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Research Article

A NOVEL APPLICATION OF EXTRACORPOREAL SHOCKWAVE ON GINGIVAL FIBROBLASTS AND OSTEOBLAST-LIKE CELLS

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Shockwave therapy, fibroblasts, cell proliferation, cell viability

A B S T R A C T

Background: The idea of treating different deformities or diseases with extracorporeal Shockwave Therapy in the maxillofacial region has been one of the emerging therapies. Shockwave therapy has been widely used for the management of urolithiasis and in traumatology.
Objectives: To evaluate the effect of the application of extracorporeal Shockwave Therapy on gingival fibroblast (PCS-201-018) and osteoblast-like cell lines (MG-63).
Methods: 20 gingival fibroblast and 20 osteoblast-like cell lines were included. They were divided as 10 cell lines each and considered as Control (no shockwave) and the remaining 10 cell lines were considered as Test group (shockwave) for both group of cell lines. Further analysed for cell-cell interaction using Scratch analysis through live imaging. Cell viability was analysed with MTT assay and ATP assay using bio-spectrometry.
Results: Encouraging results were observed for cell migration and proliferation in test cell lines. Cell viability was notably higher than the control cell lines. The calorimetric readings for intracellular ATP analysis were significantly higher for test compared to control cell lines.
Conclusion: The present in-vitro study showed promising effect of Shockwave Therapy on gingival fibroblast and osteoblast-like cell lines. This molecular study may pave way for application of shockwave therapy in situ for periodontal disease.

INTRODUCTION

There has always been a growth in the search for effective and better treatment techniques in the area of medical science. An upsurge in the application of shock waves in medicine was observed (Prabhuji *et al*., 2014). Initially, high-energy focused extracorporeal shock wave therapy (ESWT) was used to disintegrate renal stones by its physically destructive property (Graff *et al*., 1988). Nowadays, owing to its regenerative potential, ESWT is regarded as a form of mechanotherapy in regenerative medicine. ESWT has been effectively used to treat various musculoskeletal disorders (Wang CJ, 2003), chronic soft tissue wounds (Mittermayr *et al*., 2012), neurological pathologies and ischemia heart disease (Wang Y, 2010). It is proposed that shock waves could promote tissue regeneration through mechano-transduction, in which target cells can sense and adapt their biological behaviour to extracellular physical signals of shock waves (D'Agostino *et al*., 2015).

Although just being introduced into Dentistry, ESWT has already demonstrated its potential in regeneration of alveolar bone (Satishkumar *et al*., 2008), removal of tooth biofilm (Muller *et al*., 2011) and eradication of periodontal pathogens (Novak *et al*., 2008). The influence of ESWT on tooth stability after active orthodontic movement has also been assessed wherein, shock wave treatment achieved faster reduction of tooth mobility (Falkensammer *et al*., 2015). These findings suggest that ESWT might be promising non-invasive adjunctive therapy for periodontal and orthodontic treatment.

Fibroblasts play a crucial role in remodelling of the extracellular matrix by synthesizing and organizing connective tissue components. They respond to various micro environmental signal including soluble cytokines and growth factors as well as cell matrix or cell-cell interactions that control the balance between synthesis and degradation of extracellular matrix (ECM) (Atamas, 2002). Activation of gene expression for transforming growth factor β1 (TGF- β1) and collagen types I & III which are the main factors involved in regenerative process, through ESWT has shown to aid in fibroblast proliferation and differentiation (Berta *et al*., 2009). Due to inflammation, fibroblasts undergo phenotypic transition, as a result, chemotactic migration at the site of

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injury, changes in the ECM and influence the release of various mediators, IL-8, IL-6, prostaglandins, TGF- β1 etc. (Frairia and Berta, 2011).

The efficacy of Extracorporeal Shockwave Therapy on enhancing bone regeneration, inducing angiogenesis, bacterial effect, stimulation of osteoblasts, fibroblast cells and enhancing bone morphogenic proteins (BMP) are noteworthy (Show *et al*., 2020). (Li *et al*., 2010), hypothesised for the first time that ESWT was helpful for the treatment of peri-implantitis. A vast scope in the treatment of oral and maxillofacial conditions was later seen.

(Lucia *et al*., 2006) evaluated the effects of shock waves on Osteoblast-like cells (MG63) when using two different shock wave generators (electrohydraulic and electromagnetic devices) in terms of cell damage, cell viability, osteogenic phenotype expression and cytokine production.

Taking these studies into consideration, this in-vitro evaluation was done to further investigate the effects of shockwave therapy on oral gingival fibroblast and osteoblast – like cell lines.

MATERIALS AND METHODS

This is an in-vitro study involving the effect of extracorporeal shockwave therapy on human gingival fibroblasts and Osteoblast-like cell lines. The study protocol was reviewed and approved by the Institutional review board of Krishnadevaraya College of Dental Sciences and Hospital Ethical Committee (REF: KCDS/48/ Ethical Comm /2022-23), affiliated to Rajiv Gandhi University of Health Sciences, Bengaluru, Karnataka, India, followed in accordance with the ICMR 2018 and Helsinki Guidelines 2013. The study has been registered in clinical trials with the identity number- NCT05766293 and NCT042373617 for gingival fibroblasts and osteoblast-like cells respectively in the website clinicaltrial.gov.in. This study was conducted from June 2022 to January 2023.

Source of Data

Fibroblast (PCS-201-018) cell lines derived from healthy human gingival tissue and osteoblast like cell lines (MG-63) derived from bone material retrieved from human osteosarcoma bone were procured from National Centre for Cell Science, Pune, Maharashtra and Stroma Biotechnologies Private. Ltd., Bengaluru, Karnataka, India.

Sample Size

The power of the study was set at 80%, considering minimum attrition and margin of error of 20%. Accordingly, 20 cell lines were included in each group. A total of 20 cell lines were considered for gingival fibroblasts and 20 for osteoblast like cells. The sample size was estimated using the GPower software v. 3.1.9.7 [(Franz Faul, Universität Kiel, Germany)].

