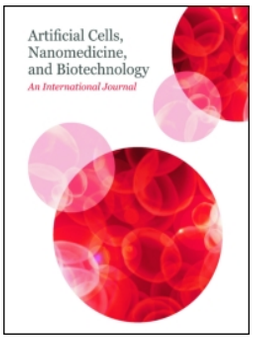

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Primary Osteoblasts Response to Shock Wave Therapy Using Different Parameters

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ABSTRACT

Over the past decade extracorporeal shock-wave therapy (ESWT) has been increasingly applied to orthopaedic and musculoskeletal pathologies, the aim of this study was to assess how the energy density of the shock waves and the number of impulses affect viability, differentiation and synthetic activity of osteoblasts. Primary sheep osteoblasts cultures were treated with ESWT with an electro-hydraulic shock wave generator by selecting three different energy levels (14–21–28 kV corresponding at 0.15–0.31–0.40 mJ/mm^2) and two different total numbers of impulses (500, 1000) for each level. At the and of treatment, cell counts and viability were recorded. Cells were then cultivated for 48 hours starting from a concentration of 1×10^4 cells/ml. The biological activity and viability were evaluated at 24 and 48 hours after treatment. No cytodestructive effects were observed in Group A, while a cytodestructive effect of ESWT was seen in cultures receiving the highest energy treatments. The different shock wave treatment induced differences in MTT assays after 24 and 48 hours, in particular the highest level showed a detrimental effect on cell respiration at both experimental times as compared to the Control Group and the protein metabolism was generally depressed by ESWT with impulses at the highest energy level. After 24 hours such effect further increased with the growing number of impulses. The lowest energy level appeared to significantly improve the metabolic parameter in primary cell cultures as compared to controls when 500 impulses were selected. The current study has demonstrated that one of the most important aspects to be considered is not the total number of impulses used but the energy level of the shock waves, thus confirming that ESWT has a dose-dependent effect on cells.

Key Words: Osteoblast; Shock wave therapy.

INTRODUCTION

Over the past decade extracorporeal shock-wave therapy (ESWT) has been increasingly applied to orthopaedic and musculoskeletal pathologies, such as epicondylitis (Haupt, 1997; Ko et al., 2001; Speed et al., 2002a), painful hell syndrome (Chen and Huang, 2001; Hammer et al., 2000; Maier et al., 2001; Rompe et al., 1996a,b), calcific tendonitis of the shoulder (Chen and Huang, 2001; Hammer et al., 2000; Loew et al., 1995, 1999; Maier et al., 2000a, 2001; Rompe et al., 1995, 1996a,b, 2001a; Speed et al., 2002b; Spindler et al., 1998; Wang et al., 2001a), chronic plantar fasciitis (Maier et al., 2000b; Odgen et al., 2001; Rompe et al., 1996c), nonunions (Birnbaum et al., 2002; Ikeda et al., 1999; Rompe et al., 2001b; Schaden

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et al., 2001; Valchanow and Michailow, 1991; Wang et al., 2001b), pseudoarthrosis (Schleberger and Senge, 1992; Vogel et al., 1997), and femoral head necrosis in adults (Ludwig et al., 2001). Many Authors have investigated the effects of ESWT on cell cultures and tissues (Augat et al., 1995; Delius et al., 1995a,b; Forriol et al., 1994; Haupt and Chvapil, 1990; Haupt et al., 1992; Johannes et al., 1994a,b; Kaulesar Sukul et al., 1993; Park et al., 1991a,b; Rompe et al., 1998; Vaterlein et al., 2000; Wang et al., 2002a; Weinstein et al., 1991; Yeaman et al., 1989) to gain a better understanting of the biological mechanism by which shock waves act. They have achieved contradictory results, even if a general consensus of agreement exists among the findings on some micromechanical effects on biochemical cell physiology (Delius and Adams, 1999; Delius et al., 1998; Endl et al., 1996; Johannes et al., 1994a; Rompe et al., 1998; Wang et al., 2001a). Shock waves have been hypothesized to enhance revascularization of avascular or minimally vascular tissues (Wang et al., 2002b), as happens in the case of femoral head necrosis in adults (Ludwig et al., 2001). Enhanced release of local growth factors and recruitment of stem cells have also been observed after shock wave treatment (Thiel, 2001). In contrast, a detrimental effect of ESWT has been seen in a human bladder carcinoma cell line following the chemical disturbance of bubble implosion affecting mitochondrial functionality. In addition, the generation of free-radicals has also been claimed as a potential cause of cell damage (Gambilher and Delius, 1992; Suhr et al., 1996). Some Authors have focused on the cytotoxic effect of shock waves and found it to be related to the high level of energy depending on the tissues and cells used (Delius et al., 1995b, 1998; Gambilher and Delius, 1992; Laudone et al., 1989; Wörle et al., 1994).

