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Influence on the number of shock waves and pressure in pulp cells and osteoblasts

Ana Laura Ramírez-Ramírez[1], Achim M. Loske[2], Francisco Fernández[2], René García-Contreras[3]*, Roberto Ruíz-Díaz[1], Gisel García-García[1]

1. Área de Ortodoncia, Escuela Nacional de Estudios Superiores, Unidad León, Universidad Nacional Autónoma de México, Boulevard UNAM 2011, 37689 León, Gto., México.
2. Centro de Física Aplicada y Tecnología Avanzada, Universidad Nacional Autónoma de México, Boulevard Juriquilla 3001, 76230 Querétaro, Qro., México.
3. Área de Nanoestructuras y Biomateriales, Escuela Nacional de Estudios Superiores, Unidad León, Universidad Nacional Autónoma de México, Boulevard UNAM 2011, 37689 León, Gto., México.

Corresponding author:
René García-Contreras
E-mail: rgarciac@enes.unam.mx

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ABSTRACT

Introduction: Biomedical applications of shock waves have been developed to treat various conditions. **Objective:** To evaluate the effectiveness of extracorporeal shock waves according to the pressure and number of waves applied to stimulate the proliferation of cultured human odontoblasts and dental pulp stem cells. **Material and methods:** Prospective, comparative, in vitro experimental study. Approximately 6×10^5 cells/ml of human bone and dental pulp cells were inoculated, and different numbers of shock waves were applied to randomly form 8 groups per

cell type. **Results:** The number of shock waves and their positive pulse pressure influence cell viability. By applying 400 shock waves at a pressure of approximately 22 MPa to osteoblasts, a 50% increase in cell viability was obtained at 48 hours. **Conclusions:** The use of low pressure and a high number of shock waves increases cellular activity.

Keywords: Shock waves, Acoustic cavitation, Osteoblasts, Pulp cells, Cell Viability.

INTRODUCTION

Biomedical applications of shock waves have been established for the safe and effective treatment of various conditions. Extracorporeal shock wave lithotripsy (ESWL) revolutionized the treatment of urolithiasis in the early 1980s. It was followed by ESWL to treat stones in the gallbladder, bile duct, pancreatic duct, and salivary gland ducts[1]. Shock waves (SHW) have also been used in different fields of medicine to stimulate healing processes[2-4]. SHW therapy stimulates healing processes by inducing neovascularization and differentiation of mesenchymal stem cells in injured tissue to allow proper healing and regeneration[4-7]. Bone marrow mesenchymal stromal cells are known to have the potential to differentiate into osteogenic and various musculoskeletal-related cell lineages[8,9]. Treatment with SHW induces such cells by increasing differentiation markers (alkaline phosphatase, osteocalcin) and transforming growth factor beta 1 (TGF-1) expression[8,9].

The interaction of SHW with matter is a broad field and, although progress has been made in understanding the phenomena involved, questions remain to be answered[1]. One of the main phenomena related to mechanotransduction is the growth and collapse of microbubbles after the passage of a SHW, called acoustic cavitation[10]. The collapse is so violent that it generates high-velocity fluid jets (microjets) and secondary SHWs that produce tissue stresses, increasing circulation and metabolism, as well as cell permeability[1].

This study aimed to evaluate the effectiveness of SHWs according to their pressure and quantity in stimulating the proliferation of a culture of human bone cells (HBC) and human dental pulp stem cells (hDPSC) by estimating cell viability through metabolic activity following the application of different doses of SHWs.

MATERIALS AND METHODS

An in vitro, prospective, and comparative study was designed using human bone and pulp cells. The samples were obtained from third molars extracted from patients attending the Surgery Clinic of the Escuela Nacional de Estudios Superiores (ENES), Unidad León, through a non-probabilistic quota sampling with a triplicate sample size of a total of three independent experiments. The inclusion criteria were human bone and pulp cells. Exclusion criteria were cells with more than 20 cell divisions and lower cell density. The elimination criteria were applied in case of contamination of the cell culture or lack of cell density.