Study Design

Gingival fibroblast and osteoblast-like cell lines were used for the study. The cell lines were delivered in T- 25 flask with Dulbecco's Modified Eagle Medium (DMEM) from the laboratory (Stroma Biotechnologies Pvt. Ltd., Bengaluru). The cell lines were transferred to vials and were infused with a fresh medium DMEM.

A total of 40 cell lines were included in this study, 20 gingival fibroblasts and 20 osteoblast like cells. They were further grouped based on the following criteria.

Criteria for Grouping

Gingival Fibroblasts

Control group: Cells not exposed to Extracorporeal Shock wave Therapy (Figure. 1).

Test group: Cells exposed to Extracorporeal Shock wave Therapy (Figure. 2).

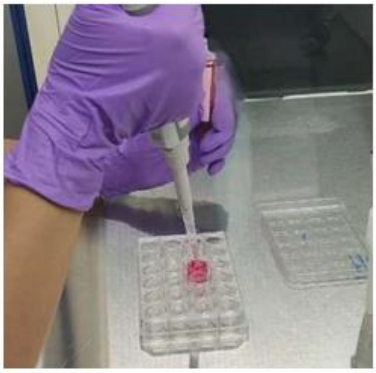


Figure 1 Gingival fibroblast cells seeded in 24 x 12 well plates (Control group)

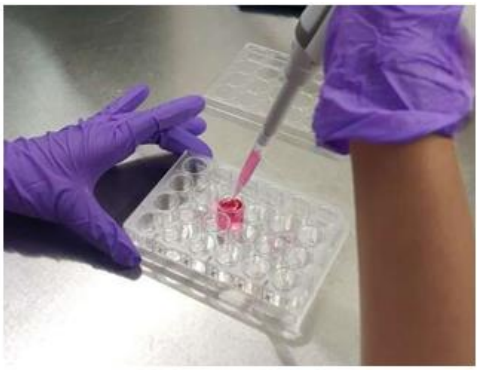


Figure 2 Gingival fibroblast cells seeded in 24 x 12 well plates (Test group)

Osteoblast-like cells

Control group: Cells not exposed to Extracorporeal Shock wave Therapy (Figure 3)

Test group: Cells exposed to Extracorporeal Shock wave Therapy (Figure 4).

Figure 3 Osteoblast-like cells seeded in 24 x 12 well plates (control group)

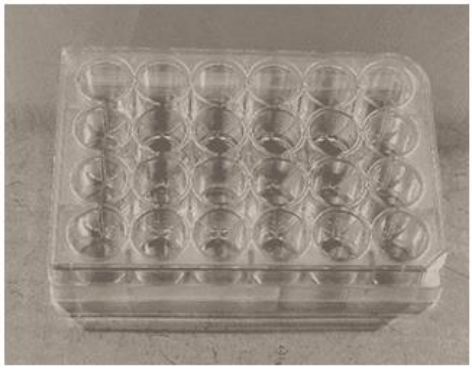


Figure 4 Osteoblast-like cells seeded in 24 x 12 well plates (Test group)

Human gingival fibroblast and osteoblast like cell and its culture

The fibroblast and osteoblast-like cell lines were stored in freezing medium, i.e., 50% Dimethyl Sulfoxide (DMSO). Revival of cells were carried out by placing them in the CO_2 incubator at 37°C in a humidified atmosphere containing 95% air and 5% CO_2 for 3 minutes. Once the cells were thawed, the fluid proportion was separated. Addition of Dulbecco's Modified Eagle Medium (DMEM) was done and centrifuged at a speed of 2000 rpm for 4 minutes. The cells from the sediment were seeded in culture flasks (T-25 flask) containing DMEM, 10% Fetal Bovine Serum (FBS) and was supplemented with 100 IU/mL penicillin and 100 µg/mL streptomycin solution. The growth medium was restored every 24 hours and observed until a confluency of 70% was attained. This procedure was repeated for three days in an anaerobic chamber.

Once the required confluency on day 3 was obtained, the fibroblast (Figure 5) and osteoblast- like cell cultures (Figure 6) were subjected to Wave therapy was considered as Test group and cell lines without wave therapy as Control group. Both the groups were assessed for cell proliferation and migration, and cell to cell interaction. This was done using Scratch assay. Rate of scratch closure was directly proportional to proliferative rate. Cell cultures were subjected for Cell viability using MTT Assay [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] and ATP (Cellular Adenosine Triphosphate) production for mitochondrial activity.

Figure 5 Microscopic image of confluent Fibroblast cells

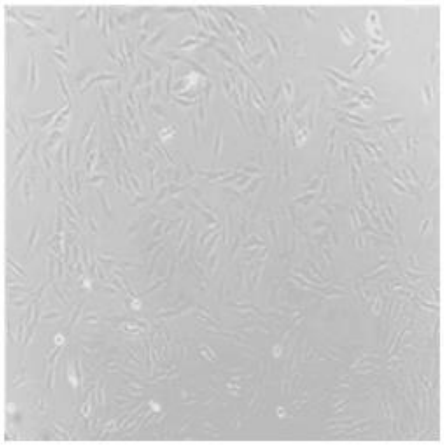


Figure 6 Microscopic image of confluent Osteoblast-like cells

Application of Extracorporeal Shock Wave

Shockwave device was modified to generate true shockwaves which are characterized by a sudden rise in pressure followed by an exponential decay. In our study, true pressure shockwaves were denoted as graded bar pressure on the panel of shockwave generator (super-wave technology Pvt, Ltd) (Figure. 7). Shock wave application was performed using 15-mm diameter shock wave transmitter (Figure. 8). The cells were seeded in sterile 24 x 12 well plates. The well plate was covered with parafilm and Shock wave treatment was given by placing the shock tube perpendicular to a point on parafilm such that the base of the shock tube comes in direct contact with parafilm. The excess medium was removed after the completion of treatment.