Few studies on the effects of ESWT on bone cells are available. Wang F.S. has suggested that ESWT may enhance bone marrow stromal cell growth and differentiation towards osteogenic cells by means of TGF-β1 production (Wang et al., 2000, 2002a,c). He observed osteogenic differentiation after using extracorporeal shock waves with 016 mJ/mm^2 energy and 500 impulses.

In a previous investigation the present authors studied the effect of ESWT on an osteosarcoma cell line (MG-63) (Martini et al., 2003). Since differences between cell lines and primary osteoblast metabolism has been reported (Torricelli et al., 2003), the present study was conducted in the ambit of a larger project currently in progress to evaluate the immediate effect of ESWT on viability, differentiation and synthetic activity of cells 24 and 48 hours after treatment. Three different energy levels (Group A:14 kV and 0.15 mJ/mm^2; Group B: 21 kV and 0.31 mJ/mm^2; Group C: 28 kV and 0.40 mJ/mm^2; Control Group: no energy) and two different numbers of total impulses (500, 1000) for each level were tested on primary osteoblasts (sheep

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osteoblasts) and results were discussed by considering also the previous experience with the osteosarcoma cell line MG-63 (Torricelli et al., 2003).

MATERIALS AND METHODS

Cell Cultures

Primary cultures of sheep bone (sOB) were isolated sterilely from small specimens of trabecular bone derived from the iliac crest of mongrel sheep, 68 ± 7 kg b.w., raised on a traditional breeding farm (Pancaldi, Budrio, Bologna, Italy). Sheep were used immediately after the death and euthanization occurred at the end of other research protocols approved by the Ethic Animal Research Committee of the University of Bologna and the National Health Minestry involving neither the donor skeletal site nor therapies affecting bone metabolism. The animals were treated in strict accordance with the International and European law on animal experimentation. The iliac crest biopsies were surgically performed under general anaesthesia and obtained in the vertical direction using a counter-rotating biopsy needle positioned 2 cm behind the anterosuperior iliac spine and 2 cm below the summit of the iliac crest.

Immediately after the biopsy, the fragments of iliac crest bone were washed with serum-free D'MEM. They were then digested in the medium with 1 mg/ml collagenase for 2 hours at 37°C. The enzymatic reaction was stopped by adding an equal volume of medium with 10% FCS, and the supernatant containing the released cells was used. Cells were centrifuged at 1000 rpm for 5 minutes, resuspended in D'MEM containing 10% FCS, seeded into a 25 cm^2 flask and cultivated at 37°C in a humidified 95% air/5% CO_2 atmosphere. At confluence, cells were released with trypsin, counted (Coulter Counter Z1, Beckman Coulter Inc., Miami, FL, USA), resuspended in medium at a concentration of 1×10^6 cells/ml in 1.5 ml test tubes and maintained at 37°C until their use for the experiment. Cells were also seeded at 1×10^5 cells/ml in chamber slides (4-wells) to test their osteoblastic phenotype and cultured in D'MEM supplemented with ascorbic acid (50 μg/ml) and β-glycerophosphate (10^{-8} M).

The expression of the osteoblastic phenotype was assessed by alkaline phosphatase activity (ALP, diagnostic kit no. 245, Sigma Diagnostic Inc. St. Louis Co.), osteocalcin (OC, Novocalcin enzyme immunoassay kit, Metra Biosystem, Quidel Corporation, San Diego, CA, USA) and Von Kossa staining which revealed the deposition of calcium in the extracellular matrix after adding $1.25(OH)_2D_3$ (10^{-9} M).

