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# Responses of intramembranous bone and sutures upon *in vivo* cyclic tensile and compressive loading

Alexandra I. Peptan, Aurora Lopez, Ross A. Kopher, Jeremy J. Mao *

*Columbia University, College of Dental Medicine, Fu Foundation School of Engineering and Applied Sciences, Department of Biomedical Engineering, 630 W. 168 St. – PH7E – CDM, New York, NY 10032, USA*

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**Abstract**

Cranial vault and facial sutures interpose between mineralized bones of the skull, and may function analogously to appendicular and cranial base growth plates. However, unlike growth plates that are composed of chondrocyte lineage, cranial and facial sutures possess heterogeneous cell lineages such as mesenchymal cells, fibroblasts, and osteoblasts, in addition to vascular-derived cells. Despite recently intensified effort, the biological responses of intramembranous bone and sutures to mechanical loading are not well understood. This study was designed to investigate whether brief doses of tensile or compressive forces induce modeling and growth responses of intramembranous bone and sutures. In different groups of growing rabbits *in vivo*, cyclic tensile or compressive forces at 1 N and 8 Hz were applied to the maxilla for 20 min/day over 12 consecutive days. Computerized histomorphometric analyses revealed that the average sutural widths of both the premaxillomaxillary suture (PMS) and nasofrontal suture (NFS) loaded in either tension or compression were significantly higher than age- and sex-matched sham controls ($P<0.01$). The average cell densities of tension- or compression-loaded PMS and NFS were significantly higher than sham controls ($P<0.01$). The average osteoblast occupied sutural bone surface loaded under tension was significantly higher than that of sham control ($P<0.05$). Interestingly, tensile loading significantly reduced the average osteoclast surface, in comparison to sham control ($P<0.05$). For the NFS, tensile loading significantly increased the average osteoblast occupied sutural bone surface, in comparison with that of sham control ($P<0.05$). Also for the NFS suture, compression significantly reduced the average sutural osteoclast surface in comparison with sham control ($P<0.05$). Taken together, the present data suggest that high-frequency cyclic forces in either tension or compression induce modeling and growth changes in cranial sutures. Due to the structural complexity of cranial vault and facial sutures, either tensile or compressive forces likely are transmitted as shear stresses and upregulate genes and gene products responsible for sutural growth.


*Keywords:* Cranial sutures; Forces; Osteogenesis; Osteoblasts; Osteoclasts

## Introduction

Regulation of bone mass and architecture by mechanical loading has gained increasing attention not only as a result of the desire to understand bone as a biologically viable structural material but also as a result of highly prevalent skeletal diseases such as arthritis, osteoporosis, and developmental deformities. Most previous work on mechanical modulation of bone architecture and bone density has focused on the appendicular skeleton. In the mechanostat model, strain amplitude was recognized as the primary determinant of bone adaptation [1]. Subsequent studies have demonstrated that strain rate is another critical variable for both periosteal and endocortical bone appositions [2–6]. In avian and sheep ulna models subjected to four-point loading, periosteal and endocortical bone appositions are increased by short daily doses of mechanical stimuli as few as 36 cycles per day [7,8]. In a rat tibia model subjected to four-point loading up to 1200 cycles per day, periosteal and endocortical bone apposition rates vary as a function of strain rates [2,9]. In the adult sheep ulna, the trabecular bone density increases more than 1/3 in response to brief daily bursts of low-amplitude, high-frequency stimulation over 1 year [10]. Cyclic mechanical strain twice as high as that in normal function applied daily to the rat ulna not only induces bone apposition but also inhibits osteoclastogenesis as evidenced by TRAP

* Corresponding author. Fax: +1 212 342 0199.
*E-mail address:* jmao@columbia.edu (J.J. Mao).

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labeling [11]. The converging message from an increasing number of experimental studies of mechanical modulation of appendicular bone appears to suggest that brief doses of cyclic mechanical stresses, especially interposed with resting periods, stimulate anabolic responses.

Craniofacial skeleton derives from both neural crest and mesenchymal cells, and mostly is formed via the intramembranous ossification. A unique feature of intramembranous skull bones is the presence of craniofacial sutures that serve both to transmit mechanical stress and to enable longitudinal growth [12,31]. Cranial sutures are soft connective tissue between mineralized intramembranous skull bones and consist of multiple cell lineages such as mesenchymal cells, fibroblast-like cells, osteogenic cells and blood vessel-borne cells. The metabolic responses of these connective tissue cells in cranial sutures to mechanical stresses are not well understood. Contrary to early belief, flat bones of the cranial vault are loaded during mastication [13–15]. The structural complexity of craniofacial bones and the presence of cranial sutures provide the basis for momentary changes in tension, shear and compression during functional activities such as mastication [13,14]. Recently, brief doses of cyclic forces at 5 N and 1 Hz have been found to induce anabolic changes in craniofacial sutures as evidenced by increasing sutural widths and increasing sutural cell density [16].

Biological changes in cranial sutures upon mechanical loading seem to be more related to modeling than remodeling, given that changes in sutural morphology readily take place [12,31]. Modeling is defined as changes in bone's shape and dimension, whereas remodeling indicates bone resorption and apposition without net change in shape [1]. Furthermore, different waveforms and frequencies of exogenous cyclic forces are expressed as corresponding waveforms and frequencies of bone strain in cranial sutures [15,16]. The objective of the present study was to investigate whether brief doses of tensile and compressive forces at a higher frequency (8 Hz) induce different metabolic responses. The rationale for the present selection of both tensile and compressive forces in a single animal model is to address a clinically held concept in dentofacial orthopedics that tension leads to bone formation, whereas compression leads to bone resorption [33]. This clinical belief leads to our motivation for a null hypothesis that tension and compression applied in age- and sex-matched animals have similar effects in sutural growth.