Figure 7 Shockwave generator

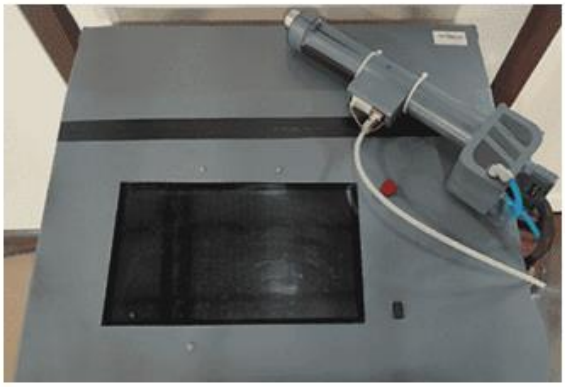


Figure 8 Shockwave transmitter

After cell confluency (70%-80%) was obtained, were treated with six pulses at a constant 2.5 bar pressure with a time gap of 2 secs between each shot. The generation of blast sound upon shock wave exposure, assured that the cells were exposed to shock waves (Figure. 9). The Control samples were maintained in the same culture conditions, without exposing to shockwaves (Şimşek *et al*., 2021).

Figure 9 Application of shockwaves

Cell Proliferation and Migration

The scratch migration assay, was conducted to determine the migration rates of cells with or without Extracorporeal Shock Wave Therapy (ESWT). Scratch wound assay of gingival fibroblast cells were performed using the live imaging system Cytosmart-Lux-2 live cell imager. The cells in the Control (without ESWT) and Test (ESWT) groups were seeded into a 24 x 12 plate at a density of 0.05×10^6 cells/plate and cultured at 37°C in a 5% CO_2 incubator. When the cells reached a confluency of 80%, the scratch was introduced using a 10µl sterile pipette tip and the images were captured every 30 minutes until complete closure was observed (Lu *et al*., 2020). Real-time cell migration status was investigated by closure of the scratch gap at regular intervals and quantified using scratch assay algorithm of Cytosmart-Lux-2 system.

Determination of Cell Viability – MTT Assay

Cell viability was determined using MTT Assay (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide). Cells were seeded in fibronectin coated 24 x 12 well plate for cultivation at 37°C. After 48 h, 20 mL of MTT 5g/L (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to each well at final concentration of 0.5mg/ml after 4 hours of shock wave treatment. Cells were cultured in a 24 x 12 well plate to 70% confluency and treated with shock wave pulses as described above. Cells were incubated in MTT medium for 3 hours at 37°C in the dark and formazan crystals were solubilized using 50% Dimethyl Sulfoxide (DMSO). Absorbance was read at 570 nm using Eppendorf Bio-spectrometer with DMSO as reference.

Cellular ATP Assay

The amount of intra/extracellular ATP due to increased mitochondrial activity is an indicator for the cellular viability. Intracellular ATP was assayed using ATP Determination Kit (Molecular ProbesTM Invitrogen detection technologies). The fibroblasts and osteoblast-like cells were cultured in 24 x 12 well plate to a 70% confluency and shock wave was applied. Immediately after the treatment the cells were trypsinized and the cell number was determined using Haemocytometer.

Cells were washed by centrifugation at 2000 rpm for 5 minutes 2 x times using Phosphate Buffered Saline (PBS) and the pellet was suspended in 100µl DI water for cell lysis. Boiling lysis was performed at 100°C for 10 minutes and the cell lysate was immediately incubated on ice. Lysate was centrifuged at 4° C at 1000 g for 1 minute and 10µl of the supernatant was assayed using the kit. Colorimetric readings were recorded for controls and shock wave treated samples and the total ATP concentration was determined using ATP standard curve (Priglinger *et al*., 2017).

Values obtained for Cell viability assessed through MTT Assay and ATP Assay were subjected to statistical analysis

Statistical Analysis

Statistical Package for Social Sciences [SPSS] for Windows, Version 22.0 Released 2013 Armonk, NY: IBM Corp., was used to perform statistical analyses. Descriptive analysis included expression of cell viability of human gingival fibroblasts and osteoblast-like cell lines in terms of mean and standard deviation for each group. Independent Student "t" test was used to compare the mean cell viability of fibroblast and osteoblast-like cell lines. Student paired "t" test was used to inter compare the mean cell viability between fibroblast and osteoblast like cell lines. The level of significance was set at P=0.05.

RESULTS

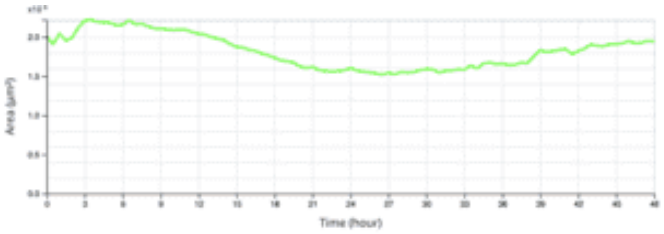
A total of twenty Fibroblast and 20 osteoblast-like cell lines were used for this study. Cell procurement was followed by culturing. Modification of equipment to deliver shockwave was done using true shockwave principle (McClure and Dorfmuller, 2003). Only, 10 Fibroblast (Test) and 10 osteoblast-like (Test) cell lines were exposed to shockwaves and remaining cell lines were not exposed to ESWT. Analysis for outcome using mean and standard deviation was done for categorical variables. Gingival fibroblasts and osteoblast-like cell lines were procured in a T-25 flask from Stroma Laboratories. Culturing of the cell lines and exposure to Extracorporeal Shockwave Therapy (ESWT) was executed in the Laboratory for Hypersonic and Shock Wave Research.

On Day 3, when appropriate confluent culture was seen, cell lines were subjected to Scratch Analysis. An exponential increase in proliferation and migration were observed in the test samples.

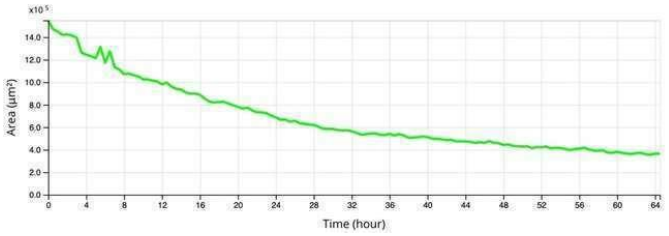
Gingival Fibroblasts Group

Observed as baseline, mid-point at 24th hour and end point till scratch closure (74th hour). Control samples demonstrated a slower rate of scratch closer [baseline (Day 3), 24th hour till scratch closure (98th hour)]. Shockwave treated cell lines closed faster (Figure. 11, Graph II) compared to the control

samples, which was represented through live imaging (Figure. 10, Graph I).