The cells were then seeded in a 1.5 ml screw-cup cryotube (48 × 12.5 mm) (Cryovial-Symport, Beloell, Quebec, Canada) at a concentration of

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1×10^6 cell/ml. The tube was carefully inspected to eliminate any air bubble trapped inside and was maintained at 37°C during all experimental time.

ESWT Procedures

An electro-hydraulic shock wave generator (Ossatron® HMT, High Medical Technologies AG, Kreuzlingerstrasse, Lengwil, CH) was used for the experiment with the same technical characteristic described in the previous experience with MG-63 cells line (Torricelli et al., 2003). The test tubes containing cell suspension were mounted into a cylindrical, degassed, water-filled container which can be attached directly to the shock wave module of the generator. The longitudinal axis of the tube coincided with that of the shock wave module and the focus of the shock wave was correctly pointed towards the tube with cell suspension.

Four tubes were randomly assigned to the groups receiving ESW treatment (A, B, C) as follows: shock wave energy of 14 kV and 0.15 mJ/mm^2 (Group A), 21 kV and 0.31 mJ/mm^2 (Group B) and 28 kV and 0.31 mJ/mm^2 (Group C) kV. All groups were divided into two subgroups (1 and 2) according to the number of impulses (500 or 1000 impulses). Two tubes were assigned to the Control Group, which received no shock wave treatment but was exposed with the device switched off to check the effects of test tube management on cell viability.

Assessment of Viability and Metabolic Parameters

At the end of treatment, cells from all tubes were counted (Coulter Z1 Counter, Miami, Florida, USA) and cell viability (Trypan blue dye exclusion) was evaluated in relation to the baseline value.

Cells from each tube were then reseeded at a concentration of 1×10^4 viable cells/ml, and were cultivated in quadruplicate, in 4-chamber slides, for 24 and 48 hours. Cell supernatant was collected after 24 and 48 hours for evaluation of cell damage and metabolism (cellular respiration, release of intracellular enzymes, osteoblast differentiation, matrix and cytokine production).

The following tests were performed: lactate dehydrogenase (LDH, diagnostic kit no. 228, Sigma Diagnostic Inc., St. Louis, USA), nitric oxide (NO, modified Griess reagent no.G4410, Sigma Diangostic Inc., St. Loius, USA), alkaline phosphatase (ALP, diagnostic kit no.245, Sigma Diagnostic Inc., St. Louis, USA), osteocalcin (OC, Novocalcin enzyme immunoassay kit, Metra Biosystem, Quidel Corporation, San Diego, CA, USA), C-terminal procollagen Type I (PICP, procolagen-C enzyme immunoassay kit, Metra Biosystem, Quidel Corporation, San Diego, CA, USA), interleukin-6 (IL-6 immunoassey R&D System, Minneapolis, MN, USA) and

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Table 1. Cell count and viability of sOB reported for shock wave energy, total impulses and experimental time (mean, n = 2).

Therapy		Viability (%)
A1	1.27	85
A2	0.89	59
B1	1.17	78
B2	0.98	65
C1	0.43	29
C2	0.21	14
Control	1.29	86

Note: A1 = 14 kV and 0.15 mJ/mm^2—500 impulses; A2 = 14 kV and 0.15 mJ/mm^2—1000 impulses; B1 = 21 kV and 0.31 mJ/mm^2—500 impulses; B2 = 21 kV and 0.31 mJ/mm^2—1000 impulses; C1 = 28 kV and 0.40 mJ/mm^2—500 impulses; C2 = 28 kV and 0.40 mJ/mm^2—1000 impulses; Control = no therapy.

Table 2. MTT test and LDH in sOB reported for shock wave energy, total impulses and experimental time (mean ± SD, n = 4).

