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Shock Wave-Stimulated Periosteum for Cartilage Repair

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13. SUPPLEMENTARY NOTES

14. ABSTRACT
The primary objective of this project is to determine if extracorporeal shock wave (ESW)-stimulated periosteum improves cartilage repair when it is used as an autograft to fill a defect in the articular surface of goats. A miniature fiber optic pressure sensor will be inserted into the tibial periosteum of 6 animals to measure the actual shock waveform in the tissue for two ESW doses (energy densities). In 12 goats, tibial periosteum stimulated by one of the 2 doses of ESWs (n=6) will be harvested, 4 days post-treatment, as an autograft for implantation into one 1 cm2 defect surgically produced in the trochlear groove of the knee joint of the same goat. Non-ESW-treated periosteum will serve as the control group (n=6). All animals will be sacrificed after 16 weeks, and the reparative tissue will be quantified histomorphometrically by determining the areal percentage of selected tissues in the original cartilage defect area.

15. SUBJECT TERMS- Shock waves, cartilage, goat

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I. INTRODUCTION

The primary objective of this project is to determine if extracorporeal shock wave (ESW)-stimulated periosteum improves cartilage repair when it is used as an autograft to fill a defect in the articular surface of goats. Periosteum, which contains cells with chondrogenic potential, has been investigated as an autograft for cartilage repair procedures. However, this approach is limited because the cambium layer of the periosteum is normally only 2-5 cells thick, and some of these cells are lost during the harvest procedure. We have recently demonstrated that extracorporeal shock waves, at doses approved by FDA for treatment of certain disorders, can stimulate up to a 10-fold increase in the thickness of rat periosteum after only 4 days.

This protocol deals with the therapeutic use of shock waves. Shock waves are pressure waves of very short duration (a few microseconds). The initial peak compressive wave is followed by a lower amplitude tensile wave. The ESWs can be produced by apparatus that focus the waves at a certain location in the body or produce waves which radiate from the shock wave head. Normally treatments apply up to 3000 shocks in a session. The "dose" of shock waves is measured in energy density. For our studies the energy densities to be used will be: 0.15 mJ/mm2 ("low") and 0.45 mJ/mm2 ("high"). This range is approved by FDA for other indications than the one we will be investigating, but it demonstrates that shock waves in this dose range have an acceptable safety profile.

During this 6-month period of the project we investigated: 1) the effects of shock waves from 2 types of ESW apparatus and at different doses (*i.e.*, energy density and number of shocks) on the periosteum of goats; 2) the specific locations at which we administer the shock waves, because the periosteum varies with respect to its thickness at various anatomic locations; and 3) a cell culture system to screen the various types of ESWs.

II. BODY

Following are the tasks comprising the Statement of Work and our related achievements

Specific Aim #1

Task 1. Measurement of the ESW pressure waveform in the periosteum of goats

1.a. Measure the pressure waveforms for select settings of the ESW apparatus in a water bath, for specific locations of sensor away from the head of the ESW device.

1.b. Insert a pressure sensor into the periosteum of the right proximal tibial of6 goats to measure the pressure waveform in the periosteum for select settings of the ESW apparatus.

The data related to these tasks were documented in the Annual Report of December, 2011

Specific Aim #2

Task 2. Histological evaluation of the thickness and number and type of cells in the ESW-stimulated periosteum and controls

2.a. Histological processing of periosteum from 12 goats which underwent ESW treatment and from 6 sham-treated goats

2.b. Histomorphometric evaluation of the thickness of the periosteum and number and type of cells making up the periosteum

The histological evaluation of the periosteal response to various types of shock waves is ongoing.

Task 3. Determination of the chondrogenic potential of ESW-stimulated cambium cells *in vitro*

3.a. Isolate cells from the enzymatically-digested ESW-stimulated periosteum and sham-treated controls, 4 days post-ESW treatment, and grow in monolayer

3.b. Produce pellet cultures of the periosteal cells in chondrogenic medium

3.c. Process pellets for histological evaluation

3.d. Histomorphometric evaluation of the pellet

We have implemented cultures of mesenchymal stem cells (MSCs) in suspension and in hydrogels as tissue simulants in an effort to: 1) enable us to compare the chondrogenic and osteogenic potential of cells exposed to ESWs *in vivo* with cells exposed to ESWs *in vitro*; and 2) to screen the effects of the various shock wave parameters *in vitro* prior to the animal evaluations. We are currently using this *in vitro* system to evaluate the effects of various types of shock waves at various doses on the cell viability and proliferation of MSCs, using platelet-derived growth factor (PDGF)-BB as a control stimulation.

