et al. 2009).

Collagen type I

In view of the increased expression of messenger RUNX2, we also assessed the influence of the pretreated PRP on type I collagen expression. By real time-PCR, we observed a significant increase at 72 h in ESW-PRP–treated osteoblasts. No significant alteration was found in both untreated PRP and the controls (Fig. 3).

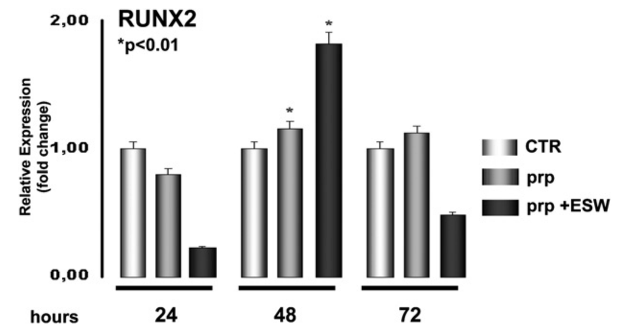

Fig. 2. Results of real-time PCR for the transcription factors of RUNX2. In the osteoblasts added with ESW-stimulated PRP, there was a significantly increased RNA expression of this gene, with respect to the untreated PRP and medium ESW-treated osteoblasts. RUNX2 at 48 h after the treatment was doubled in value, and at 72 h returns approximately to the starting level.

Osteocalcin

Osteocalcin was also modulated by the pretreated PRP: at 72 h, real-time PCR showed a significantly higher mRNA expression of OC in the osteoblasts added with

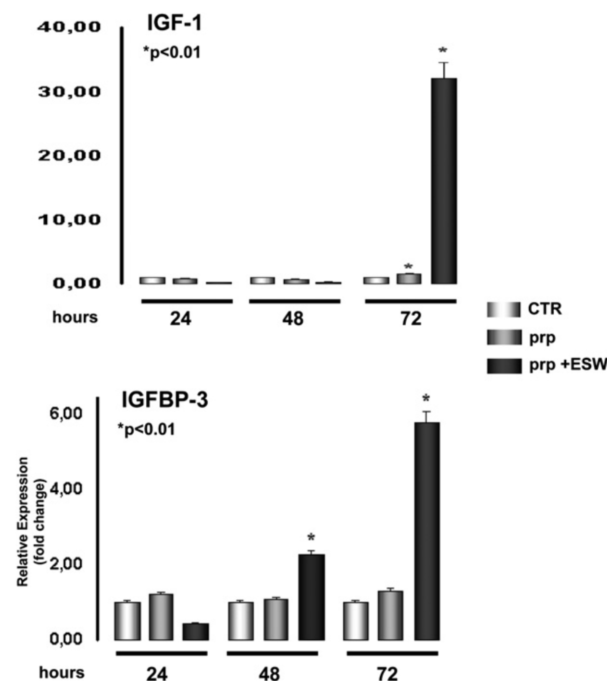

Fig. 1. Results of real-time PCR for the transcription factors of IGF-1 and IGFBP3. In the osteoblasts added with ESW-stimulated PRP, there was a significantly increased RNA expression of all the examined genes, with respect to the untreated PRP and medium ESW-treated osteoblasts. The expression of IGF-1 was increased at 72 h from stimulation. At 48 h, the expression level of the IGFBP3 increased and at 72 h is about five-fold higher in ESW-pretreted-PRP osteoblasts respect to both the untreated PRP osteoblasts and the ESW–α-MEM–treated osteoblasts.