Cell isolation and culture

Approximately 6×10^5 cells/mL of HBC and hDPSC were used. The primary culture of HBC and hDPSC was obtained from fresh biopsies of bone tissue and dental pulp, free of pulp and periapical pathology. The protocol for isolation and culture of HBC and hDPSC was approved by the Bioethics Committee of the ENES León Unit of the UNAM (registration CE_16/004_SN).

Application of shock waves

Different doses of SHW and pressure levels were applied with a constant of duration of two seconds. In addition, eight groups per cell type were used (Table 1). All cells were suspended in a culture medium (GIBCO™ DMEM (DMEM High Glucose 1X)) supplemented with 10% fetal bovine serum, 1% antibiotic (Penstrep, Sigma-Aldrich®) and glutamine (GIBCO™, Grand Island, NY, USA) and 1 mL of cell suspension was placed in sterile 41 mm long by 13 mm diameter polyethylene pipettes (Thermo Fisher Scientific, Waltham, MA, USA), heat sealed. The application of the SHWs was performed in the Multifunctional Generator of the Center for Applied Physics and Advanced Technology (CFATA) of UNAM, an experimental setup based on a Piezolith 2501 shock wave generator (Richard Wolf GmbH, Knittlingen, Germany), consisting of 3,000 piezoelectric crystals placed on a spherical aluminum shell with a radius of curvature of 345 mm (Figure 1). To generate a shock wave, the piezoelectric elements receive a high-voltage discharge. Due to the inverse piezoelectric effect, each element undergoes an expansion, emitting a compression wave that propagates through the water. Due to non-linear effects and the superposition of the contribution of each element, a shock wave is generated in the vicinity of the center (focus). The piezoelectric elements, sealed with a polymer layer, form part of the bottom of an acrylic tank (base: 675×675 mm; height: 450 mm). A precision xyz positioner (±0.5 mm) was installed at the top of the tank to center the samples in the focus. Depending on the voltage, the pressure of the positive pulse of the SHW varies. Temperature and water level were set at 37°C and 80 mm above the focal plane, respectively. The control group went through the same process but without receiving SHW.

Table 1.
Experimental parameters

Group	Pressure (MPa)	Number of shock waves
Control		
1	9.9±0.2	200
2	9.9±0.2	800
3	21.7±0.3	200
4	21.7±0.3	400
5	21.7±0.3	800
6	42.0±1.1	200
7	42.0±1.1	800

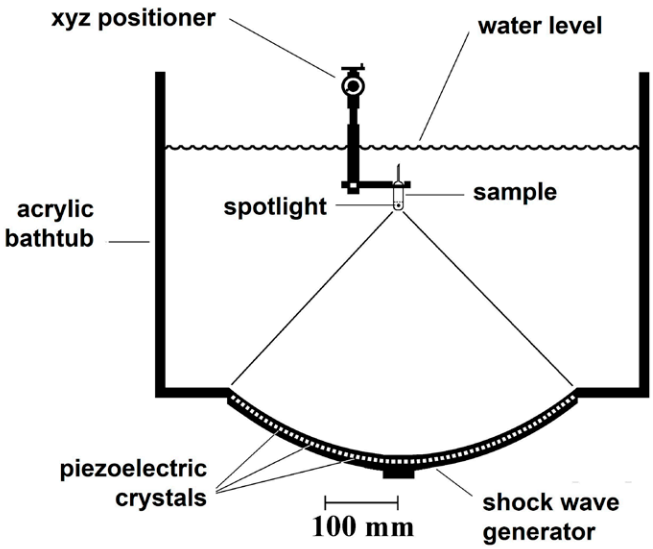

Figure 1. Diagram of the shock wave generator.

Counting cell viability and proliferation

After applying the SHWs, the medium was removed from the pipettes, washed with 0.5 mL of PBS (pH 7.4) and the cells were detached and re-suspended with 1 mL of trypsin (Sigma-Aldrich®, Toluca, Edo. Mex., Mexico). All cells were inoculated with culture medium (DMEM) supplemented with 10% fetal bovine serum, 1% antibiotic, and glutamine in a 24-well plate and incubated at 37°C with 5% CO_2 and 95% humidity.

