

# Arteries Respond to Independent Control of Circumferential and Shear Stress in Organ Culture

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**Abstract**—Arteries respond to changes in *global* mechanical parameters (pressure, flow rate, and longitudinal stretching) by remodeling to restore *local* parameters (circumferential stress, shear stress, and axial strain) to baseline levels. Because a change in a single global parameter results in changes of multiple local parameters, the effects of individual local parameters on remodeling remain unknown. This study uses a novel approach to study remodeling in organ culture based on independent control of local mechanical parameters. The approach is illustrated by studying the short term effects of circumferential and shear stress on remodeling-related biological markers. Porcine carotid arteries were cultured for 3 days at a circumferential stress of 50 or 150 kPa or, in separate experiments, a shear stress of 0.75 or 2.25 Pa. At high circumferential stress, matrix synthesis, smooth muscle cell proliferation, and cell death are significantly greater, but matrix metalloproteinase-2 (MMP-2) and pro-MMP-2 activity are significantly less. In contrast, biological markers measured were unaffected by shear stress. Applications of the proposed approach for improved understanding of remodeling, optimizing mechanical conditioning of tissue engineered arteries, and selection of experimentally motivated growth laws are discussed.

**Keywords**—Remodeling, Vasomotor response, Carotid artery, Biological markers.

## INTRODUCTION

Remodeling is the process by which arteries respond to sustained changes in their global mechanical environment, characterized by blood flow, arterial pressure, and longitudinal stretching. At the macro-level, remodeling manifests as changes in arterial dimensions and mechanical response which do not result from the deformation caused by altered loads. The effect of the mechanical environment on arterial remodeling has been studied in animal models and perfusion organ

culture where arteries were subjected to controlled changes in blood flow rate, arterial pressure, or longitudinal stretch ratio for a period of several days to months.<sup>6,11,12,14,16,21,23,27,28</sup>

Results from animal models have shown that arteries respond to altered blood flow by an acute vasomotor response directed to restore the flow-induced shear stress at the arterial lumen to homeostatic levels, followed by a chronic remodeling process.<sup>15,16,26,41</sup> Though animal investigations keep the artery under conditions that are close to physiological conditions, there are difficulties in precisely and continuously controlling and monitoring the mechanical environment and remodeling outputs. In addition, other factors such as neuronal stimulation and the local hormonal and metabolic environment might affect smooth muscle cell activity and wall remodeling. To focus solely on the effects of mechanical environment, organ culture systems were used.<sup>6,11,12</sup> They provide conditions supporting arterial metabolism and maintaining arterial function for a period of several days up to four weeks.<sup>4,6,11,12</sup> Organ culture systems allow independent control of the magnitude and frequency of pressure and flow rate to better evaluate their contribution to remodeling outputs. Because remodeling in response to alterations in pressure and flow takes more time than the period during which current organ culture systems can maintain smooth muscle cell viability, remodeling response is estimated by the trends of the change in the geometrical dimensions or by specific biological markers which are indicators of the proliferative, synthetic, or degradative activity of vascular cells.<sup>2,6,11,12,28,29,38</sup>

Similar to animal studies, results from organ culture investigations support the conclusion that remodeling induced by alterations in global mechanical parameters is an adaptive response that maintains the local shear stress at the intima and circumferential stress and axial strain in the media to their baseline values. Stresses and strains represent the local mechanical environment of

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the endothelial and smooth muscle cells. It is the changes in the local mechanical factors that affect cellular processes such as cell proliferation, apoptosis, hypertrophy, migration, and matrix synthesis and degradation, for which the combined effect ultimately leads to observed geometrical and structural responses of remodeling.

Both animal and organ culture studies performed so far have revealed many aspects of the normal and pathologic remodeling of arteries from different species, age groups, and locations within the vasculature.<sup>4,9,10,14,20,27,28,41</sup> However, the methodology used in these studies cannot address several important issues. Past studies cannot quantify the remodeling response caused by a controlled change in a single parameter of the local mechanical environment such as circumferential stress, flow-induced shear stress, axial stress, or pulsatile circumferential stress or strain, while keeping the remaining local parameters unchanged. In general, a change in a single global parameter, which is the experimental design used in past investigations, leads to a change of several local parameters and, therefore, the remodeling response is a result of the combined changes of all local parameters. For instance, a change in the mean arterial pressure causes changes in circumferential stress, pulsatile circumferential strain, and shear stress at the endothelium. Though under physiological conditions, the arterial pressure, flow, or longitudinal stretch might vary, understanding the contribution solely of a single local mechanical factor to which the endothelial and smooth muscle cells are exposed can provide insights on the mechanisms of arterial remodeling. Quantification of the independent effects of the altered shear stress, circumferential stress, and circumferential pulsatile strain can reveal which parameter has a major remodeling effect, whether they synergistically contribute to the remodeling outputs, and whether there exists a causal link between a deviation from baseline value of certain local mechanical parameter and observed remodeling outputs.

Focusing on the effects on remodeling caused by alterations in global mechanical parameters, past studies did not sufficiently analyze the contribution of arterial dimensions and mechanical properties on remodeling outputs. The size and mechanical properties of vascular tissue relate changes in global mechanical environment of an artery to changes in local mechanical environment of arterial cells via equations of equilibrium. Therefore, past studies could not determine the extent of the “remodeling capacity” of endothelial and smooth muscle cells or the amount and reorganization of the extracellular matrix responsible for an observed mode of remodeling.

It is not possible to realize independent control of the local mechanical environment of the endothelial

and smooth muscle cells in animal experiments. However, a novel approach in design and perform investigations in organ culture systems can address the issues mentioned above. This study illustrates this approach in studying the effects of circumferential and shear stress on arterial remodeling of porcine carotid arteries.

## METHODS

### *Experimental System and Specimen Preparation*

The organ culture system used has been described in detail and validated in earlier studies.<sup>6,12</sup> Validation included studies of endothelial and smooth muscle cell functionality after up to 7 days in culture. The system consists of a flow system (pump, pulse dampener, reservoir, and resistance clamp), measurement devices (camera and pressure transducer), and a vessel chamber connected by silicone tubing (Cole-Parmer). The system was sterilized by autoclave prior to each experiment.