Graph I Scratch closure in control group – Fibroblasts



Graph II Scratch closure in Test group – Fibroblasts

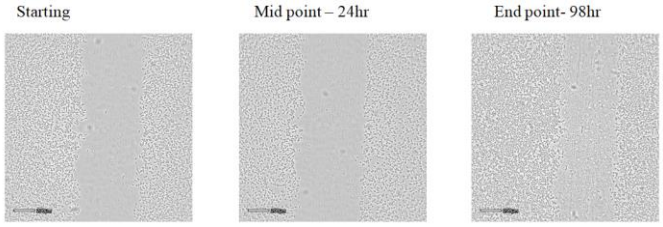


Figure 10 Scratch closure in Control group – fibroblasts

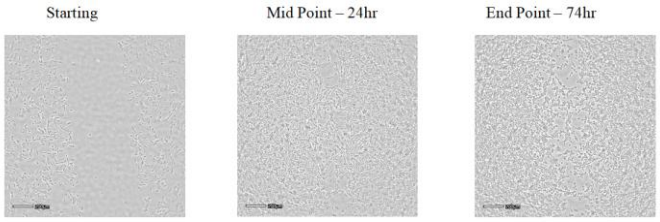


Figure 11 Scratch closure in Test group – fibroblasts

Osteoblast-like Group

Observed as baseline, mid-point at 12th hour and end point till scratch closure (27th hour). Control samples unexposed to ESWT were subjected to scratch analysis at baseline (Day 3), 20th hour till scratch closure (60th hour). Shockwave treated cell lines closes faster (Figure 13, Graph IV) as compared to the control samples, which was represented through Live imaging (Figure 12, Graph III).

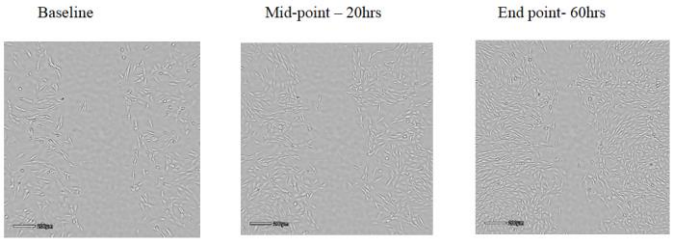
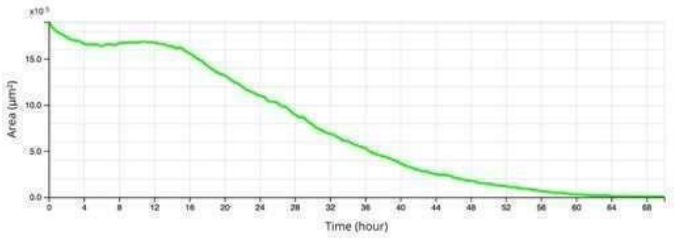


Figure 12 Scratch closure in control group – osteoblast-like cells



Graph III Scratch closure in control group – Osteoblast –like cells

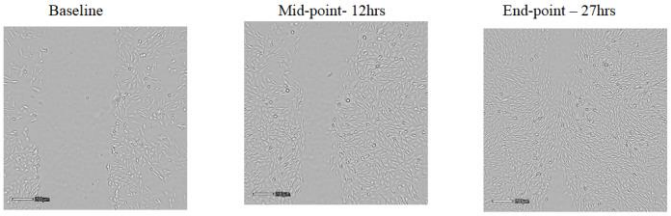
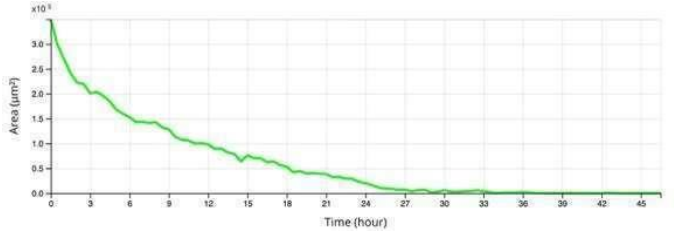


Figure 13 Scratch closure in test group- osteoblast-like cells



Graph IV Scratch closure in Test group – Osteoblast like cells

Intra group comparison in Control cell lines and Test cell lines by MTT Assay (Abs. 570nm)

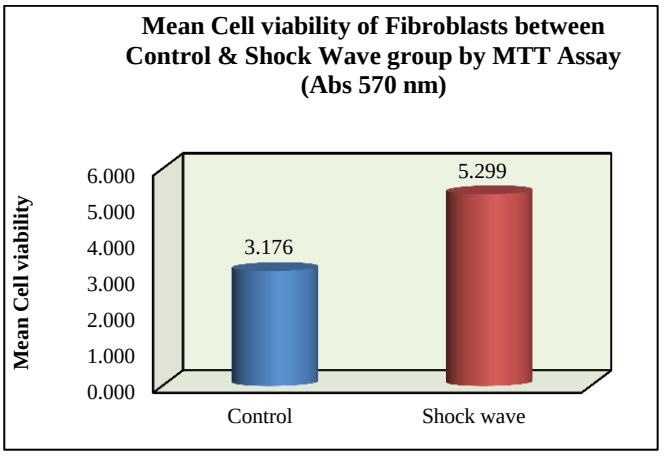
Gingival Fibroblasts

Comparison between the intervals was done using Independent Student't' test. The mean cell viability of control samples was 3.176±0.502 and for Post shockwave fibroblast cell lines showed 5.299 ± 0.402 which was statistically highly significant (P<0.001) compared to control. Mean difference between both groups was -2.123, which was also statistically highly significant (Table 1, Graph V).