Materials and Methods

A gelatin-hydoxyphenylpropionic acid (Gtn-HPA) hydrogel was prepared as a 3-dimensional matrix to be seeded with the ESW-stimulated and non-stimulated cells to investigate selected cell behavior. The 2% (by wt.) gel was cross-linked with 0.1 U/ml horseradish peroxidase (HRP) and 1.2 mM hydrogen peroxide (H_2O_2). Bone marrow-derived mesenchymal stem cells (MSCs) serving as periosteal cell surrogates were isolated from bone marrow aspirates of adult Spanish goats (10^5cells/ml bMSCs in the experiments). Focused ESWs (Piezoclast EMS) were used at the following doses:

ESW1: 0.1mJ/mm^2 x 500 impulses

ESW2: 0.4mJ/mm^2 x 500 impulses at 8Hz.

ESW treatment was applied to bMSCs in suspension in a test tube, and the cells subsequently seeded into the hydrogel. bMSC viability in the Gtn-HPA gel containing control and treated bMSCs was determined using a fluorescent live/dead assay (Calcein AM/EthD-1) at day 1.

The capability of the cells to proliferate was evaluated using a proliferation assay. The bilayer hydrogel construct consisted of a Gtn-HPA gel bottom layer containing 1000ng/ml PDGF-BB and a collagen gel seeded with 10^5 cells/ml bMSCs on top. MSC proliferation was measured using DNA PicoGreen assay at days 0, 1, 4, 7 and 14.

For a differentiation assay, Gtn-HPA-encapsulated bMSCs were induced into osteogenesis via osteogenic medium for 21 days; cryosections were then stained with Von Kossa and Alizarin Red to examine mineralization.

Results

Cell Viability after ESW stimulation

MSCs encapsulated within the Gtn-HPA gel survived the HRP and H_2O_2 induced cross-linking process (blank group in Fig. 1). bMSCs stimulated by ESWs and PDGF-BB also remained viable in the gel (Fig. 1). While there was a decrease (of about 10%) in the number of viable ESW-stimulated cells after the first 24 hours, the large variability questions the meaningfulness of the reduction in viability.

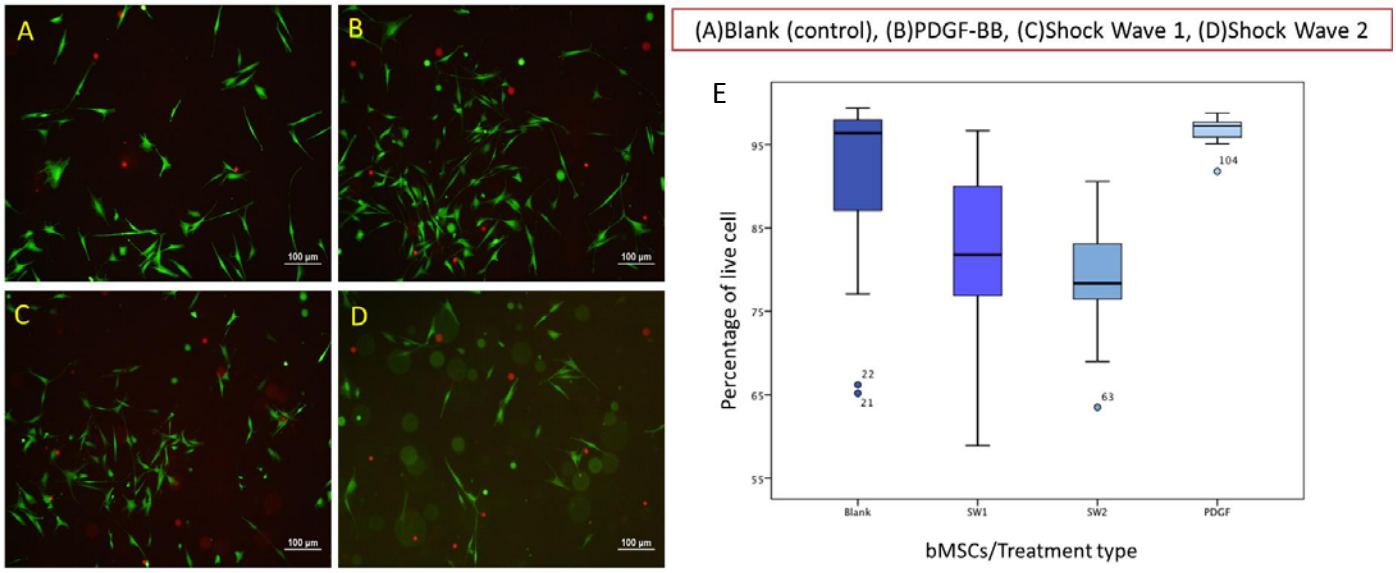

Fig. 1 (A-D), Live/dead fluorescence images of Calcein AM (green) indicating live MSCs and AthD-1 (red) indicating dead MSCs seeded in a GTN-HPA gel, 24 hours post-stimulation with ESWs and PDGF-BB as a control. (E), 24-hour viability assay, bMSCs in 2 % GTN-HPA of different groups (n=4).