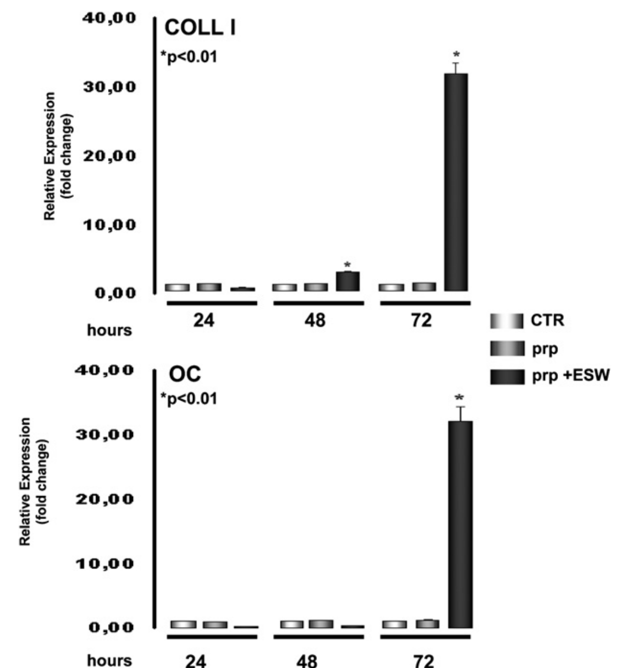

Fig. 3. Results of real-time PCR for the transcription factors of OC and type 1 collagen. In the osteoblasts added with ESW-stimulated PRP, there was a significantly increased RNA expression of all the examined genes, with respect to the untreated PRP and medium ESW-treated osteoblasts. The expression of OC and collagen type 1 was increased at 72 h from stimulation.

the pretreated PRP compared with those added with the untreated PRP, with respect to the controls (Fig. 3).

IGFBP-3

In osteoblasts added by PRP pretreated by ESW, an increased protein level of IGFBP3 at 48 and 72 h from ESW-PRP stimulation was found (Fig. 4). This was confirmed by real-time PCR (Fig. 1).

In fact, the increased expression of messenger RNA of IGFBP-3 was observed at 48 h and, still more significantly, at 72 h compared with osteoblast PRP, with respect to the controls.

IGFBP-4 and IGFBP-5

When we performed the Western blotting analyses, we found a significant reduction in the protein levels for IGFBP-4 at 24, 48 and 72 h and for IGFBP-5 at 48 and 72 h in the osteoblasts added with the pretreated PRP compared with those added with the untreated control PRP (Fig. 4). We supposed the pretreated PRP influenced the protein expression of IGFBP-4 and IGFBP-5.

DISCUSSION

ESW are a sequence of single sonic pulses emitted by an appropriate generator to a specific target area at an energy flow density ranging from 0.03–0.55 mJ/mm^2 (Rompe et al. 1998). This noninvasive, safe therapy was first applied in the field of urology, where it is used to pulverize kidney stones (lithotripsy) (Ogden et al. 2001). Since the early 1990s, ESWT has been used in the treatment of several chronic tendinopathies (Chung