Table 1 Intragroup comparison in control and test group by MTT Assay for gingival Fibroblasts

Comparison of mean Cell viability of Fibroblasts between Control & Shock Wave group by MTT Assay (Abs 570 nm) using Independent Student t Test

Parameter	Groups	N	Mean	SD	Mean Diff	p-value
Cell Viability of Fibroblasts	Control	10	3.176	0.502	-2.123	<0.001*
	Shock wave	10	5.299	0.402		



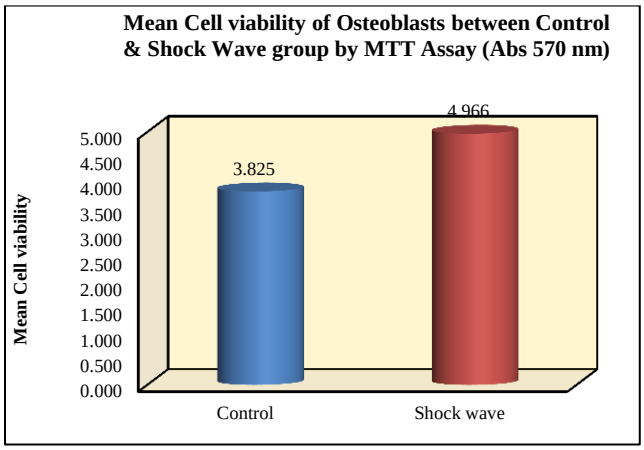
Graph V Intragroup comparison in control and test group by MTT Assay for gingival Fibroblasts

Osteoblast – like cells

The mean cell viability of control samples was 3.825±0.48 and for Post shockwave Osteoblast-like cell lines showed 4.966 ± 0.54 which was statistically highly significant (P<0.001) compared to control. Mean difference between both groups was -1.141, which was also statistically highly significant (Table 2, Graph. VI).

Table 2 Intragroup comparison in control and test group by MTT Assay for osteoblast like cells

Comparison of mean Cell viability of Osteoblasts between Control & Shock Wave group by MTT Assay (Abs 570 nm) using Independent Student t Test						
Parameter	**Groups**	**N**	**Mean**	**SD**	**Mean Diff**	**p-value**
Cell Viability of Osteoblasts	Control	10	3.825	0.475	-1.141	<0.001*
	Shock wave	10	4.966	0.535		



Graph VI Intragroup comparison in control and test group by MTT Assay for osteoblast like cells

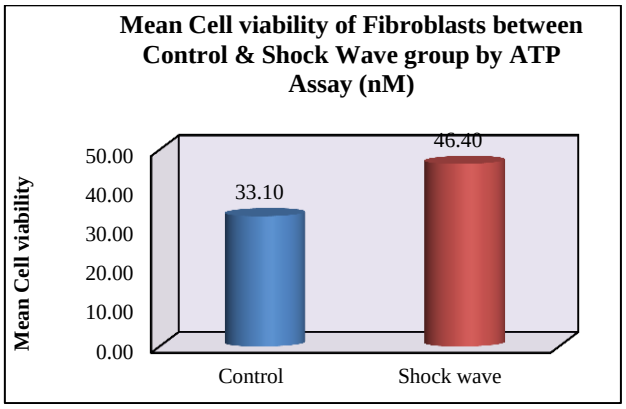
Intra Group Comparison in Control and Shockwave by ATP Assay (nm)

Gingival Fibroblasts

Comparison between study intervals was done using Independent Student't' test. Mean cell viability through ATP assay of 33.10±9.31 for untreated group and 46.40±5.93 for the test samples was seen. Shockwave group was significantly higher as compared to control (P<0.001). Mean difference between both groups was -13.300, was statistically highly significant (P<0.001) (Table 3, Graph VII).

Table 3 Intragroup comparison in control and test group by ATP Assay for gingival Fibroblasts

Comparison of mean Cell viability of Fibroblasts between Control & Shock Wave group by ATP Assay (nM) using Independent Student t Test						
Parameter	**Groups**	**N**	**Mean**	**SD**	**Mean Diff**	**p-value**
Cell Viability of Fibroblasts	Control	10	33.10	9.31	-13.300	0.001*
	Shock wave	10	46.40	5.93		



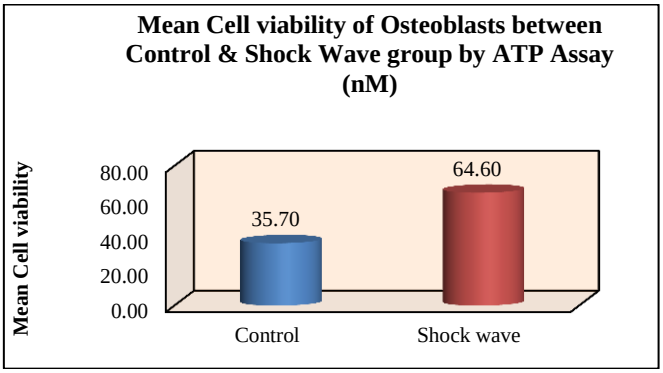
Graph VII Intragroup comparison in control and test group by ATP Assay for gingival Fibroblasts

Osteoblast-like cells

Mean cell viability through ATP assay of 35.70±13.14 for untreated group and 64.60±8.87 for the test samples was seen. Shockwave group was significantly higher as compared to control (P<0.001). Mean difference between both groups was -28.900, was statistically highly significant (P<0.001) (Table 4, Graph. VIII).

