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A Quantitative Method for the Application of Compressive Forces to Bone in Tissue Culture

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A quantitative method for the application of compressive forces to long bones in culture is described. Using this method it was found that a physiological pressure of 80 g/cm sq. applied to tibiae of 16-day-old chick embryos reduced glucose consumption to 50% of controls. Twenty four hours after the release of pressure glucose utilization again increased, approaching control levels. A pressure of 80 g/cm sq. also stimulated thymidine incorporation into DNA. The same pressure decreased the size of the extracellular fluid pool by 8%, but had no effect on the rate of equilibration of this pool with ^{22}Na.

Key words: Bone remodeling — Organ culture — Pressure.

Introduction

Eighty years ago Wolff recognized that living bone will change its internal architecture to counteract the mechanical forces exerted upon it [14]. This environmental effect on development and tissue remodeling is still poorly understood. Current theories, based on evidence from a variety of experimental systems, relate the effect of mechanical stimuli on bone remodeling to: 1) electric (piezoelectric) events [2, 5]; 2) changes in the oxygen supply to the tissue [3, 9]; 3) the ionic activity of calcium ions altered because of pressure-dependent salt solubility [10]. Evaluation of these hypotheses requires an experimental system which would allow quantitative, reproducible application of forces to living bone under near physiological conditions.

Several classic studies relating mechanical effects to bone differentiation have been performed in tissue culture. Glücksmann was probably the first to use bone cultures for this purpose [6]. He concluded that mechanical stress stimulates osteogenesis *in vitro*, thus excluding vascularization *per se* as a main regulating factor. Effects of compressive and tensile forces at different concentrations of oxygen, on the differentiation of connective tissue into bone and/or cartilage in culture were later extensively studied by Bassett and his collaborators [1]. More recently Hall reviewed some of the contradictory evidence on the subject [8]. In none of these studies was there quantitative assessment of magnitude, direction, duration or frequency of the forces applied, and comparison to physiological conditions was not possible. The purpose of this paper is to present a simple method of applying quantitated compressive forces to long bones in culture at controlled durations and frequencies. A similar approach was previously used by Solomons *et al.*, who studied the effect of tensile forces on the washout curves of ^{32}P from *in vivo* labeled rat femora [13].

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Materials and Methods

Tibiae from embryonic or newborn chicks or rats were aseptically dissected free of muscle, under a laminar air flow hood. The tibiae from each animal were immediately placed into 5 cm petri dishes and kept in culture medium at 37° until all bones were dissected. The accuracy of the dissections yielded tibial pairs which were within 4.3% (coefficient of variation) of each other with respect to their dry weights after 24 h at 110°.

The culture medium, which was exposed to atmospheric air and was changed every 24 h, had the following composition: Minimum Essential Medium (Eagle) with Gey's balanced salt solution supplemented with fetal calf serum (10%), antibiotic-antimycotic mixture (1%), ascorbic acid (0.15 mg/ml), zinc (1.00 μg/ml), manganese (0.03 μg/ml) and Hepes buffer (0.02 M), pH 7.40 (Grand Island Biological Company, Grand Island, New York). Each tibia was cultured in a sterile disposable 17 × 100 mm polypropylene tube inserted in a rotating drum (Labline) within a vapor saturated 37° incubator (Forma Scientific, model 3149).

The apparatus for the application of pressure (Fig. 1) was made from glass tuberculin syringes (Eisele & Co., Nashville, Tenn.) with individually matched polished pistons. A 1 cm segment of the piston lubricated with mineral oil transmitted pressure to the bones. The tibiae were mounted between the piston and a titanium support frame attached to the syringe barrel by spring action above the lips of the syringe. The bone was held in place by the pressure exerted along its long axis. The syringe was fixed in the center of the culture vial by stainless steel spiderleg-shaped wires attached to a bracelet collar mounted on the syringe. Air pressure was transmitted to the pistons through a 3-way teflon stopcock mounted on the needle end of the syringe and connected through a ID 1/8″ (3.125 mm), ED 1/4″ (6.25 mm) tygon tube to a manifold system consisting of 5.5 aquarium gang valve (1971 Penn Plax, Inc.). This, in turn, was connected to an air tight 360° swivel at the center