Cell viability was estimated at 48 h and 7 days by cell viability assay with tetrazolium salt (MTT) at 0.2 mg/mL (Sigma-Aldrich®) and by cell counting in a hematocytometer. Cells were incubated for 7 hours and formazan crystals were dissolved with dimethyl sulfoxide (CH_3SOCH_3, DMSO, J.T Baker, USA). The formazan was transferred to a 96-well plate and analyzed on a microplate reader (Thermo Scientific Multiskan, GO, USA) at a wavelength of 570 nm. Each assay was performed in triplicate of three independent experiments. For hematocytometer counting, the culture medium was removed from the wells of all the aforementioned groups, and the cells were washed twice with PBS. Subsequently, 0.2 mL of trypsin was added to each well for cell monolayer detachment and placed for 5 minutes in an incubator (Binder™, Tuttlingen, Germany). Afterward, samples were analyzed using a 1/10 mm deep Neubauer chamber (BOECO, Hamburg, Germany), where a coverslip was placed and viable ones were counted under a phase contrast microscope (Leica, DMIL LED Trinolocular, Massachusetts, USA).

Statistical analysis

The analysis was performed in triplicate for each group of three independent experiments (n=6/gp). Data were analyzed with Shapiro-Wilks normality tests and Tukey's post-hoc ANOVA test ($p<0.05$; 95% reliability coefficient).

RESULTS

Cell proliferation

HBC cells with a pressure of 21.7±0.3 MPa and 400 SHW showed an increase in cell viability of 50% ($p<0.001$) at 48 hours of incubation (150±3.3%, $p<0.01$), compared to the control (Table 2). In the hematocytometer count, similar results were observed with a higher number of cells at 48 hours, showing no significant differences concerning the control group (660,000±254,558 cells/mL, $p>0.05$). In the case of hDPSCs, neither group showed greater proliferation ($p>0.05$) compared to the control. After seven days of incubation, no cell type and group increased cell proliferation by either method. Mortality was dependent on pressure and number of SHW, with a 70% and up to 100% decrease in cell viability after 48 hours using the maximum number of SHW and the maximum pressure contemplated in the study. The MTT assay proved to be more efficient in estimating cell viability compared to the Neubauer chamber count.

Table 2.
Cell viability of osteoblasts and pulp cells in MTT and Neubauer assays

Group	MTT (%) 48 hrs	Statistic Significance	MTT (%) 7 days	Statistic Significance	Neubauer, 48 hrs (cells/mL)	Statistic Significance	Neubauer, 7 days (cells/mL)	Statistic Significance
HBC Groups								
Control	**100±1.9**		**100±0.03**		**260,000±84,852**		**740,000±84,852**	
1	62±1.9	0.005	46±3.9	0.032	220,000±8 4,852	0.683	260,000±84,852	0.029
2	137±46.7	0.466	75±3.8	0.070	1,020,000±311,126	0.395	420,000±141,421	0.137
3	96±10.1	0.686	41±2.3	0.017	340,000±28,284	0.395	480,000±226,274	0.329
4	150±3.3	0.006	51±0.8	0.007	660,000±254,558	0.244	220,000±84,852	0.025
5	105±1.0	0.108	21±0.9	0.005	520,000±226,274	0.329	100,000±28,284	0.039
6	61±1.5	0.002	20±0.2	0.0002	220,000±28,284	0.624	40,000±6,568	0.016
7	44±0.1	0.015	33±2.3	0.016	60,000±14,142	0.177	40,000±0	0.054
hDPSC groups								
Control	**100±13.5**		**100 ± 4.3**		**120,000±56,568**		**440,000±113,137**	
1	71±0.7	0.199	25±2.4	0.006	80,000±0	0.502	240,000±113,137	0.219
2	80±2.5	0.272	85±7.7	0.168	220,000±84,852	0.316	140,000±28,284	0.148
3	76±0.1	0.244	63±3.3	0.013	160,000±0	0.506	200,000±56,568	0.157
4	67±0.1	0.178	36±0.5	0.028	180,000±84,852	0.503	40,000±28,284	0.109
5	75±3.6	0.210	82±1.3	0.085	380,000±197,989	0.297	260,000±84,852	0.223
6	59±1.6	0.143	36±0.5	0.028	40,000±14,142	0.280	40,000±14,142	0.121
7	61±1.3	0.149	34±0.22	0.029	0±0	0.204	0±0	0.114

MTT = tetrazolium salt,
HBC = osteoblasts ("*human bone cells*"),
hDPSC = *human dental pulp stem cells*.