The media reservoir and vessel chamber were filled with sterile perfusion medium (~500 mL) and bathing medium (~150 mL) composed of Dulbecco's Modified Eagles Medium (Sigma) supplemented with sodium bicarbonate (3.7 g/L, Sigma), L-glutamine (2 mM, Sigma), L-proline (0.4 mM, Sigma), ascorbic acid (50 mg/L, Sigma), antibiotic-antimycotic solution (1%, Sigma), and calf serum (10%, HyClone). Dextran (6.5%, Sigma) was added to the perfusion media to increase its viscosity to physiologic levels (4 cP).

Bilateral porcine common carotid arteries were harvested from 6- to 7-month-old farm pigs (100–150 kg) at a local abattoir, rinsed with Dulbecco's phosphate buffered saline (PBS, Sigma) and transported to the laboratory in ice-cold PBS.

Segments having unloaded lengths of 3–5 cm were prepared under a laminar flow hood. Excess connective tissue was removed, leaks were identified using gentle inflation with air, and side branches ligated as needed. Care was taken during inflation to preserve the endothelium. A 2 mm thick ring section was cut from each end of the vessel and used to measure the unloaded cross-sectional area, outer diameter, and wall thickness. Measurements were made using a calibrated image of the backlit rings.

After assembly under a laminar flow hood, arteries were oriented in the *in vivo* flow direction and mounted on the vessel chamber cannula using a single purse-string suture. Warm bath and perfusion media (37 °C) were added and the entire flow loop transferred to an incubator (Forma Scientific) maintained at 5% CO<sub>2</sub> and 37 °C for the duration of the experiment.

Flow rate was controlled using a peristaltic roller pump (Cole-Parmer) in series with a pulse dampener (Cole-Parmer). Mean perfusion pressure was controlled by a resistance clamp and was measured using a pressure transducer (Harvard Apparatus). A CCD camera (Marshall Electronics) with an adjustable zoom lens (Leica) was used to measure the outer diameter.

### Methodology of Controlling Local Mechanical Environment<sup>33</sup>

To achieve prescribed values of circumferential and shear stress, we used a combination of an analytical and an experimental approach. First, the values of the required pressure and flow rate were determined neglecting the possible influence of flow rate on the active response of the artery. Later, precise adjustment was performed experimentally by an iterative procedure.

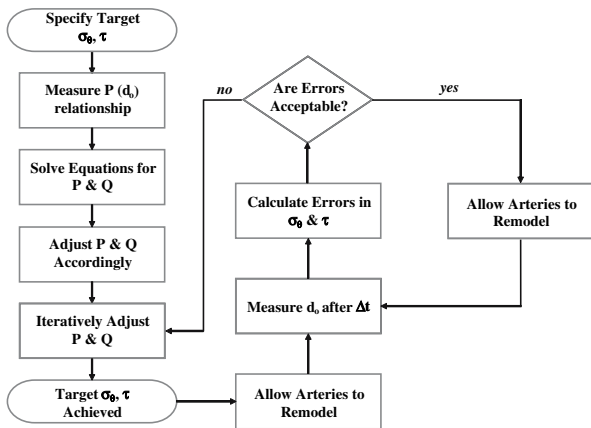
The flow chart (Fig. 1) describes the process used to control circumferential and shear stress in organ culture. The artery was assumed to be a circular cylinder of uniform thickness made of an elastic and incompressible material. The vessel is subjected to fully developed Poiseuille flow of a Newtonian fluid.

At a given time,  $t$ , the mean circumferential wall stress is given by the Law of Laplace

$$\sigma_{\theta} = \frac{P(d_o - 2h)}{2h} \quad (1)$$

where  $P$  is the transmural pressure,  $d_o$  is the outer diameter at the loaded state, and  $h$  is the wall thickness at the loaded state. The flow-induced shear stress at the luminal surface of the artery is

$$\tau = \frac{32\mu Q}{\pi(d_o - 2h)^3}, \quad (2)$$



**FIGURE 1. Overview of the process for independently controlling circumferential stress and shear stress at a fixed axial stretch ratio in perfusion organ culture.**

where  $\mu$  is the viscosity of the culture medium and  $Q$  is the flow rate. It follows from the condition of material incompressibility that at time  $t$  the total volume of the unloaded and the deformed configurations are equal

$$(D_o - H)H = (d_o - h)h\lambda_z, \quad (3)$$

where  $D_o$  is the unloaded outer diameter,  $H$  is the unloaded wall thickness.  $\lambda_z$  is the axial stretch ratio defined as  $l/L$ , where  $l$  is the *in situ* vessel length and  $L$  is the unloaded length.

A functional relationship between pressure and outer diameter,  $P = f(D_o)$ , can be determined by conducting a pressure–diameter test while the artery is held at a fixed  $\lambda_z$  and fitting to a simple quadratic function

$$P = Ad_o^2 + Bd_o + C, \quad (4)$$

where  $A$  and  $B$  are coefficients determined for a best fit to the data.

Given the target values of  $\sigma_{\theta}$ ,  $\tau$ , and knowing the undeformed dimensions,  $D_o$  and  $H$ , as well as the axial stretch ratio  $\lambda_z$ , Eqs. (1)–(4) can be solved for  $P$ ,  $Q$ ,  $d_o$ , and  $h$ . The values of pressure and flow are accepted as a first guess to achieve the prescribed values of circumferential and shear stress. Once the artery is subjected to this pressure and flow rate, it is possible to measure the deformed outer diameter and to calculate the existing circumferential and shear stress from Eqs. (1)–(3). At this point, the realized values of circumferential and shear stress may not equal their target values due to the conditions under which the pressure–diameter relationship, Eq. (4), is obtained. The mechanical behavior of the artery *in vivo* is a result of the passive response of the elastin and collagen as well as the active response of the vascular smooth muscle. In this study, we performed the inflation test in the absence of flow. Though Eq. (4) might account for the myogenic response of the vascular muscle, it does not account for the effect of flow on the muscular tone. It is well known that flow-induced shear stress modulates the vascular tone through release of relaxing or constricting factors by the endothelial cells.<sup>22</sup> Provided the difference between the existing and target values of the stresses is bigger than the admissible error, an iterative experimental approach is applied to achieve the target values of circumferential and shear stress by iteratively imposing small variations of pressure and flow.