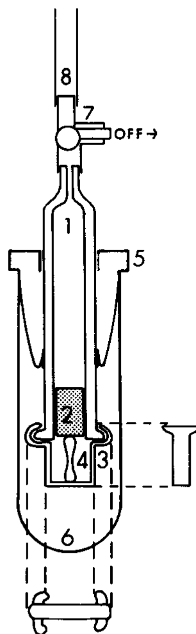

Fig. 1. Diagram of the apparatus for pressure application. (*1*) glass tuberculin syringe barrel; (*2*) 1 cm sagittal section of the glass plunger; (*3*) titanium support frame; (*4*) the excised bone; (*5*) two rings connected by three stainless steel wire springs, to keep the syringe in the center of (*6*) 17 × 100 mm polypropylene tissue culture tube; (*7*) 3-way teflon stopcock; (*8*) tygon air conduit

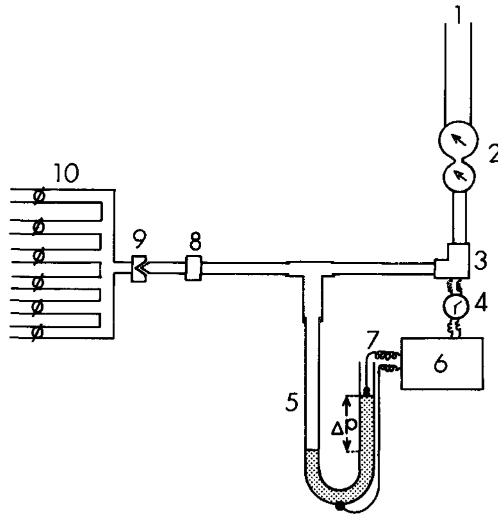

Fig. 2. Diagram of the air delivery system. (*1*) compressor-fed air line; (*2*) reducing valve; (*3*) electro-magnetic on/off valve, controlled by (*4*) clock for programming pressure application schedule, and by (*5*) mercury manometer, via (*6*) relay; according to the setting of (*7*) sliding contact wire; (*8*) air filter; (*9*) air-tight 360° swivel mounted on the rotating drum; (*10*) gang valve for distribution of pressure to multiple units

of the rotating drum. Air pressure reached the valve through a 0.45 μ Millipore filter to prevent contamination in case of air leakage into the culture tube.

The pressure was delivered by a compressor at 5 atm and reduced through a regulator valve (Airco, Style No. 9102) to 0 to 50 p.s.i. To assure additional control of air pressure in terms of level and duration the following feedback circuit was introduced into the system (Fig. 2): An electromagnetic valve (Detroit Controls, S60-2X) was inserted between the air reducing valve and the swivel. The valve was controlled by a signal transmitted by a mercury manometer connected in parallel with the main air pressure line. A drop in pressure would disconnect the electrical circuit established through the mercury column, and open the valve via an electronic relay (Precision Scientific Co., Chicago, Ill., Cat. No. 62690). A clock was introduced into the electric circuit to deliver pulses at selected intervals and durations.

Results

The Magnitude of the Compressive Force. Air pressure in the system was shown on the mercury manometer. Due to air compressibility, tube elasticity, and the possibility of air leaks and piston friction, the pressure delivered by the pistons was less than the manometer reading. Therefore, each piston was calibrated under experimental conditions using a force displacement transducer (FtU3, Grass Instrument Co., Quincy, Mass.). The relationship between the monitored and the delivered pressure in the range of applied pressures, is shown in Fig. 3. At low pressures the relationship was linear, whereas with increased pressure the loss in the transmitted force increased progressively.

The minimum pressure that would be exerted on the tibia of a standing newly hatched chick through a cross-sectional plane at the level of the metaphyseal plate was calculated to be 100–200 g/cm^2, assuming that a force equivalent to

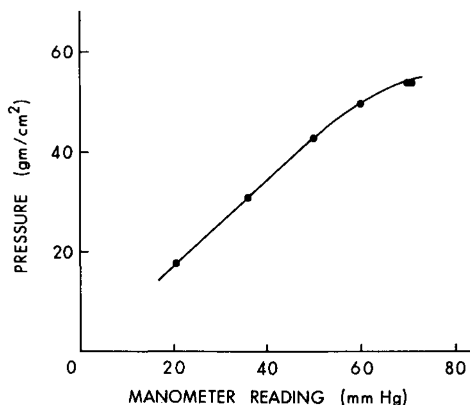

Fig. 3. Relationship of pressure actually applied by piston, to air pressure read on manometer connected in parallel with air delivery system

half the bird's weight was applied normally to that plane. If the tibia of a 16-day-old-embryo were to support half the weight of the embryo at that age the pressure exerted in the same plane would be about 50 g/cm^2. The physiological pressures exerted during movement would unboubtedly be higher than the calculated "static" pressures because the bird hops and carries its weight on one leg. A similar calculation for the newborn rat indicated a pressure of about 60 g/cm^2.