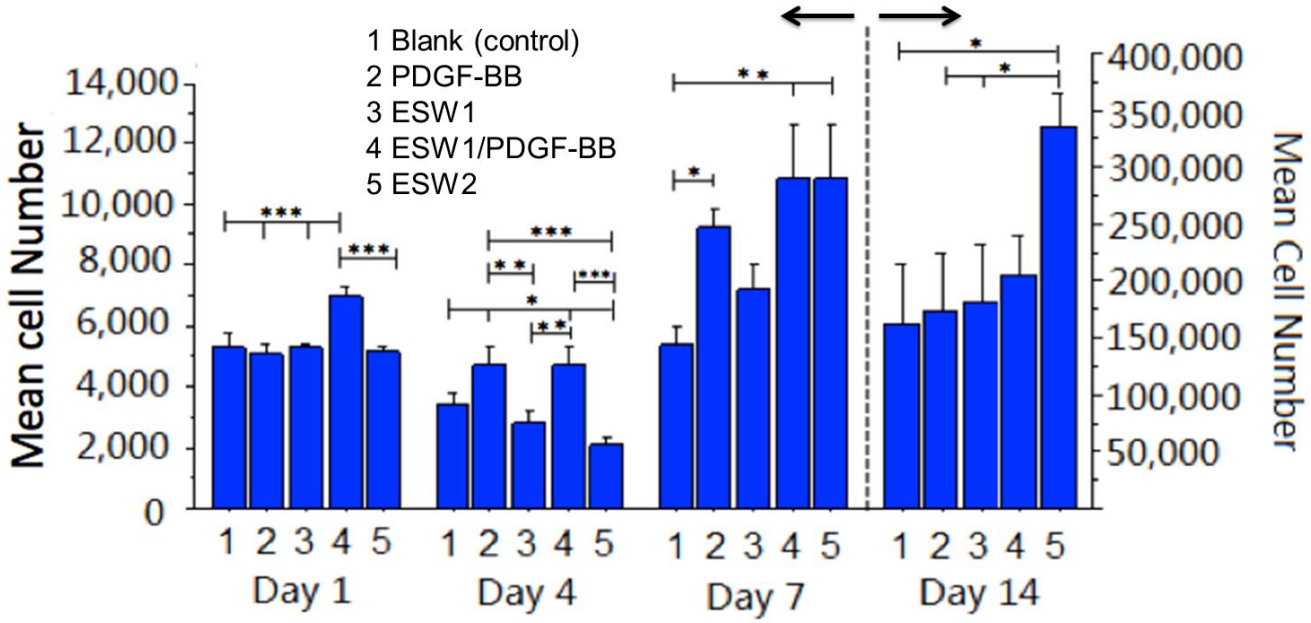

Fig. 2. Effect of different stimulants on bMSC proliferation in Gtn-HPA hydrogel constructs at days 1, 4, 7, and 14. (n=6).

MSC Proliferation

Of note, after 7 days, MSCs stimulated by ESW2 alone proliferated slightly more than cells stimulated by PDGF-BB (Fig. 2). By 14 days the ESW2-stimulated cells exceeded by 2-fold the number of cells stimulated by PDGF-BB. There were no additive or synergistic effects of the combination of ESWs and PDGF-BB on cell proliferation.

MSC Osteogenic Differentiation in the Gel

Gtn-HPA hydrogels supported the differentiation of MSCs into osteogenic-like cells. Gtn-HPA cross-linking supported the intercellular communication and mineral formation within different layers of the gel construct. Work in progress is assessing the effects of ESW treatment n osteogenesis using this model.

Discussion

This set of experiments demonstrated that Gtn-HPA hydrogels could provide a supportive environment for MSC survival, proliferation, and differentiation of cells stimulated by ESWs. A notable finding was that after 14 days, ESWs alone stimulated a 2-fold greater proliferation of MSCs compared to PDGF-BB. ESW treatment of bone marrow (and periosteum) could increase greatly the number of osteogenic cells for boner reconstruction. Gtn-HPA hydrogels are commended by their tunable cross-linking and preservation of the bioactivity of growth factors such as PDGF-BB.

Specific Aim #3

Task 4. Evaluation of the cartilage repair induced by ESW-stimulated periosteum in a goat model

4.a. ESW stimulation of the periosteum in the proximal tibia

4.b. Harvest of the ESW-treated periosteum and sham controls after 4 days, and implantation into chondral defects in the trochlear groove of the same animals

4.c. Sacrifice of the goats 16 weeks post-implantation, and processing of tissue for histological evaluation.

4.d. Histomorphometric evaluation of the cartilage repair
This aim is to be achieved in future experiments.

III. KEY RESEARCH ACCOMPLISHMENTS

- The use of a standardized cell culture system to screen the effects of various types of ESW at select doses has begun.
- ESWs of select magnitude increase the stimulation of MSCs greater than 2-fold compared to a known mitogen, PDGF-BB.

IV. REPORTABLE OUTCOMES

The results described above in Task 3 were reported at the International Symposium on Osteology in Monaco, May 2-4, 2013.

V. CONCLUSIONS

These results demonstrate the range of tissue responses that can be induced by ESWs.

VI. REFERENCES

None

VII. APPENDICES None