Table 4 Intragroup comparison in control and test group by ATP Assay for osteoblast like cells

Comparison of mean Cell viability of Osteoblasts between Control & Shock Wave group by ATP Assay (nM) using Independent Student t Test						
Parameter	**Groups**	**N**	**Mean**	**SD**	**Mean Diff**	**p-value**
Cell Viability of Osteoblasts	Control	10	35.70	13.14	-28.900	<0.001*
	Shock wave	10	64.60	8.87		



Graph VIII Intragroup comparison in control and test group by ATP Assay for osteoblast like cells

Comparison of Mean cell Viability between MTT and ATP Assay

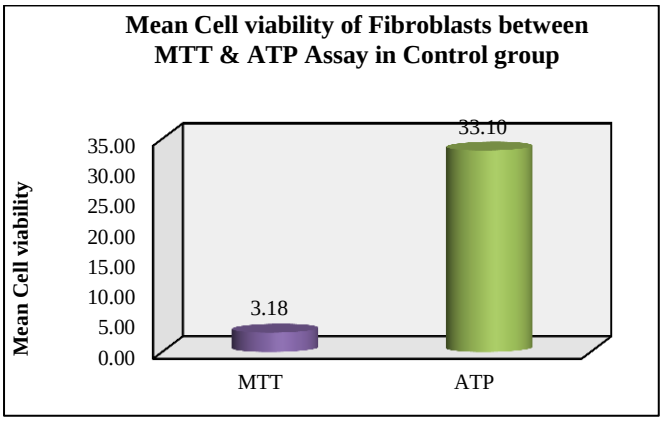
Gingival Fibroblasts

In Control Group,

Mean cell viability as determined by ATP assay showed significantly higher value of 33.10±9.31 as compared to MTT Assay with 3.18±0.50. Mean difference in the cell viability of Fibroblasts between two assays was statistically highly significant at P<0.001 (Table 5, Graph IX).

Table 5 Comparison of mean cell viability of Fibroblast cells between MTT and ATP Assay (Control Group)

Comparison of mean Cell viability of Fibroblasts between MTT & ATP Assay in Control group using Student Paired t Test						
Group	**Assay**	**N**	**Mean**	**SD**	**Mean Diff**	**p-value**
Control	MTT	10	3.18	0.50	-29.924	<0.001*
	ATP	10	33.10	9.31		



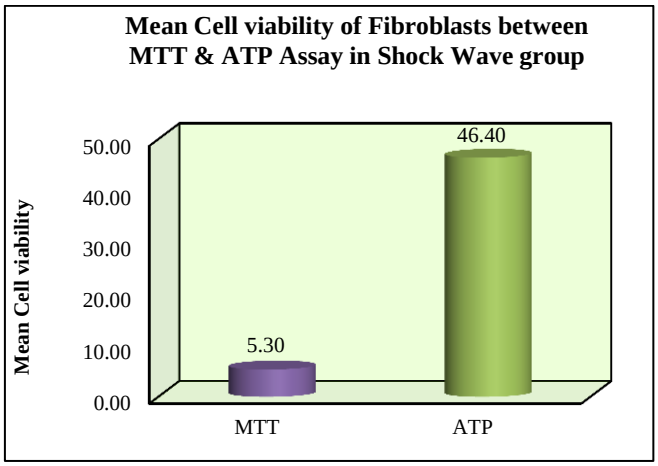
Graph IX Comparison of mean cell viability of Fibroblast cells between MTT and ATP Assay (Control Group)

In Shockwave Group

Mean cell viability as determined by ATP assay showed significantly higher value of 46.40±5.93 as compared to MTT Assay with 5.30±0.40. Mean difference in the cell viability of Fibroblast cells between two assays was statistically significant at P<0.001 (Table 6, Graph X).

Table 6 Comparison of mean cell viability of fibroblast cells between MTT and ATP Assay (Test Group)

Comparison of mean Cell viability of Fibroblasts between MTT & ATP Assay in Shock Wave group using Student Paired t Test						
Group	**Assay**	**N**	**Mean**	**SD**	**Mean Diff**	**p-value**
Shock wave	MTT	10	5.30	0.40	-41.101	<0.001*
	ATP	10	46.40	5.93		



Graph X Comparison of mean cell viability of fibroblast cells between MTT and ATP Assay (Test Group)

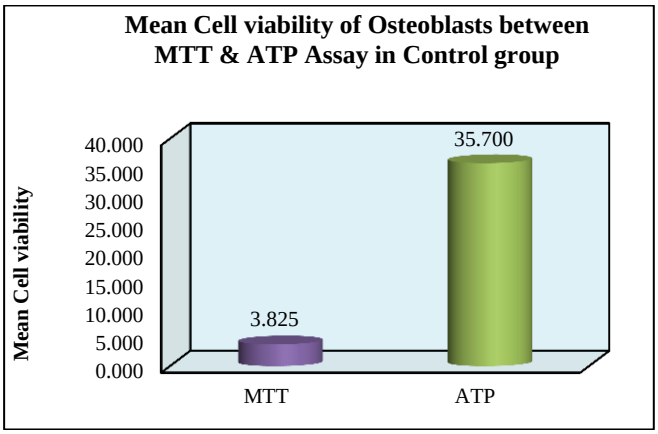
Osteoblast-like cells

In Control Group

Mean cell viability as determined by ATP assay showed significantly higher value of 35.70±13.14 as compared to MTT Assay with 3.825±0.48. Mean difference in the cell viability of Osteoblast-like cells between two assays was statistically highly significant at P<0.001 (Table 7, Graph XI).

Table 7 Comparison of mean cell viability of Osteoblast-like cells between MTT and ATP Assay (Tontrol Group)

Comparison of mean Cell viability of Osteoblasts between MTT & ATP Assay in Control group using Student Paired t Test						
Group	Assay	N	Mean	SD	Mean Diff	p-value
Control	MTT	10	3.825	0.475	-31.875	<0.001*
	ATP	10	35.700	13.141		



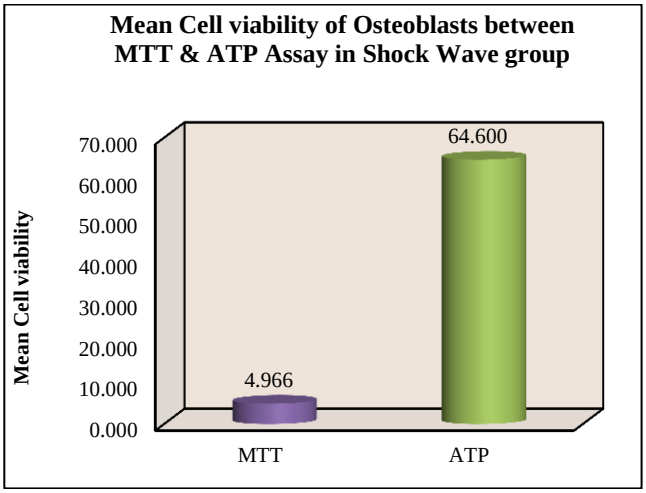
Graph XI Comparison of mean cell viability of Osteoblast-like cells between MTT and ATP Assay (Tontrol Group)

In Shockwave Group

Mean cell viability as determined by ATP assay showed significantly higher value of 64.60±8.87 as compared to MTT Assay with 4.97±0.54. Mean difference in the cell viability of Osteoblast-like cells between two assays was statistically significant at P<0.001 (Table 8, Graph XII).