In general, remodeling leads to changes in arterial dimensions and mechanical properties, and, thereby, the mechanical response of the vessel. Therefore, to maintain the prescribed values of circumferential and shear stress over time, the pressure and flow in the organ culture system must vary accordingly. The approach depends on the technical ability to determine

the deformed wall thickness of the artery during an organ culture experiment. If it is possible to monitor the outer diameter and the wall thickness, the actual values of the circumferential and shear stress can be calculated at any moment and the pressure and flow can be iteratively adjusted as described above. A similar procedure is applicable if the duration of the experiment is relatively short and no significant changes in undeformed dimensions of the cultured artery are observed. Then, the current wall thickness is calculated from the measured outer diameter using the condition of material incompressibility, Eq. (3). This is typically the case in the experiments performed in this study. Finally, if the unloaded arterial dimensions vary but wall thickness cannot be continuously recorded, it is necessary to interrupt the experiment after a certain time interval and to measure directly the current undeformed wall thickness and outer diameter. This is the case during first 15 h of experiment performed in this study.

#### *Experimental Protocol*

Three 3–5 cm segments were cut from bilateral porcine carotid arteries. Two of the segments were paired and used as experimental arteries while the remaining segment was used as a control to correct for changes in unloaded dimensions that occur during culture. The control vessel was required because preliminary experiments showed that the measured unloaded outer diameter and wall thickness change during the first 15 h of culture and then remain constant.<sup>39</sup> For circumferential stress studies, experimental arteries ( $n = 10$ ) were subjected to a circumferential stress of 50 kPa (Case A) or 150 kPa (Case B). Shear stress and the axial stretch ratio were held at physiologic levels of 1.5 Pa and 1.5, respectively.<sup>11,16,41</sup>

For shear stress studies, experimental arteries ( $n = 6$ ) were paired and subjected to a shear stress of 0.75 Pa (Case C) or 2.25 Pa (Case D) while circumferential stress and the axial stretch ratio were held at physiologic levels of 100 kPa and 1.5, respectively.<sup>19,28</sup> The control artery was cultured under the high circumferential or shear stress conditions because preliminary experiments showed that changes in unloaded dimensions were independent of loading conditions. For each experiment, the axial stress of both experimental vessels was equal because, at the physiologic axial stretch ratio, axial stress does not vary with pressure.<sup>37,40</sup> All experiments were run for 3 days.

The flow loops were placed in the incubator and the flow rate was set at the minimum flow rate of 30 mL/min for approximately 1 h to allow the vessels to acclimate to the organ culture environment. The vessels were then elongated in equal increments every

10 min to a physiologic stretch ratio of 1.5 over a period of 30 min.

The inflation test was then conducted to determine the pressure–outer diameter relationships. During testing, flow was stopped and the flow loop was clamped such that the vessel and the pressure transducer were isolated. Arteries were preconditioned by increasing pressure from 50 to 200 mmHg at a rate of about 5 mmHg/s for 10 cycles. Diameter measurements were taken at pressures of 50, 125, and 200 mmHg. The outer diameter measurement was acquired using a C program and the pressure data was acquired using Quick DataAcq software (Data Translation, Inc.).

Equations (1)–(4) were solved for the pressure and flow rate required to achieve the prescribed values of circumferential and shear stress. The pressure and flow rate were then adjusted to their target values over a 30-min period. Pressure and flow rate were then adjusted iteratively until the prescribed values of pressure and flow rate were achieved. Circumferential and shear stress were calculated every 30 s using a C program. The experiment was monitored regularly and the pressure and flow rate were adjusted when the time averaged errors in circumferential or shear stress exceeded approximately 5%. Results are plotted as the time averaged value of each parameter ( $\sigma_\theta$ ,  $\tau$ ,  $P$ ,  $Q$ ,  $d_o$ , and  $h$ ) over 5 h.

The control artery was cultured for approximately 15 h and then the unloaded diameter and wall thickness were measured. From these measurements, the ratio of the unloaded dimensions at 15 h to their initial value was calculated. The corrected values of unloaded outer diameter and wall thickness for both experimental arteries were then calculated using the ratios measured for the control artery assuming the ratios are the same for all specimens. Following this, the inflation test was conducted for the experimental arteries and Eqs. (1)–(4) were solved again for the pressure and flow rate required to achieve the prescribed stress values. Pressure and flow rate were adjusted accordingly and control of circumferential and shear stress was resumed for the remainder of the experiment.

At the end of culture, approximately 5 mm of each end of the vessel was discarded to avoid end effects and possible tissue damage due to suturing the tissue on the cannulae. The artery was then divided into sections for measurement of matrix synthesis, matrix metalloproteinase activity (MMP) activity, cell proliferation, and cell death.

#### *Biological Markers*

Matrix synthesis was measured using a <sup>3</sup>H-proline incorporation assay based on the method of Peterkofsky



and Prockop.<sup>30</sup> Static culture, which minimizes tritium use, was used to determine the response, relative to controls, to the stimulus applied in organ culture. Following culture, ring segments, approximately 4 mm in length, were statically incubated in DMEM supplemented with <sup>3</sup>H-proline (10  $\mu$ Ci/mL) for 18 h. The incubation period of 18 h was determined to be optimal from preliminary studies. Following radiolabelling, tissue segments were washed in quench solution [PBS supplemented with sodium sulfate (0.8 mM) and L-proline (1.0 mM) (Sigma)] four times for 30 min. Tissue segments that were subjected to a series of 3 freeze-thaw cycles were used as negative controls to insure that <sup>3</sup>H-proline incorporation was due to cellular uptake and not passive diffusion. Samples were lyophilized and digested in 0.2–0.4 mg/mL proteinase K (Sigma) in 100 mM ammonium acetate solution (Sigma) overnight at 60 °C. The radioactivity was measured using a scintillation counter (Tri-Carb, Perkin-Elmer) and results were normalized to the tissue sample's dry weight.