	MTT assay (OD 550 nm)		LDH (IU/L)	
Therapy	24 hours	48 hours	24 hours	48 hours
A1	0.22 ± 0.03*†	0.19 ± 0.0*†	6.55 ± 1.0	5.25 ± 0.49
A2	0.17 ± 0.01†	0.17 ± 0.01†	7.15 ± 1.48	6.85 ± 0.49
B1	0.17 ± 0.01	0.17 ± 0.02	6.50 ± 0.85	6.00 ± 0.14
B2	0.17 ± 0.02	0.15 ± 0.06	7.20 ± 0.57	6.60 ± 0.71
C1	0.05 ± 0.00*†	0.06 ± 0.01*	8.00 ± 2.26	6.35 ± 0.64
C2	0.04 ± 0.00*†	0.09 ± 0.06*	9.05 ± 1.20	6.65 ± 1.20
Control	0.17 ± 0.01	0.17 ± 0.01	6.00 ± 0.99	6.10 ± 0.65
Kruskal Wallis' Anova exact test	$P < 0.001$	$P < 0.01$	Ns	ns.

Note: A1 = 14 kV and 0.15 mJ/mm^2—500 impulses; A2 = 14 kV and 0.15 mJ/mm^2—1000 impulses; B1 = 21 kV and 0.31 mJ/mm^2—500 impulses; B2 = 21 kV and 0.31 mJ/mm^2—1000 impulses; C1 = 28 kV and 0.40 mJ/mm^2—500 impulses; C2 = 28 kV and 0.40 mJ/mm^2—1000 impulses; Control = no therapy.

*$P < 0.05$. Mann Whitney U exact test with Monte Carlo Method—Difference between experimental group and control.

†$P < 0.05$. Wilcoxon's exact test with Monte Carlo Method—Difference between 500 and 1000 impulses within the same experimental group.

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Transforming Growth Factor-β1 (TGF-β1; Quantikine human TGF-β1 immunoassay, R&D System, Minneapolis, MN, USA). The MTT test was done to evaluate cell viability: 80 μl of MTT solution (5 mg/ml in phosphate buffer) and 720 μl of medium were added to each well and plates were incubated in the same conditions for a further 4 h. After the supernatants had been discarded, the dark blue crystals of formazan were dissolved by adding DMSO and quantified spectrophotometrically at 550 nm. Results are reported as optical density (OD).

Statistical Analysis

Because of the lack of homoscedastic findings and small data sets (Levene's test), the non-parametric exact tests were used together with the Monte Carlo methods for probability calculation. The Kruskal–Wallis Anova was done using mean ranks to compare various different treatments. It was then followed by the Mann–Whitney U test to compare each treated sample with its control. The Wilcoxon's U test was performed on dependent samples to compare cultures at the two intervals of time. The

Table 3. ALP and NO in sOB reported for shock wave energy, total impulses and experimental time (mean ± SD, n = 4).

	ALP (IU/L)		NO (μM)	
Therapy	24 hours	48 hours	24 hours	48 hours
A1	28.25 ± 0.50	25.73 ± 0.61	3.33 ± 0.21	3.75 ± 0.31*
A2	27.55 ± 0.96	25.48 ± 0.89	3.15 ± 0.06	3.45 ± 0.13*
B1	26.35 ± 0.41	24.95 ± 0.79	3.15 ± 0.13	3.15 ± 0.24
B2	26.55 ± 0.41	24.83 ± 0.70	3.00 ± 0.08	3.15 ± 0.47
C1	26.55 ± 1.02	25.00 ± 0.77	3.10 ± 0.22	3.28 ± 0.21
C2	24.90 ± 0.62*	25.20 ± 0.73	2.95 ± 0.06	3.20 ± 0.24
Control	27.05 ± 0.68	25.87 ± 1.22	3.03 ± 0.10	3.20 ± 0.10
Kruskal Wallis' Anova exact test	$P < 0.001$	Ns	$P < 0.01$	$P < 0.1$

Note: A1 = 14 kV and 0.15 mJ/mm^2—500 impulses; A2 = 14 kV and 0.15 mJ/mm^2—1000 impulses; B1 = 21 kV and 0.31 mJ/mm^2—500 impulses; B2 = 21 kV and 0.31 mJ/mm^2—1000 impulses; C1 = 28 kV and 0.40 mJ/mm^2—500 impulses; C2 = 28 kV and 0.40 mJ/mm^2—1000 impulses; Control = no therapy.

*$P < 0.05$. Mann Whitney U exact test with Monte Carlo Method—Difference between experimental group and control.