Table 8 Comparison of mean cell viability of Osteoblast-like cells between MTT and ATP Assay (Test Group)

Comparison of mean Cell viability of Osteoblasts between MTT & ATP Assay in Shock Wave group using Student Paired t Test						
Group	**Assay**	**N**	**Mean**	**SD**	**Mean Diff**	**p-value**
Shock wave	MTT	10	4.966	0.535	-59.634	<0.001*
	ATP	10	64.600	8.872		



Graph XII Comparison of mean cell viability of Osteoblast-like cells between MTT and ATP Assay (Test Group)

DISCUSSION

Periodontal disease leads to destruction of periodontal ligament and adjacent supporting alveolar bone (William RC, 1990). Loss of attachment is the hallmark of periodontal disease. ESWT has successfully promoted regeneration of alveolar bone lost following experimentally induced periodontal disease (Satishkumar *et al.*, 2008). Since pocket formation is a site-specific disease, in future application of ESWT requires a prior knowledge of the effect of shockwaves at cellular level. To increase the efficiency of acoustic waves, technical improvements are still ongoing for this non-invasive intervention. The present study is an attempt undertaken with the aim to evaluate the effect of extracorporeal shockwave therapy on fibroblast cells and Osteoblast-like cells.

Acoustic wave was initially introduced in urology for lithotripsy. Shockwaves are generated extracorporeally (electrohydraulic, piezoelectric, or electromagnetic). The resulting energy is focused by concentrating reflectors and is noninvasively transmitted inside the body to induce therapeutic effects at a target area (Novak P, 2014).

Shockwave treatment has been successfully treating acute and chronic conditions in medical field. There is sparsity of literature pertaining to the use of extracorporeal shockwave therapy in oral cavity. It has been hypothesized that ESWT could promote the regeneration of alveolar bone (Satishkumar, 2008). Shockwave is dose dependent, and improves symptoms over time. Many studies have emphasized the beneficial effects of shockwave therapy (Prabhuji MLV *et al*., 2014).

(Huang ZL *et al*., 2010) evaluated the effect of extracorporeal shock wave on proliferation, differentiation, adhesion and migration of osteoblasts in Sprague-Dawley (SD) rats. Migration of osteoblasts was assessed through wound healing assay. The mean migration of osteoblasts in the shock wave treated group at 12 and 24 hours after wound formation was greater suggesting that shock wave could promote the migration of osteoblasts. Six points were photographed at 12 and 24 hours after the scar was made under a microscope at 40 × magnification, and the mean migration rate was determined and analysed using the Image Pro Plus 6.0 software. In our study, culture plates were selected for scratch analysis. 10-µL pipette was used to perform scratch on the cultured plate at 3rd confluent day. Scratch analysis was done using live imaging microscope at midpoint 12 hours and till end point of 27 hours for the shockwave group and 20 hours and 60 hours for the control group to demonstrate closure. Our study was similar in the analysis of migratory activity for the osteoblast-like cells to the above-mentioned study. Both the studies have demonstrated greater closure in shockwave treated group.

In this study, true pressure shockwaves were generated (super-wave technology Pvt, Ltd). Previous studies (Şimşek *et al*., 2021); Basoli *et al*., 2020) on fibroblasts, have used radial shockwave transmitter / Ortho-Gold100 Shock wave device with varying pulses of shockwave and energy flux densities unlike our study. This study is the first of its kind involving primary gingival fibroblast cell lines from healthy gingival tissue and osteoblast like cells, being subjected to a specifically developed shockwave therapy.

(Martini L, 2006) studied the early effect of ESWT on osteoblast-like cells (MG-63) using different types (electromagnetic and electrohydraulic) of shockwave generators. Similar to their study, we have included MG-63 osteoblast-like cells in the present study. Cell culture and exposure to shockwave therapy (procedure) was almost same in both the studies. Contrary to the above study, we have applied a newly developed handheld shockwave device using true shockwaves mechanism unlike their study checking the effect of shockwaves using two different shockwave generators on MG-63 cell lines.

(Jelaska *et al*., 2000) Studies on fibroblast in-vitro and in-vivo confirm that shockwave stimulate fibroblastic activity. It is now scientifically established that ESWT activate fibroblast proliferative rate, collagen synthesis and elevation of growth factors. Studies show data can be related to increased blood flow observed in ESW treated tissue. (Frairia and Berta, 2011) Fibroblasts activity have been evaluated through bi-culture and tri-cultures with shockwave application in-vitro, with formation of multilayer epithelium layer of oral mucosal equivalents. (Peters *et al*., 2022) Our study also showed positive effects of ESWT at a molecular level.

In Martini L *et al*., study (2006) cell viability assessment was done through MTT assay. In their study, evaluation clearly demonstrates the influence of type of device and electro flux density (EFD) used at 24- and 72-hours post shockwave therapy. Similarly, our study also evaluated using MTT assay at 72 hours. In the current study, cell viability has been assessed using MTT and ATP Assay. Direct conversion of electrical pulses to acoustic waves was done with the modified equipment to the handheld transmitter which was demonstrated as acoustic bars on the display panel. Only MTT assay was similar in both the studies.