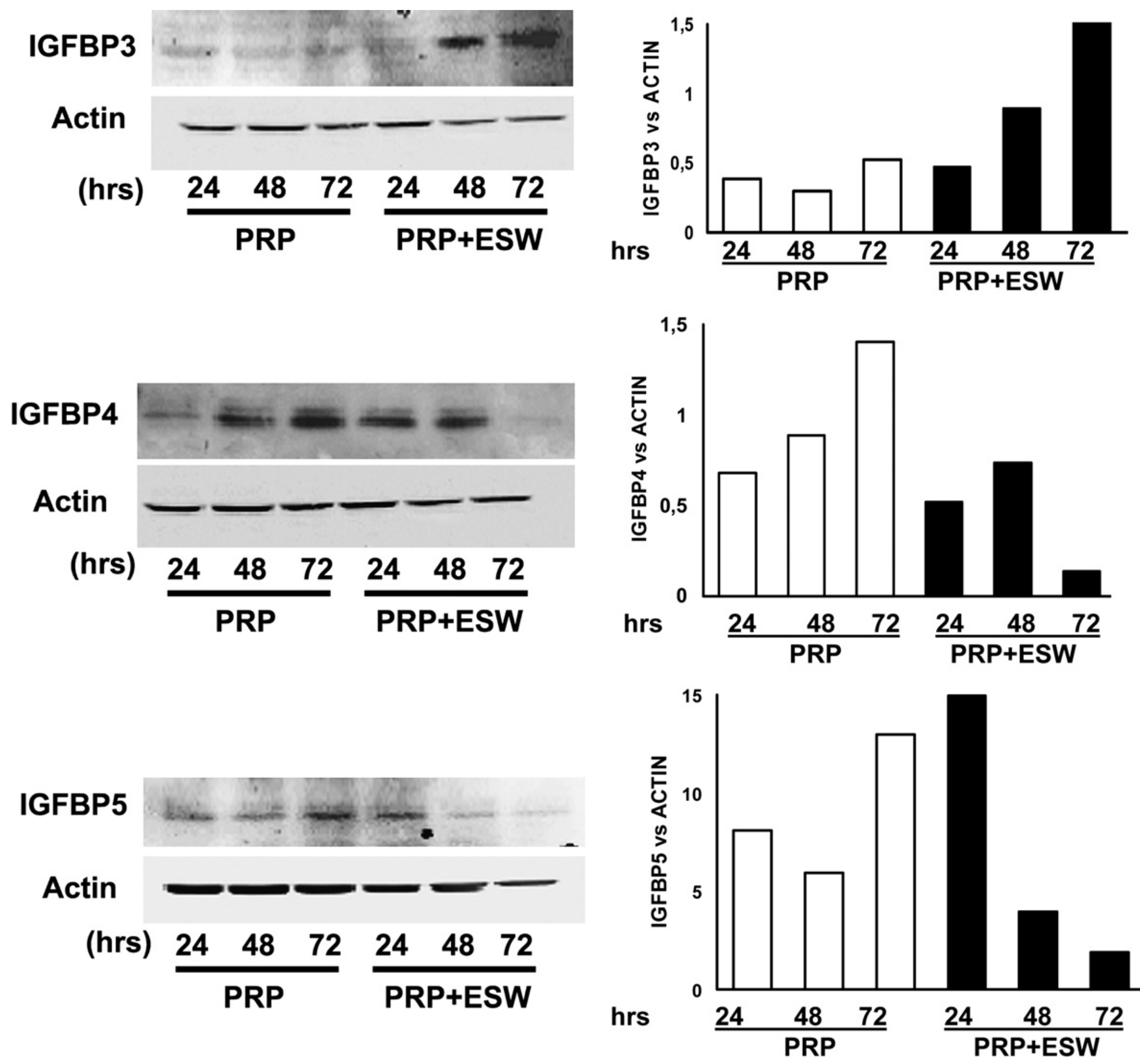

Fig. 4. Results of the Western blot analysis of mouse osteoblast extract using antibodies against IGFBP-3, IGFBP-4 and IGFBP-5. Compared with the osteoblasts added with untreated control PRP, in the osteoblasts added with the ESW-stimulated PRP, at 48 and 72 h there were higher protein lof IGFBP-3 and lower concentrations of IBGFP-4 and IGFBP-5.

and Wiley 2002). In some studies, ESWT has shown promising results in the treatment of rotator cuff tendinopathy, extensor tendinopathy of the elbow and chronic plantar fasciitis (van Leeuwen et al. 2009; Kearney and Costa 2010; Rebuzzi et al. 2008; Johnson et al. 2007; Vulpiani et al. 2007; Ho 2007). In particular, treatment of the tendon and muscle tissues was found to induce a long-term tissue regeneration effect, in addition to a more immediate analgesic and antiinflammatory action (Mariotto et al. 2005). In keeping with this, an increased neoangiogenesis was observed in the tendons of dogs after 4–8 weeks of ESWT (Wang et al. 2002). Furthermore, clinical observations indicate an immediate increase in blood flow around the treated area (Wang et al. 2003). Nevertheless, although ESW is now widely used in therapy, the cellular mechanisms by which they enhance fracture healing and tendon repair have not yet been completely clarified. Experimental evidence shows that shock waves could promote growth and differentiation of bone-marrow stromal cells to osteoprogenitors (Wang et al. 2002). Furthermore, ESW induces positive effects on proliferation and collagen type I expression in osteoblast-like cells (Martini et al. 2005). In murine osteoblast primary cultures, ESWT induced both proliferation and differentiation of osteoblasts (Tamma et al. 2009).

Interest is now being shown in investigating the reciprocal influence exerted by several cell lines after ESW treatment. In ischemic muscle tissue, an increased expression of VEGF has been observed on the endothelial cell surface, resulting in the recruitment of endothelium progenitor stem cells inducing neoangiogenesis (Aicher et al. 2006). Mariotto et al. (2005) found that nitric oxide production by the endothelial cells exerts an antiinflammatory action on the surrounding cells. In a previous study, we demonstrated a reduced RANKL/OPG in the osteoblasts after SW treatment, which downregulated the osteoclasts and inhibited osteoclastogenesis (Tamma et al. 2009).