The activity of MMP-2 and MMP-9 were measured using SDS-PAGE zymography<sup>17</sup> using a method similar to that of Chesler *et al.*<sup>2</sup> Snap-frozen tissue samples were homogenized in ice-cold lysis buffer [10 mM sodium phosphate pH 7.2, 150 mM sodium chloride, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 0.2% sodium azide (Sigma)] using a mechanical tissue homogenizer (Ultra-Turrax 25, IKA). The protein concentrations of the samples were measured using a modified Lowry protein assay.<sup>24</sup> Equal amounts of protein were loaded in each lane of the gel. The gels were 10% polyacrylamide with 1.0 mg/mL of gelatin. Electrophoretic migration was carried out at 4 °C and, following migration, the proteins in the gel were renatured in a series of 2 incubations in 2.5% Triton X-100 for 15 min each. The gels were then incubated overnight at 37 °C in an assay buffer [50 mM Tris-HCl pH 7.4, 10 mM calcium chloride, 50 mM sodium chloride, and 0.05% Triton X-100 (Sigma)]. Following incubation, gels were stained with Coomassie Brilliant Blue (Sigma). Proteins having gelatinolytic activity were visualized as clear lysis bands while the rest of the gel was stained blue. Prestained molecular weight markers were used to determine the molecular weight of the lysis bands. ScionImage was used to quantify the zymography results based on the pixel intensity and size of the lysis band.

Tissue samples were fixed overnight in 10% neutral buffered formalin (Fisher) and then processed for paraffin embedding. Samples were cut into 5  $\mu$ m thick sections, deparaffinized, and stained using the protocols described below.

Hematoxylin and eosin (H&E) staining was used to compare the morphology of arterial tissues following

culture. Sections were stained for H&E using an auto-stainer (Leica). Hematoxylin stains cell nuclei purple and eosin stains connective tissue pink. Following staining, coverslips were mounted over the sections using Cytoseal 60 mounting medium (Richard-Allan Scientific). Sections were imaged using a Nikon Eclipse E800 microscope.

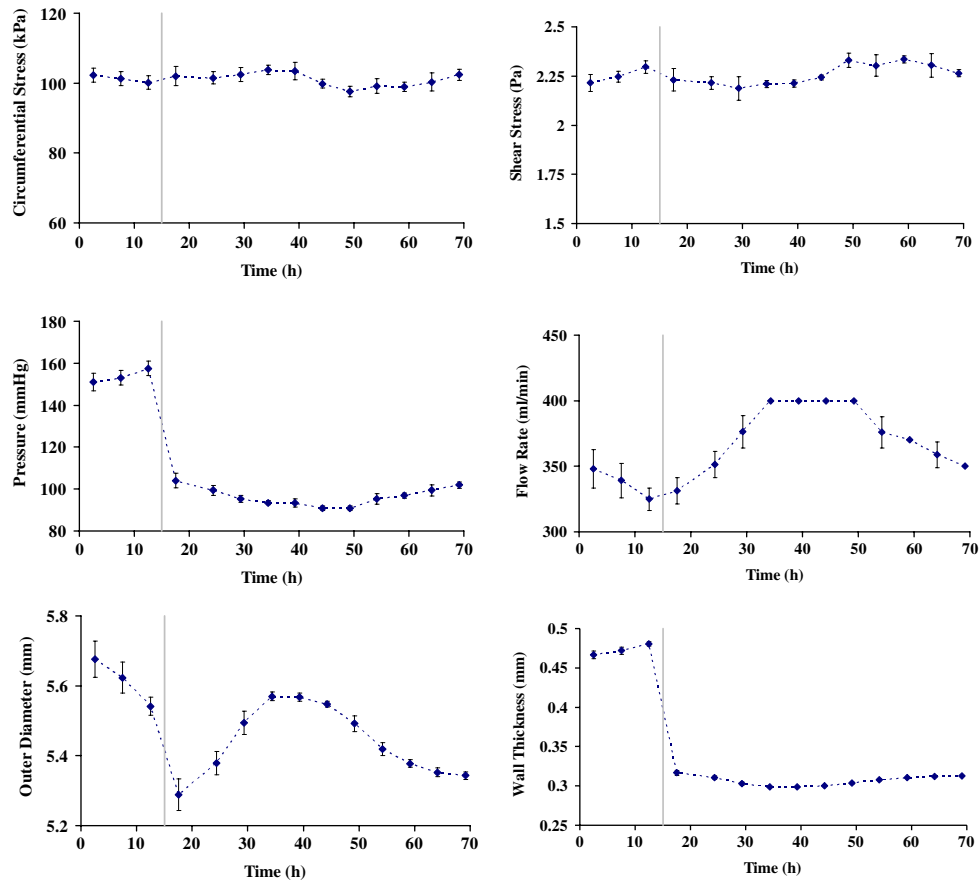
Cell proliferation was measured by using a 5-bromo-2'-deoxy-uridine (BrdU) incorporation assay described previously.<sup>6,12</sup> BrdU was added to the perfusion media at a final concentration of 10  $\mu$ M for the last 24 h of culture. Following deparaffinization, BrdU epitopes were unmasked using heat induced epitope retrieval.<sup>34</sup> Sections were permeabilized using 0.5% Triton X-100 in PBS for 20 min at 37 °C. Slides were then incubated in primary and secondary antibodies included in the labeling kit (BrdU Labeling and Detection Kit II, Roche). Sections were incubated in 0.25  $\mu$ g/mL 4'-6-diamidino-2-phenylindole (DAPI, Sigma) in PBS for 5 min to label cell nuclei. Coverslips were then mounted using Gel/Mount (Biomedica). Sections were later visualized at 10 $\times$  magnification using fluorescent microscopy. Images were taken at four equally spaced locations around the circumference of the artery for two non-serial sections. Total nuclei and BrdU-positive nuclei were counted using ImagePro Plus software (Media Cybernetics) and the ratio of labelled nuclei to total nuclei was calculated for each sample. Proliferation was calculated for each cell type within the arterial wall based on the shape and location of the cell.

Results are plotted as mean  $\pm$  standard error of the mean unless stated otherwise. Paired Student's *t*-tests were used to compare the difference between the means of the experimental groups. A *p*-value of less than 0.05 was considered statistically significant.