(Satishkumar *et al*., 2008) checked the effect of shockwave therapy for alveolar bone regeneration on rat model of Periodontitis. This study has mentioned sparsity of dental literature regarding usage of ESWT and its effects on the healing of periodontal tissue and alveolar bone resorption that result from chronic inflammation of periodontitis. Here, application of unfocused shockwaves at EFD 0.1mJ/mm^2 with single application of 100, 300, or 1000 impulses was done with the pulse rate applied, 5 pulses per second. Similarly, the present in-vitro study has been undertaken with the goal to evaluate the effect of ESWT on Fibroblast and osteoblasts like cells, at cellular level. Contrarily, we have used newly developed shockwave device with a handheld transmitter using true shockwaves generation principle. We have applied ESWT at 6 pulses with 2 second interval in multiple application at 2.5 bar pressure. (Satishkumar *et al*., 2008) concluded ESWT can be used as adjunct in regeneration of periodontal tissue in the management of periodontal bone loss. They have also evaluated oral microbial samples through Polymerase Chain Reaction (PCR) analysis and radiographically assessed the alveolar bone resorption.

(Muller P *et al*., 2011) assessed the potential of shockwave device to remove calculus and biofilm and to kill bacteria on extracted teeth. They compared shockwave device versus magnetostrictive ultrasonic scaler in the efficacy of calculus and biofilm removal. Focused shockwave was used to remove biofilms by three log steps but was not able to reliably remove calculus. Randomly selected teeth were treated with shockwave device (Duolith, Storz Medical AG, Tägerwilen, Switzerland) at an energy density of 0.4mJ/mm^2 and a frequency of 3Hz. The device was moved perpendicularly on the root surface in very small elliptical pattern. The working end of the tip was submerged in the saline solution (cavitation). Muller *et al* (2011) in-vitro study has assessed the application of shockwave on teeth wherein our study used true shockwaves wherein electrical energy is converted to pressure energy and generated shockwave on Fibroblasts and osteoblast-like cells.

Foldager *et al*., (2012) published in a narrative review on application of extracorporeal shockwave in orthopaedics using focused versus unfocused shockwaves. This review concluded that shockwave does not delineate focused and unfocused shockwaves but proposed, using Total Energy Dose (TED) allowed for integration of the EFD and the number of pulses, which has been proposed as a good predictor correlating the

variables to tissue response. Our study has utilized this concept in the latest device from Laboratory for Hypersonic and Shock Wave Research to deliver focused shockwave which is characterized by a sudden rise with an exponential fall in the transmitted shockwave (TED) on Fibroblasts and Osteoblast-like cell lines.

Altuntas EE *et al* (2012) evaluated the effect of shockwave therapy on healing of subcondylar mandibular fractures in albino rats. A single ESWT of 500 pulses, 4 bars, and 1Hz was applied in their study. Ultrasound transmission gel was used as a contact medium between the ESW device and the skin of rats. This study checked the effect of ESWT in-vivo. Contrarily, in our in-vitro investigation on the effect of ESWT, 6 pulses with 2.5 bar pressure applied with a 2 second time interval on Fibroblasts and Osteoblast-like cells. Altuntas *et al*., concluded that shockwave therapy would contribute to prevent complications such as ankyloses, fibrosis, and hypermobility occurring because of prolonged fixation. The above-mentioned study proposed the application of ESWT in clinical application wherein our study demonstrates the effect of ESWT at molecular level.

Datey A *et al*., (2016) conducted an initial study on extracted teeth to check the efficacy of desensitizing agents with shockwave treatment. In this in-vitro study, a hand held shockwave generator with focused shockwave generated in a particular region was used where the energy in the shockwave emanating from the open end of the polymer tube used was estimated to be around 1.25 joules. This is similar to the our study wherein the electrical energy was directly converted to true shock waves and pressure transferred to a hand held transmitter represented as bars on the display panel of the shockwave device.

Datey A *et al*., (2019) studied on investigating the effect of shockwaves to cure multispecies chronic periodontitis in a humanized rat model. Tracing paper (95 GSM) was used as the diaphragm in the shock tube and generation of shockwave using hand held device, 2.5 fill pressure, peak amplitude and steady times of the shockwave generated were 14 bars and 20µs respectively. They observed the effect of shockwave on dense complex biofilms and concluded that shockwave therapy disrupted biofilm into multiple isolated colonies of microorganisms on exposure. This is an investigation further to elucidate the effect of shockwaves in a clinical scenario unlike our study used parafilm as impedence for transmitting true shockwaves investigating its effect on Fibroblasts and osteoblastic activity.

Song WP *et al*., (2020) hypothesized the effect of shockwave therapy to be helpful in the osseointegration of dental implants. He has elucidated that the effect of shockwaves on calculus does not support the present hypothesis that it promotes osseointegration and has referred to (Rompe *et al*., 1998) study on rabbits. Rompe *et al*., (1998) has graded shockwaves according to electro flux density (EFD) for the first time and found that high energy shockwaves (EFD > 0.6mmJ/mm2) had a destructive effect on tissues and could be used in the treatment of gravel, while medium and low energy shockwave had a positive effect on tissue healing. Our study has generated true shockwaves without electrical manipulation, this creates impedence and pressure that can directly be transmitted to the hand device to deliver shockwaves.

In the present study, Gingival fibroblasts and Osteoblast-like cell lines were procured for the investigation. Source of the Osteoblast-like cells were derived from human osteosarcoma bone. Periodontal disease being site specific, osteoblast obtained from these sites were an appropriate option. Further studies, evaluating the effect of shockwaves on fibroblasts are important as these cells (osteoblasts and fibroblasts) make up the bulk of periodontal structure. Shockwave effect on humanized animal models form the next step of evaluation before applying ESWT in clinical scenario.

CONCLUSION

The present results showed promising effect of Extracorporeal Shockwave Therapy (ESWT) on gingival fibroblast cells in cell migration through faster scratch closure and cell viability confirmed through Bio-spectrometry than control group. In the present study, modified extracorporeal device using true shock waves was utilized. This device is simple in execution where pressure is directly transferred and controlled through a hand held transmitter. In future, ESWT would be a non-invasive treatment with least complications in oral treatment. This molecular study may pave way for application of ESWT in clinical scenario. It can be a potent adjunct to conventional periodontal therapy. As an emerging treatment aspect, ESWT may be a useful option for management of variety of periodontal ailments.

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