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same test was used for comparison of samples receiving the same energy treatment but differing in the number of impulses.

RESULTS

Table 1 shows cell counts and viability at the end of the experimental shock wave treatment. No cytodestructive effects were observed in Group A. On the other hand, the cytodestructive effect of ESWT was seen in cultures receiving the highest energy treatments (Group C).

Table 2 shows the MTT and LDH mean values after 24 and 48 hours obtained from the cell cultures reseeded at 1×10^4 in 4-chamber slides. The different shock wave treatment induced differences in MTT assays after 24 and 48 hours. Only the highest level (28 kV and 0.40 mJ/mm^2) showed a detrimental effect on cell respiration at both experimental times as compared to the Control Group. After 24 hours such effect further increased with the growing number of impulses. On the other hand, the lowest level (14 kV and

Table 4. OC and PICP in sOB reported for shock wave energy, total impulses and experimental time (mean ± SD, n = 4).

	OC (ng/ml)		PICP (ng/ml)	
Therapy	24 hours	48 hours	24 hours	48 hours
A1	30.00 ± 3.46*	27.75 ± 2.63*	1.92 ± 0.02*	1.87 ± 0.08†
A2	27.00 ± 1.15	27.00 ± 1.63	1.68 ± 0.06*	1.70 ± 0.06*,†
B1	27.00 ± 2.31*	26.00 ± 2.00	1.90 ± 0.05*	1.82 ± 0.05*,†
B2	28.00 ± 1.15*	26.50 ± 1.00	1.98 ± 0.05	1.63 ± 0.55†
C1	25.00 ± 2.31	23.00 ± 2.71	1.25 ± 0.49*	1.01 ± 0.18*,†
C2	24.00 ± 1.15	23.00 ± 1.63	1.35 ± 0.09*	1.35 ± 0.34*,†
Control	25.00 ± 1.15	23.67 ± 1.15	2.05 ± 0.08	1.91 ± 0.04
Kruskal Wallis Anova exact test	$P < 0.006$	$P < 0.01$	$P < 0.001$	$P < 0.001$

Note: A1 = 14 kV and 0.15 mJ/mm^2—500 impulses; A2 = 14 kV and 0.15 mJ/mm^2—1000 impulses; B1 = 21 kV and 0.31 mJ/mm^2—500 impulses; B2 = 21 kV and 0.31 mJ/mm^2—1000 impulses; C1 = 28 kV and 0.40 mJ/mm^2—500 impulses; C2 = 28 kV and 0.40 mJ/mm^2—1000 impulses; Control = no therapy.

*$P < 0.05$. Mann Whitney U exact test with Monte Carlo Method—Difference between experimental group and control.

$^\dagger P < 0.05$. Wilcoxon's exact test with Monte Carlo Method—Difference between 500 and 1000 impulses within the same experimental group.

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0.15 mJ/mm^2) appeared to significantly improve the metabolic parameter in primary cell cultures as compared to controls when 500 impulses were selected. The cells did not show any significant increase in LDH dosage.

Determination of ALP and NO (Table 3) demonstrated that the protein metabolism was generally depressed by ESWT with impulses at the highest energy level (28 kV and 0.40 mJ/mm^2) in compared to the Control Group. A decrease in ALP activity in Group C2 was observed. The treatment with low energy level appeared to have a stimulating effect on the L-arginine/nitric oxide synthetase (NOS) pathway of sOB cultures versus controls, after 48 hours, as evidenced by the determination of NO free radicals (Damolius and Hauschka, 1997).

According to the ostecalcin and procollagen I (PICP) parameters, the synthesis of bone matrix showed significant differences in relation to the treatment received. Low-energy treatments generally enhanced osteocalcin production at 24 and 48 hours when compared to controls (Table 4). The PICP production was generally negatively affected by ESWT (Table 4).

TGF-β cytokine decreased significantly when cells received the highest energy density, i.e. 28 kV (Table 5). IL-6 did not show any differences for primary cells (Table 5).

Table 5. IL-6 and TGF-β1 in sOB reported for shock wave energy, total impulses and experimental time (mean ± SD, n = 4).