## RESULTS

### *Control of Local Mechanical Environment*

The time course of each mechanical parameter is shown for a representative case to illustrate the method of controlling the local mechanical environment (Fig. 2). For this case, the prescribed values of circumferential stress and shear stress were 100 kPa and 2.25 Pa, respectively (Case D). The unloaded dimensions and pressure-diameter relationship of the artery were determined at the onset of the experiment and again after approximately 15 h of culture. This process, referred to as the adjustment period, is shown in Fig. 2 as a shaded vertical line at 15 h. For the case presented (Fig. 2), the unloaded diameter and wall thickness of the artery reduced by 36.3 and 1.7%, respectively, after 15 h of culture. Analysis of the



**FIGURE 2.** Time course of mechanical and geometrical parameters from a representative experiment (Case D). The prescribed values of  $\sigma_{\theta}$ ,  $\tau$ , and  $\lambda_z$  were 100 kPa, 2.25 Pa, and 1.5, respectively. Each point represents the average measurement of each parameter over 5 h. The shaded vertical line indicates the adjustment period during which the current unloaded dimensions were determined and the inflation test was conducted. Prior to this period, circumferential and shear stress were calculated using the initial unloaded dimensions. Following this period, the stresses were calculated using the updated unloaded dimensions. Results are plotted as mean  $\pm$  SD.

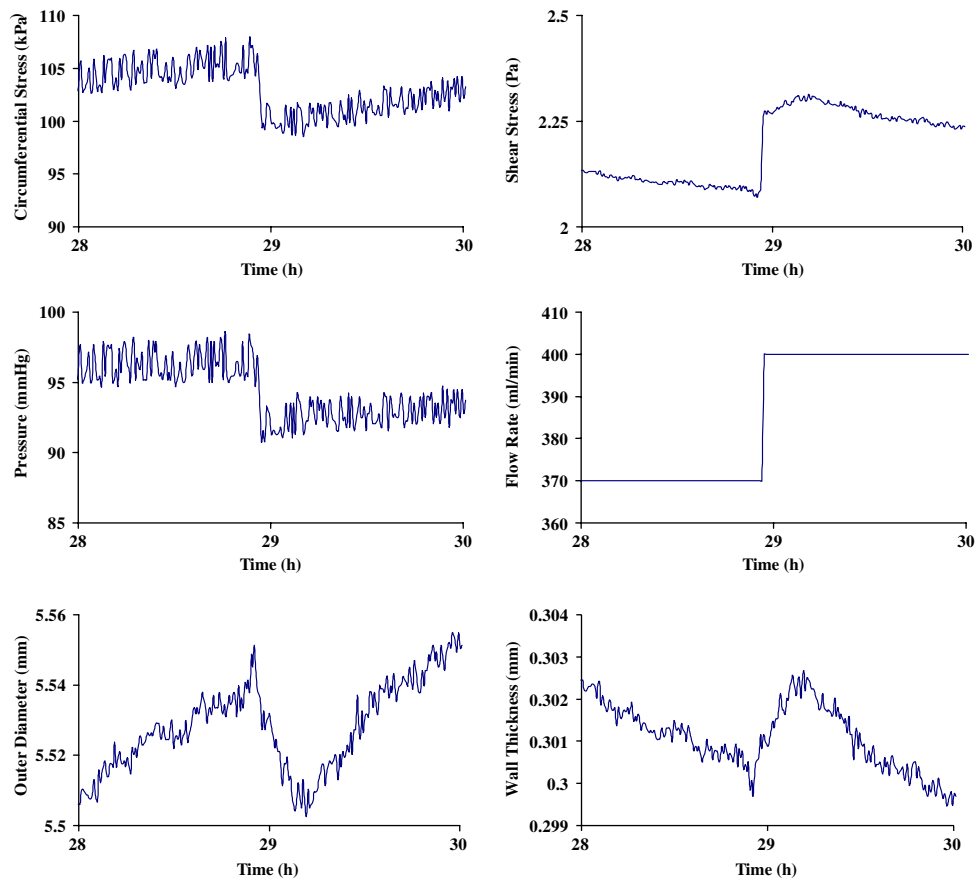
results will be focused on data recorded after the adjustment period because, prior to this, observed changes in geometry may result from the transient changes of the unloaded dimensions.

The vessel experienced non-monotonic changes in outer diameter during culture. Following the adjustment period, the outer diameter of the artery progressively increased for about 15 h and then gradually decreased for the remainder of the experiment. To account for these changes in outer diameter, pressure and flow rate were adjusted up to 12.7 and 20.7% to maintain circumferential and shear stress.

The time course of mechanical parameters measured continuously are shown during an iterative adjustment of pressure and flow rate (Fig. 3) for the representative experiment (Case D). The progressive increase in arterial outer diameter resulted in deviations of circumferential stress and shear stress from their prescribed values. In order to restore circumferential and shear stress, pressure was reduced by approximately 6.3% and flow rate was increased by

7.5%. During this iterative adjustment, circumferential stress was reduced by approximately 6.8% and shear stress was increased by approximately 8.6%, which restored the stresses to their prescribed values. The outer diameter of the artery reduced rapidly for approximately 20 min following adjustment of pressure and flow rate and, then, the outer diameter continued to increase as it did prior to the iterative adjustment.

Similar time courses were observed for arteries under each loading condition (data not shown). In general, the pressure and flow rate required to maintain the target values of circumferential and shear stress varied considerably both between and within experiments. For example, to achieve a circumferential stress of 50 kPa, pressure ranged from 70 to 120 mmHg during the first 15 h of culture and from 50 to 65 mmHg thereafter. To achieve a circumferential stress of 150 kPa, pressure ranged from 180 to 200 mmHg during the first 15 h of culture and from 110 to 210 mmHg thereafter.



**FIGURE 3.** Detailed view of mechanical parameters during an iterative adjustment of pressure and flow rate for the representative experiment (Case D).