The pressure applied to the bone in the present experiment was a variable which could be changed to cover the entire range of physiological conditions and beyond.

The Effect of Pressure on the Extracellular Fluid Space. An important consideration is the effect of the pressure on the exchange of solutes between the medium and the tissue. To investigate this question pairs of 16-day-old-chick-embryo tibiae were incubated with and without pressure, 80 g/cm^2, in media containing the radioisotope ^{22}Na, at 37°, for varying times. Immediately after removal from the medium the tibiae were blotted dry and placed in a NaI (Tl) gamma scintillation counter. The kinetics of ^{22}Na uptake are presented in Fig. 4. At all times the pressure reduced the uptake of ^{22}Na. Statistical analysis using the Wilcoxon matched-pairs signed rank test revealed that the difference between experimental and control values was significant at the $p < 0.02$ level. Single-compartment kinetic analysis [12] in which the data were fit to the expression $(1 - u_t) = \exp(kt)$, where u_t represents the ratio of the uptake at time t to the uptake at equilibrium and k is the rate-constant of equilibration, revealed that the difference was due to the size of the equilibrating Na pool (extracellular fluid space) and not to different rates of equilibration. The rates were 0.347 min^{-1} and 0.376 min^{-1}, respectively, corresponding to half times of equilibration of about 2 minutes for both the control and the pressurized tibiae. The fit of these constant to the data can be estimated by comparing the solid lines in Fig. 4 (calculated) to the experimental points. The pressure caused a reduction of 8% in the size of the extracellular fluid space.

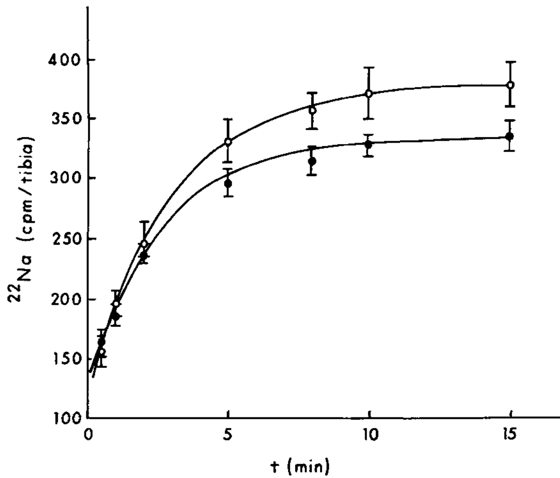

Fig. 4. Rate of extracellular fluid equilibration with medium measured by ^{22}Na uptake. ○-control tibiae, ●-contralateral tibiae exposed to a pressure of 80 g/cm sq. Each point represents the mean and standard error of the mean of four determinations. The lines represent the theoretical functions $^{22}\mathrm{Na}_t = {}^{22}\mathrm{Na}_{eq}(1-e^{kt})$, where $^{22}\mathrm{Na}_t$ represents the radioactivity uptake at time t, $^{22}\mathrm{Na}_{eq}$ the radioactivity uptake at equilibrium and k the equilibration constant. The k values calculated by linear regression were 0.347 min^{-1} and 0.376 min^{-1} for the control and pressurized tibiae, respectively. Only the size of the extracellular fluid pool showed a statistically-significant reduction with pressure

The Effect of Pressure on Glucose Consumption. In the absence of bacterial contamination glucose consumption is an adequate measure of tissue viability. Seven pairs of 16-day-old chick-embryo tibiae were incubated with and without pressure. The culture media were exchanged at 24 h intervals and glucose was assayed using the oxidase-peroxidase method (Glucostat, Worthington Biochemical Corp., Freehold, New Jersey 07728). As seen in Fig. 5, after higher consumption during the first day, utilization stabilized at about 1 mg of glucose per explant per day in the controls and 0.4 mg per day in the tibiae exposed to pressure. The difference was highly significant ($p < 0.001$ by Student's t test for paired samples). After removal of the pressure on day 4 the lower glucose consumption persisted for 24 hours, after which it rose to approach that of the controls. Paralleling the rise in glucose consumption a corresponding fall in pH was observed which was statistically significant ($p < 0.001$ by Student's t test for matched pairs). After removal of the pressure the difference in pH decreased (see legend to Fig. 5).