DISCUSSION

It is essential to understand the reaction of bone and pulp cells to shock waves, however, in most of the reported studies, there is no consensus regarding the most important physical parameters. In the case of in vitro studies, differences among experimental setups make comparisons between published results difficult[11]. For example, the biological effects produced with the pressure profile emitted by the equipment used in this study are not directly comparable with those that result when using another model, even if the magnitude of the positive pressure pulse and the number of pulses applied are equivalent. Nevertheless, general conclusions can be drawn.

The results of the present study demonstrated that the amount of SHW and the positive pulse pressure influence cell proliferation. Applying 400 SHW with a pressure of 21.7 MPa to human osteoblasts, an increase in cell viability of 50% ($p<0.001$) was observed at 48 hours post-treatment. Using the maximum number of SHW and the maximum pressure contemplated in the study, there was a 70 and up to 100% decrease in cell viability from 48 hours. This is in accordance with a study reported by Tam *et al*. in which the highest energy density induced a significant decrease in viable cells of 90%[12].

Treatment with low-intensity SHW resulted in increased cell proliferation, increasing the number of viable cells by 44.4% ($p<0.001$). High-pressure SHW increased the number of viable cells by 21.7%. This difference was statistically significant ($p=0.011$). With the high-pressure treatment there was no significant change by keeping the number of waves applied constant[12]. This result is also reported in a study published by Martini *et al*.[9]. At 48 hours, cell viability decreased by approximately 54% in the group receiving higher pressure SHW compared to the other groups ($p<0.0005$), suggesting that SHW pressure alone affects osteoblast viability, regardless of the total number of waves applied[9]. Because Tam *et al*.,[12] and Martini *et al*.,[9] used an electromagnetic and electrohydraulic SHW generator, respectively, it is not convenient to make a direct comparison with the parameters adjusted in our piezoelectric generator. Other authors have stated that the biological effects are generally proportional to the total energy absorbed by the tissue, but problems have arisen when comparing different studies in which only the energy flux density and the number of pulses were specified[13-15].

When using low-pressure level and high SHW number, we found an increase in cell viability compared to the control at 48 hours, coinciding with the results published by other authors[12,16-18]. However, on day 7 we detected a decrease in all groups as compared to the control group. This fact is not consistent with the results published by Tam *et al*.[12] and Kusnierczak *et al*.,[19] who detected a long-term effect on the number of viable cells, cell proliferation, and mineralization of human periosteal cells between the third and eighth day. Because periosteal cells play an important role in bone healing, it is hypothesized that a SHW treatment could exert a delayed stimulatory effect on periosteal cells. Although our results showed differences from those of Tam *et al*.,[12] in the long term they agree that a higher amount of low-intensity SHW favors cellular activities compared to a low amount of high-intensity SHW.

Future perspectives will focus on increasing the sample size of the study, on the detection of pro-inflammatory proteins such as interleukins (IL), IL-1B, IL-6, tumor necrosis factor alpha (TNF-α), alkaline phosphatase, the identification of mineralizing crystals and determining the capacity of SHW to accelerate cell differentiation to osteogenic, adipogenic and chondrogenic lineages of human stem cells. In addition, it is suggested that the study be conducted for at least 30 days since this is the time it takes for an inflammatory effect to heal, and depending on the number of days, cell activity varies.

CONCLUSIONS

Low pressures and high SHW amounts showed a greater increase in cell viability (50%) of human osteoblasts at 48 hours without showing an increase in hDPSC. In the future, the use of shock waves could be used for biomedical applications in dentistry.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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