We have performed an experiment comparing the arterial responses to maintaining either constant global or constant local mechanical parameters. At the onset of the experiment two arteries were subjected to pressure and flow that produce circumferential stress of 150 kPa and shear stress of 1.5 Pa. The pressure and flow applied to one artery were kept constant for 72 h. Calculations based on recorded geometrical dimensions at 15 h show that the circumferential stress decreased by 22.7% and the shear stress increased by 200%. Thereafter the stresses varied by 7.1 and 15.5%, respectively. The pressure and flow applied to the second artery were varied accordingly and after the adjustment period at 15 h the circumferential and shear stress were maintained within 3.4 and 4.5% of their target values.<sup>39</sup>

#### *Response of Biological Markers of Remodeling*

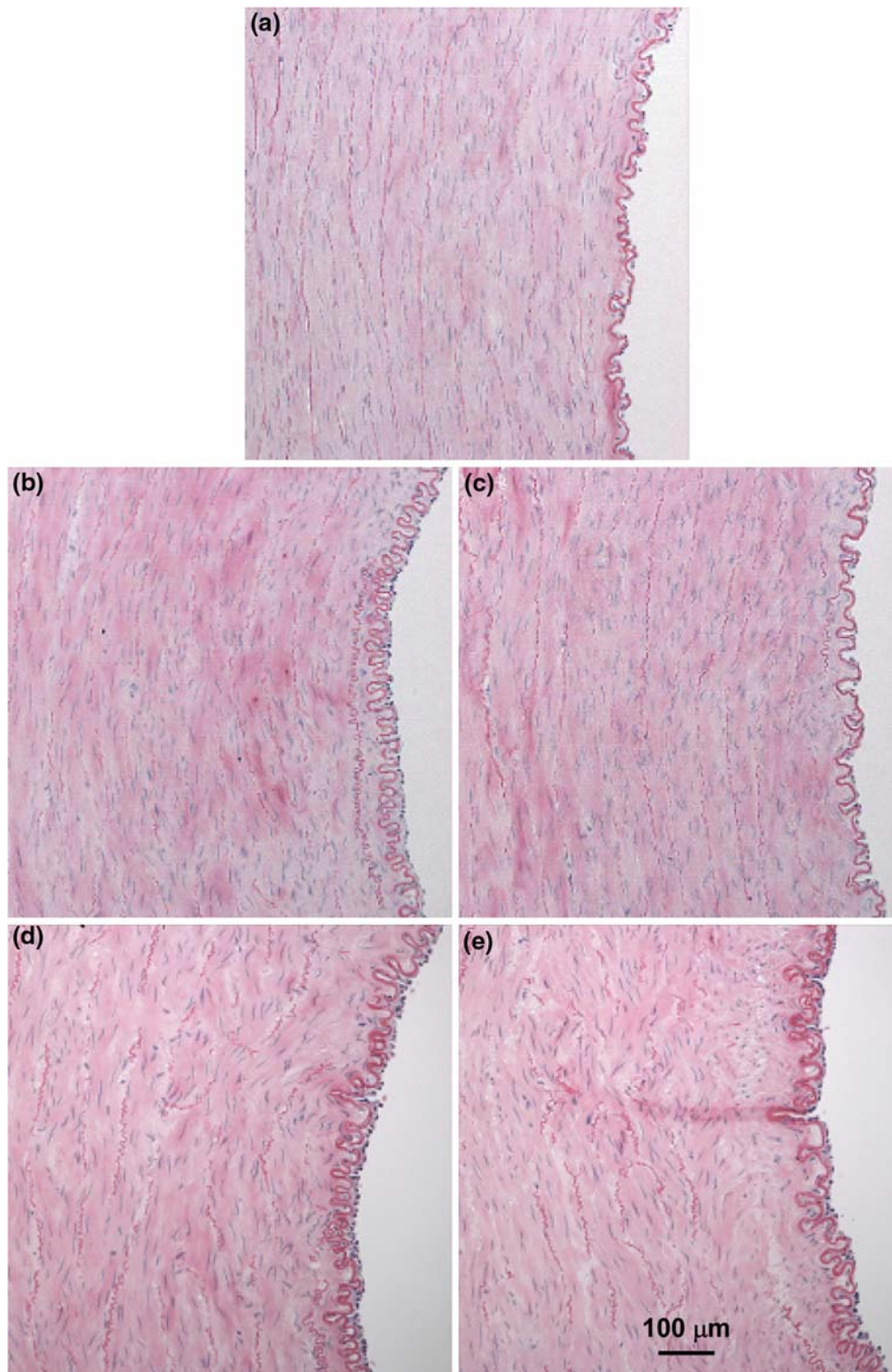
The morphology of all experimental arteries in this study was similar to that of fresh arteries (Fig. 4). For each loading condition, the arteries generally maintained an intima comparable to that of fresh arteries

with an intact endothelial cell layer and internal elastic lamina. In the media, the smooth muscle cells are oriented circumferentially and the extracellular matrix retains its structural integrity.

The mean value of <sup>3</sup>H-proline incorporation for arteries ( $n = 10$ ) subjected to high circumferential stress was significantly greater than for low circumferential stress vessels ( $p < 0.04$ ) (Fig. 5). In contrast, there were no significant differences in the <sup>3</sup>H-proline incorporation levels of high and low shear stress arteries ( $n = 6$ ).

Mean relative MMP-2 and pro-MMP-2 activity levels in arteries exposed to high circumferential stress were significantly less than in arteries exposed to low circumferential stress, respectively ( $p < 0.05$ ) (Fig. 6). Changes in circumferential stress did not significantly affect the levels of pro-MMP-9 activity. Shear stress did not significantly affect the activity of MMP-2, pro-MMP-2, or pro-MMP-9.

In general, the percentage of proliferating cells was greater in the intimal and adventitial layers than for in the media (Fig. 7). In experiments varying circumferential stress, there was an approximately fourfold

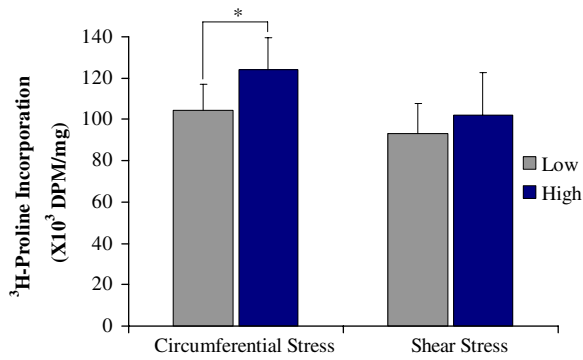


**FIGURE 4.** Comparison of tissue morphology of transverse sections for arteries using hematoxylin and eosin staining. Representative samples of a fresh (a) artery and vessels subjected to low circumferential stress (b), high circumferential stress (c), low shear stress (d), high shear stress (e) are shown. The lumen is on the right in each image.

increase in proliferation for cells in the media and an approximately 50% increase for cells in the adventitia at high circumferential stress relative to low circum-

ferential stress arteries ( $p < 0.05$ ). In shear stress experiments no significant difference in proliferation for cells in any region.