PRP has significant potential in the treatment of diseased cartilage, tendons, ligaments and bone (Foster et al. 2009). *In vitro*, there is a dose-response relationship between the platelet concentration and the proliferation of human adult mesenchymal stem cells, the proliferation of fibroblasts, and the production of type I collagen (Liu et al. 2002). PRP has also been shown to be osteopromotive rather than osteoinductive (Ranly et al. 2007). Few basic science or clinical studies have yet focused on examining the role of PRP in bone healing after orthopedic trauma (Gandhi et al. 2006). Mesenchymal stem cell proliferation can be upregulated by PRP. Furthermore, PRP induces a rapid increase in mRNA production for both bone morphogenetic protein-2 and RUNX2, growth factors involved in osteogenesis (Simman et al. 2008).

In this work we have verified that after ESW stimulation the platelets, too, cooperate in the osteogenic actions exerted on the bone tissue. We suppose SW application on the platelets increases the release of the growth factors. They accelerate tissue healing, regulating chemotaxis, proliferation, differentiation, debris clearance from the tissues, angiogenesis and extracellular matrix deposition (Anitua et al. 2004). ESW may be able to trigger these platelet activities.

Our data showed that osteoblasts stimulated by ESW-treated PRP showed an increased expression of IGF-1 mRNA after 72 h compared with the untreated PRP control osteoblasts. This result indicates that SW administration can enhance the expression of IGF-1 in osteoblasts, contributing to upregulation of osteoblast activity.

Having observed that ESW-PRP upregulated IGF-1 expression, we proceeded to assess the effect of the pretreated PRP on IGFBP-3 in the osteoblasts. There was an increase in both IGFBP-3 protein and mRNA already after 48 h from the stimulation, indicating that a system regulating IGF-1 activities is triggered by ESW-PRP treatment.

Our data showed a significant downregulation of IGFBP-4 and IGFBP-5 protein expression after 72 h of ESW-PRP treatment. These results confirm that IGFBP-4 and IGFBP-5 interfere with IGF action in osteoblasts and provide a framework for discerning mechanisms of collaboration between signal transduction pathways activated by BMPs and IGFs in bone (Mukherjee and Rotwein 2008).

We found that at 48 h, there was a relative increase in mRNA for RUNX2 in the ESW-PRP osteoblasts compared with the controls. This could be a direct effect of the stimulated PRP, not mediated by IGF-1, because it appeared earlier than the increased expression of the latter.

To continue this study of the effects of SW-pretreated PRP on osteoblast differentiation, we assessed the expression of collagen type I by real-time PCR. The experiments showed a significant increase in collagen type I after 72 h from stimulation in the osteoblasts with added pretreated PRP compared with untreated control PRP.

The OC gene encodes for a bone-specific protein that is induced in osteoblasts with the onset of mineralization of extracellular matrix, at late stages of differentiation (Owen et al. 1990). Bone-specific expression of the OC gene is principally regulated by the RUNX2 transcription factor (Ducy et al. 1997).

In fact, RUNX2 binds to OSE2, a *cis*-acting element activating the OC promoter, but OSE2 has been found in the promoter regions of all the major genes expressed by the osteoblast (Ducy et al.1997). Because the treatment

had also upregulated RUNX2 expression in the osteoblasts, we studied the effects on OC expression. The OC mRNA expression assessed by real-time PCR was found to be significantly higher at 72 h in the osteoblasts with added pretreated PRP compared with untreated PRP and the controls.

In conclusion, the results of our study provide the rationale for associating SW treatment with the new bioengineering techniques. In fact, we have ascertained that ESW can activate platelet-enriched plasma, because the PRP used in the experiments had not been previously activated by the administration of thrombin or calcium gluconate, so the effects induced by the ESW-PRP can be imputed purely to the physical stimulation actions produced by the SW. At this point, it would be interesting to know what effects could be produced on PRP by combining chemical stimulation with thrombin or calcium gluconate and physical stimulation with SW. We believe that combining SW with the administration of stem cells could also have important effects.

In this work we have widened the knowledge of SW-induced platelet activation on the osteoblasts, an aspect that had not been previously studied in other research. Our results show that after SW stimulation the platelets can modulate the osteoblast differentiation process by upregulating the expression of the gene markers of osteoblast differentiation, such as RUNX2 and OC. ESWT also had a positive effect on collagen type I, an osteoblastic marker and specific bone matrix molecule. Finally, we observed that the IGF-IGFBP system was regulated and stimulated to promote osteoblastic activities, after ESWT.

These positive results encourage us to proceed with clinical applications to verify the possibility of combining, *in vivo*, the PRP infiltration method with ESW to treat musculoskeletal disorders.

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