	IL-6 (pg/ml)		TGF-β1 (pg/ml)	
Therapy	24 hours	48 hours	24 hours	48 hours
A1	8.10 ± 0.28†	8.30 ± 0.14	777.75 ± 194.77	691.25 ± 38.38
A2	7.85 ± 0.49†	7.55 ± 0.92	749.50 ± 56.34	700.00 ± 32.91
B1	8.35 ± 1.06	7.70 ± 0.28	760.00 ± 36.51	703.75 ± 22.87
B2	8.35 ± 0.78	8.65 ± 0.21	740.00 ± 42.43	696.25 ± 25.62
C1	8.60 ± 0.42	7.75 ± 0.35	597.00 ± 41.10*	621.25 ± 20.97*
C2	8.50 ± 0.85	8.00 ± 0.42	602.50 ± 21.02*	648.75 ± 10.31*
Control	8.15 ± 0.64	7.90 ± 0.58	746.25 ± 20.56	693.75 ± 27.80
Kruskal Wallis Anova exact test	ns	Ns	$P < 0.01$	$P < 0.05$

Note: A1 = 14 kV and 0.15 mJ/mm^2—500 impulses; A2 = 14 kV and 0.15 mJ/mm^2 —1000 impulses; B1 = 21 kV and 0.31 mJ/mm^2—500 impulses; B2 = 21 kV and 0.31 mJ/mm^2—1000 impulses; C1 = 28 kV and 0.40 mJ/mm^2—500 impulses; C2 = 28 kV and 0.40 mJ/mm^2—1000 impulses; Control = no therapy.

$^\dagger P < 0.05$. Wilcoxon's exact test with Monte Carlo Method—Difference between 500 and 1000 impulses within the same experimental group.

*$P < 0.05$. Mann Whitney U exact test with Monte Carlo Method—Difference between experimental group and control.

DISCUSSION

The present paper investigated the immediate effects of ESWT on cell viability and the early effect on metabolic activities by measuring various biochemical parameters in relation to the energy levels of shock waves and the total number of impulses applied to sheep primary osteoblast cultures. The present physical parameters (total energy and number of impulses) were selected on the basis of clinical and experimental studies promoting cell proliferation (Wang et al., 2000, 2002c).

Various Authors have investigated these aspects not only in normal but also in pathological cell cultures, focusing mainly on the ''cytotoxic'' effects of shock waves on different malignant cell lines and the possibility to increase cell membrane permeability to molecules by means of the cavitation mechanism. The transient shock wave induces the formation of dimples on cell membranes and enhances not only the efficiency of the drug but also cell death by cell organelles and metabolism alterations (Kambe et al., 1997; Randazzo et al., 1988; Smits et al., 1991).

The physical characteristics of ESWT and the mode of application seem to have some effect on the biological outputs. Many studies have shown a relationship between cavitation and cell injury. Lifshitz has conducted an in vitro study with degassed vacuum to reduce the number of cavitation nuclei in cell medium and observed a reduced shock wave damage (Lifshitz et al., 1997). Smits has demonstrated that cell damage is eliminated if cells are suspended in gelatine (Smits et al., 1991). Delius has shown that cell damage can be greatly reduced by increasing the hydrostatic pressure during the shock wave treatment of cells (Delius et al., 1995b, 1998). On the whole, all of these experiments have confirmed that a reduction in the cavitation effect of shock waves leads to less cell damage.

Cytodestructive effect was observed immediately after treatment and, in particular, the groups treated with the highest energy level (28 kV and 0.40 mJ/mm^2) showed a notable reduction in terms of total number of cells and viability (Table 1) when compared to controls. The ESWT at low energy level showed no immediate cytodestructive effect and also appeared to have a short-term effect on cell metabolism such as cell respiration, assessed using the MTT method. The low-energy ESWT (14 kV and 0.15 mJ/mm^2) had a stimulating effect on cell respiration, whereas the high density depressed it. No significant changes in LDH values were observed either at 24 or at 48 hours in all treated groups.

Regarding the synthetic activity, osteoblasts treated with 14 kV appeared to have a slightly activated metabolism, as evidenced by the higher ALP, NO, OC and PICP values. Depression of specifically differentiated metabolic

activity was observed to be depending on the high energy shock wave therapy with increasing impulses.