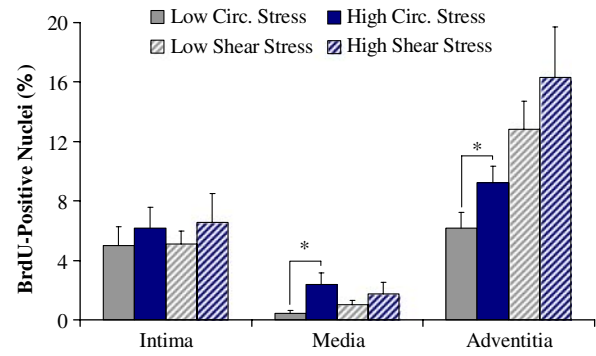




**FIGURE 5.** Matrix synthesis was measured by <sup>3</sup>H-proline incorporation for arteries. For one set of experiments, arteries were exposed to low (50 kPa, Case A) or high (150 kPa, Case B) circumferential stress ( $n = 10$ ). For the other experiments, arteries were either exposed to low (0.75 Pa, Case C) or high (2.25 Pa, Case D) shear stress ( $n = 6$ ). Incorporation is measured in units of disintegrations per minute (DPM) per mg of tissue dry weight.  $p < 0.05$ .

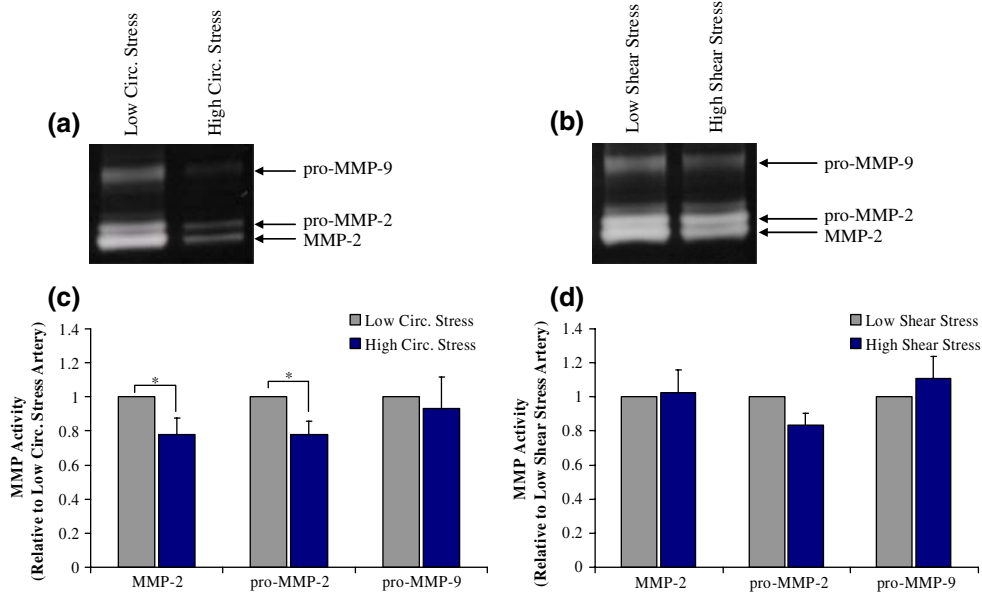
## DISCUSSION

We developed a novel approach to independently controlling circumferential and shear stress in perfusion organ culture. To illustrate the method, we have studied the effects of circumferential and shear stress on several biological markers of remodeling. The motivation for this work is that remodeling outputs caused by changes in the global mechanical environment can be better analyzed and predicted on the basis of the remodeling response to independently controlled local mechanical parameters.



**FIGURE 7.** Percentage of proliferating cells detected by BrdU incorporation during the final 24 h of culture. Arteries were subjected to low and high levels of circumferential ( $n = 8$ ) or shear ( $n = 6$ ) stress.  $p < 0.05$ .

The significant changes of unloaded arterial dimensions that occurred during the early stage of culture were not caused by matrix synthesis or cell proliferation due to the short timeframe, but likely due to other processes that take place during culture. The change in unloaded dimensions probably results from the loss of water content due to forced diffusion in a manner similar to that described by others in which water extrusion occurred when a vessel is subjected to radial compression.<sup>3</sup> The reduction of unloaded cross-sectional area of the arterial specimen was recorded in all experiments and was reported previously.<sup>5</sup> Therefore, if the condition of material incompressibility is used to calculate the current wall thickness from the measurements of the deformed outer diameter, it is



**FIGURE 6.** Effect of circumferential ( $n = 9$ ) and shear stress ( $n = 6$ ) on MMP-2/9 gelatinolytic activity measured using SDS-PAGE zymography. Representative zymograms for arteries exposed to high and low levels of circumferential stress (a) and shear stress (b) showing MMP-2 (67 kDa), pro-MMP-2 (72 kDa), and pro-MMP-9 (92 kDa) activity as clear lysis bands. Gelatinase activity was determined by densitometric analysis for circumferential stress (c) and shear stress (d) experiments.  $p < 0.05$ .

important to account for such changes in unloaded dimensions. The finding that unloaded arterial dimensions change during culture further suggests that better comparison between results obtained in animal studies and in organ culture systems should be performed at equivalent stresses rather than at equivalent pressures and flow rates.

Following the adjustment period, when no change in unloaded dimensions occurs, the control of circumferential and shear stress is achieved by significantly varying pressure and flow rate to account for changes in arterial geometry. A plausible explanation for the observed variation of the vessel outer diameter is a change in vascular tone. When shear stress is kept at levels higher than the baseline values, the artery initially dilates, which is consistent with the results of *in vivo* studies on flow-induced remodeling.<sup>16,41</sup> However, the subsequent diminishing of the outer diameter (Fig. 2) indicates that the vascular tone tends to increase over time. The response may result from the presence of FCS in the culture media in accordance with the finding that an addition of 10% FCS in the culture media induces a vasoconstrictive response in arteries in perfusion culture for 4 days.<sup>1</sup> Therefore, the non-monotonic changes in outer diameter observed in this study likely result from a balance of the competing effects of a flow-induced relaxation of vascular smooth muscle and the contractile stimulation of FCS. The relative magnitude or timescale of each of these responses is unknown. Similar responses were observed in most arteries for all experimental conditions, although the order of dilation and constriction responses was not consistent between different experimental states.