The Effect of Pressure on Thymidine Incorporation. In an experiment similar to that descibe above, six pairs of tibiae were incubated for four days in media containing ^{14}C-thymidine. At the end of the experiment the specific activity of ^{14}C-thymidine was measured in the metaphyseal plates and epiphyses of each tibia. The extraction and determination of DNA were done according to the method of Burton [4]. The radioactivity in the DNA extracts was counted in a liquid scintillation counter was corrected for quenching. In the tibiae exposed to pressure

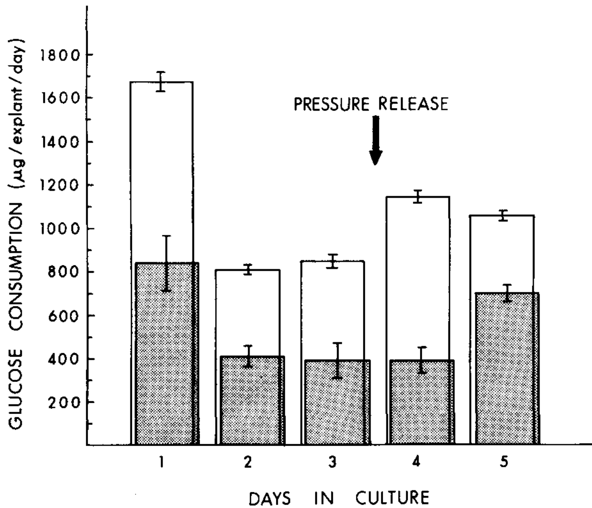

Fig. 5. The effect of pressure on glucose consumption of chick tibia explants in culture. The medium was exchanged every 24 h and the glucose consumption was calculated from the drop in glucose concentration. One of each pair of tibiae was exposed to a constant pressure of 80 g/cm sq. until day 4 when the pressure was released. Gray bars represent tibiae exposed to pressure, white bars are controls. The increased glucose consumption in the control tibiae was paralleled by a decrease in pH. The pH differences on subsequent days were: (*1*) 0.46 ± 0.05 (SE); (*2*) 0.50 ± 0.04; (*3*) 0.36 ± 0.03; (*4*) 0.48 ± 0.02; (*5*) 0.32 ± 0.03

the specific activity was 13790 ± 2180 (SE) cpm per nanogram DNA whereas in the controls it was 11940 ± 1910 (SE). The difference was significant at the $p < 0.05$ level (Student's t test for paired samples).

Discussion

We have described a method for the application of pressure to long bones in culture. The intensity of the force and the frequency of delivery can be controlled experimentally. The other variables include the species from which the bones are excised, and the age of the tissue prior to culture as well as all the modifications which can be imposed on the culture media.

In this preliminary study, designed to characterize and test the technique, we observed that the application of pressure caused a significant reduction in glucose utilization. This may reflect either a decrease in cellular availability of glucose due to impaired tissue diffusion or cell-membrane transport, or a shift in the pattern of glucose utilization. The small force and the absence of an effect on the rate of sodium equilibration strongly suggest that the differences are not due to glucose diffusion. Moreover, the correlation between the drop in pH of the medium and the glucose consumption would indicate that glycolysis is the main metabolic pathway repressed by the application of pressure. Grobstein suggests that changes in the metabolic pathways of the tissue may represent an early sign of cyto-differentiation [7]. Interestingly, a decrease in lactic dehydrogenase relative to

malic dehydrogenase was found to coincide with the transition from cartilage to bone during induced ectopic bone formation [11]. This would be compatible with a shift from glycolysis to the tricarboxylic acid pathway and with lower glucose consumption due to the higher energy yield of the latter.

As seen in Fig. 5, twenty four hours after removal of the pressure, the glucose utilization again increased to approach that of the controls. The longer lag period required for this change relative to the fast drop in consumption may be due to the *in vitro* conditions. However, we believe that the time difference indicates that these changes are due to complex irreversible chemical processes, like those associated with cytodifferentiation.

The small but significant increase in the specific activity of thymidine produced by the application of pressure would indicate stimulation of DNA replication, assuming equal distribution of the tracer in the pre-incorporation pool. Localization studies, including histological and autoradiographic examination, could show if bone formation is indeed the process stimulated.

It would be important to know if there is a link between the mechanical stimulus and the chemical signal which could be read by the cell. If we assume that this sequence of events involves piezoelectric transduction, a biochemical change in the tissue should be produced by piezoelectricity and set into motion the changes leading to cytodifferentiation. The tissue culture method described here offers a potentially fruitful approach for investigating such changes.

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