As to ALP, which is a marker for osteoblastic differentiation, primary osteoblasts were depressed only with 28 kV and 1000 impulses and for a very short time. The absence of differences between groups at 48 hours provided evidence of the attenuation of the cytotoxic effect. The ALP data may demonstrate that differentiation was slowed down by the high-energy exposure and, in general terms, during the first 24 hours following treatment.

NO was positively affected by ESWT at low energy. These results are consistent with other findings suggesting that ESWT may increase NO production by means of the cavitation mechanism, probably due to direct effect exerted on the molecules present in the tissues (Miller and Thomas, 1996). NO is a labile free radical generated physiologically through the L-arginine/nitric oxide synthase (NOS) pathway, which combines molecular oxygen with the terminal guanidine nitrogen of L-arginine in a reaction yielding citrulline as a coproduct (Daghight et al., 2002). NO determination was performed because NO is known to mediate many biological functions, such as vasodilatation, inflammation and neurotransmission, and to modulate bone cell metabolism, thus stimulating osteoblast proliferation and inhibiting bone resorption (Damolius and Hauschka, 1997; Diwan et al., 2001; Kanamaru et al., 2001; Ralston et al., 1995; Watanuki et al., 2002). The latter is hypothesized to be mediated by high levels of osteoblast-produced NO inducing apoptosis of osteoclast progenitors in bone marrow cells and inhibiting the resorptive activity of mature osteoclasts (Kanamaru et al., 2001; van't Hof and Ralson, 1997).

The highest OC values obtained for low energy (14 kV) versus controls after 24 hours, as well as the PICP values (a precursor of collagen type I and index of activated deposition of bone matrix), would confirm that a lower energy level positively affects bone cell metabolism and matrix production by osteoblasts. PICP revealed the negative effect of a higher energy level better than OC.

Finally, IL-6 was very slightly affected by the shock wave treatment. This cytokine is produced by osteoblasts, and plays a role in bone reabsorption by inducing osteoclast recruitment (Chenoufi et al., 2001). No stimulation was observed in primary ovine cells for low energy but only depressive effects for high energy.

The main advantage of in vitro models is the use of human-derived cells avoiding the effects of interspecies differences. However, at this time many difficulties are related to the supply of fresh human specimens, which are required in large quantities to minimize the well-known source of biological variability of patients (Torricelli et al., 2003). Animals can in

fact be selected in relation to their age, sex, weight, nutrition, environmental conditions, therapies. Primary osteoblasts from sheep were chosen firstly because sheep osteoblasts have been seen to behave similarly to human osteoblast in other studies on interspecies differences between rat, sheep and human primary osteoblasts (Torricelli et al., 2003), and secondly because sheep is used in routine orthopaedic research (Martini et al., 2001).

When comparing the present data with those previously obtained using an osteosarcoma cell line (MG-63), the aforementioned effects were seen for both cell types, even though the primary osteoblasts appeared to be less responsive to cell damage and depression or stimulation of metabolism parameters (Martini et al., 2003).

Osteosarcoma cell lines are widely used for in vitro studies (Torricelli et al., 2003). The expression of a great number of cytokines, growth factors and their receptors have been found to be similar to those observed in primary cultures (Torricelli et al., 2003). The resulting altered gene expression responsible for the tumorigenic phenotype may therefore bring about the various different responses to external stimula (Skjodt and Russel, 1994; Torricelli et al., 2003).

In conclusion, the current findings demonstrate that the effects of ESWT depend on the energy density selected, and the effect of the number of impulses appears to be overruled by that of the energy level. Shock waves in fact had an immediate cytodestructive effect when the energy selected was 28 kV. The detrimental effect on the metabolism parameters was observed over time and reached its lowest value after 24 hours. On the contrary, ESWT at low energy density showed a positive effect on cell metabolism in terms of enzymes, bone matrix proteins and cytokine activity.

The present results support the clinical application of ESWT using either low energy when the target is osteoblast stimulation or high energy level and high number of total impulses when tissue destruction or bone tissue recruitment are to be achieved.

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