The variation of pressure required to maintain the target values of circumferential stress, both within and between experiments, underscores the importance of controlling local mechanical parameters in organ culture. Within an experiment, there is a dramatic change in the pressure required to maintain the desired circumferential stress before and after the adjustment period. This results from the transient changes in unloaded dimensions that occur during the first 15 h of culture. Between experiments, the variation in the pressure required to maintain the desired circumferential stress results from the variations in geometry from animal to animal. In both cases, if the variations in geometry are not accounted for, arteries subjected to identical pressures could experience different levels of circumferential stress and flow-induced shear stress.

We detected significant difference in each biological marker between arteries cultured at high circumferential stress (Case A) and arteries cultured at low circumferential stress (Case B). The increases in matrix synthesis at higher circumferential stress are consistent

with the finding that protein synthesis increases in strips of rabbit pulmonary artery cultured at elevated wall stress under no flow conditions for 4 days.<sup>18</sup> Further, this finding is consistent with the previous reports that, in response to sustained hypertension, remodeling results in thickening of the arterial wall.<sup>7,23,36</sup> Though an increase in pressure induces an adaptive remodeling response that restores the baseline values of circumferential stress, it is accepted that remodeling related responses are driven by the elevated circumferential stress.<sup>23</sup> In this study, the stress is kept permanently elevated and the remodeling outputs could be limited by the synthetic and proliferative capacity of smooth muscle cells, an issue which needs further investigation.

The effect of circumferential stress on MMP-2 and pro-MMP-2 activity is consistent with the finding that MMP-2 activity in arteries subjected to a pressure of 200 mmHg is about 70% less than that of arteries held at a 100 mmHg over a 48 h culture period.<sup>2</sup> However, in that study, MMP-9 levels were significantly greater in hypertensive arteries which was not observed in this study where pro-MMP-9 activity was unaffected by circumferential stress. The reason for the discrepancy in the MMP-9 results is unclear, but could be due to the differences in culture time. Because MMPs are involved in matrix degrading processes,<sup>8</sup> the artery is essentially acting to limit tissue degradation and, therefore, contributing to a net increase in tissue content by reducing MMP-2 activity in response to elevated circumferential stress.

The increases in proliferation of medial and adventitial cells at high circumferential stress is consistent with the increase in proliferation reported for arterial strips under elevated wall stress in static culture.<sup>18</sup> The observation that cell proliferation is higher in intimal and adventitial cells than in medial cells is also consistent with previous findings.<sup>6</sup>

In contrast to circumferential stress, we found that the flow-induced shear stress does not, in the short term, have a significant affect on the measured biological markers of remodeling. The results for MMP-2 activity in response to changes in shear stress are consistent with the finding that MMP-2 activity was not statistically different in arteries cultured at 0.15 and 1.5 Pa after 48 h in culture, a response that was independent of pressure.<sup>2</sup> In that study, MMP-9 was not affected by shear stress at a pressure of 100 mmHg, but increased with shear stress at a pressure of 200 mmHg.<sup>2</sup>

The apparent lack of remodeling response to changes in shear stress may be due to several factors. Given the wide range of physiologic shear stresses, it is possible that the values of shear stress used in this study were not extreme enough to induce a remodeling

response. Normal physiologic shear stresses can range from 1.0 to 7.0 Pa depending on location within the vasculature.<sup>25</sup> In this study, arteries were exposed to shear stresses of 0.75 and 2.25 Pa, values that are 50% less and greater than 1.5 Pa, the level of shear stress that is typically considered physiologic in experiments. In addition, remodeling in response changes in flow is generally a slower process compared to pressure induced remodeling. It is possible that 72 h culture period is not sufficient to indicate detectable changes caused by wall remodeling. Finally, it is possible that remodeling response depends on the magnitude of circumferential stress experienced by the smooth muscle cells after flow-induced changes in the shear stress sensed by the endothelial cells. It is speculated that abnormal circumferential stress in combination with flow-induced alterations in the contractile state of the vascular smooth muscle might elicit the remodeling response.<sup>31</sup>

The limitations of this study result from technical constraints associated with the organ culture system. This study focuses on the case when the pressure and flow rate are varied in a manner to maintain prescribed steady values of circumferential stress and flow-induced shear stress. Control over pressure and flow to maintain prescribed pulsatile values of stresses requires a more sophisticated closed loop control system.

Lack of continuous and non-destructive monitoring of the deformed wall thickness limits the duration of the experiment to a period within which the unloaded arterial dimensions remained unchanged and wall thickness is determined from the condition for conservation of total volume. During the first 15 h of culture, circumferential and shear stress were calculated using the initial unloaded dimensions because the unloaded dimensions of the artery change during this period. Although this results in errors in calculating circumferential and shear stress, it was not possible to improve the accuracy of our stress calculations using the current organ culture system.

In conclusion, this study proposes a novel approach to study arterial remodeling in organ culture based on response to controlled changes in the local mechanical environment of endothelial and smooth muscle cells. The feasibility of the method is illustrated by studying the effects of the circumferential wall stress and luminal shear stress on set of selected biological markers associated with remodeling. We found that circumferential and shear stress differentially affect several biological markers of remodeling.

The proposed approach can be used for the design and realization of different types of experiments focused on the effects of local mechanical parameters on arterial remodeling. It is expected that the results from such experiments can promote a better

understanding and prediction of the behavior of arteries during normal physiological conditions and at certain pathological states. The results from the proposed approach can be beneficial for tissue engineering of arterial grafts. Understanding the effects of local mechanical factors on remodeling can offer a scientific basis for a design of optimal mechanical conditioning in bioreactors of constructs with embedded cells. Finally, the results obtained can be useful for mathematical modeling of arterial remodeling. Mathematical modeling of arterial remodeling requires motivated selection of local mechanical parameters, which deviation from homeostatic values are assumed to elicit specific modes of remodeling. So far, the proposed stress-grow laws<sup>13,35</sup> and remodeling rate equations<sup>31,32</sup> are based on an intuitive selection of the driving mechanical factors, rather than on their experimentally motivated ranking. The results of the novel experimental design can identify which are the significant mechanical parameters that should be taken into account when growth laws are postulated.

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