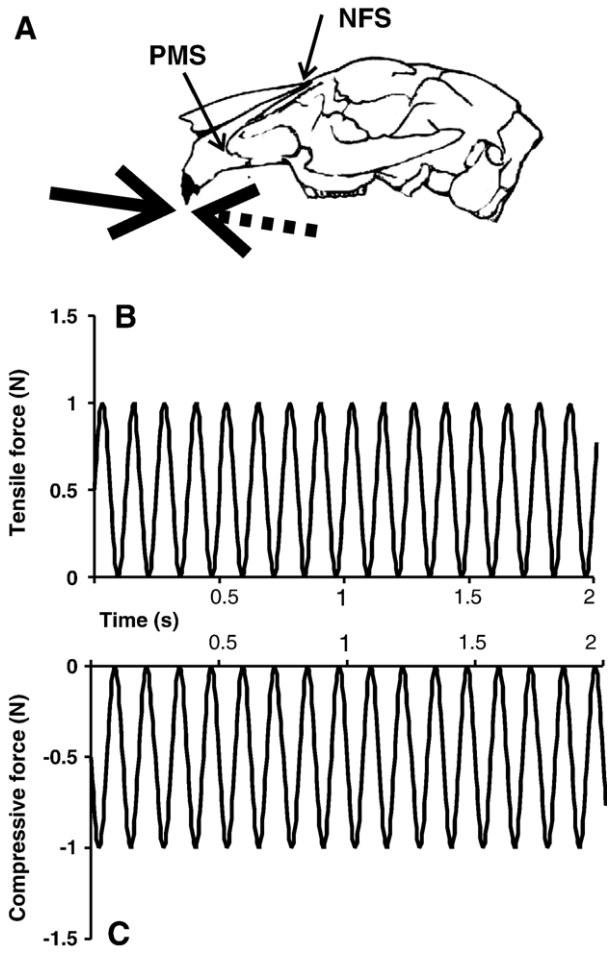


Fig. 1. Schematic and photographic illustrations of the rabbit craniofacial skeletal system and force application. (A) Schematic diagram of the rabbit skull in the sagittal plane and the orientation of the premaxillomaxillary suture (PMS) and nasofrontal suture (NFS). The solid arrow pointing right indicates compressive forces, whereas the dashed arrow pointing left indicates tensile forces. (B and C) Representative traces of force application over 2 s time course: B, cyclic tensile forces; C, cyclic compressive forces, both oscillating at 8 Hz and with a peak magnitude of 1 N.

## Material and methods

### *Chronic delivery of mechanical stimuli*

A total of twenty-one 6-week-old male New Zealand White rabbits with a mean body weight of 1.125 kg were randomly allocated to sham control ($N=5$), tensile force ($N=8$) and compressive force ($N=8$) groups. Under general anesthesia by intramuscular injection of a cocktail containing 90% ketamine (100 mg/ml; Aveco, Ford Dodge, IA) and 10% xylazine (20 mg/ml; Mobay, Shawnee, KS), tensile and compressive forces with the same peak magnitude of 1 Newton (N) were pre-programmed with a computerized servohydraulic system (858 Minibionix II, MTS, Eden Prairie, MN) and delivered on the maxillary incisors for 20 min/day over 12 days (Fig. 1A). The rabbit was placed in a supine position in a custom-made device that provided rigid fixation of the skull. The direction of mechanical loading was maintained constant by securing the maxilla to the fixation device. In two different animal groups, sine-wave compression or tension was applied at 8 Hz for 20 min/day, leading to 9600 cycles per day (cpd). The selection for 9600 cycles per day is based on our previous work demonstrating that brief doses of cyclic forces, such as 9600 cycles per day, were effective in inducing sutural growth changes. After daily force applications, the rabbits recovered and resumed food intake. A sham control group of age- and sex-matched rabbits were treated the same as the two mechanically loaded groups with the exception of mechanical loading. The rabbits were housed in a temperature-controlled room (23–25 °C) and given standard amounts of food and water. The present animal protocol was approved by the institutional animal care and use committee.

### *Tissue harvest and preparation*

Following the last episode of mechanical loading on Day 12, the rabbits were euthanized by pentobarbital overdose. After euthanasia, the skull was bisected in the midsagittal plane. The premaxillomaxillary suture (PMS) and nasofrontal suture (NFS) were selected for the present study due to their relevance to the understanding of calvarial growth, as well as their different proximity to the point of mechanical loading. The PMS suture is immediately

adjacent to mechanical loading, whereas the NFS suture is distant to the point of force application on the premaxilla. The PMS and NFS suture were dissected en bloc with at least 4 mm of surrounding bone. The specimens were trimmed, dehydrated and demineralized in 50% formic acid and 20% sodium citrate and embedded in paraffin. Sequential 8-μm sections were cut in the parasagittal plane and stained with hematoxylin and eosin.

### *Computer-assisted histomorphometry*

Quantitative histomorphometric analysis of specimens was performed using an image-analysis system (Image Pro Plus). All measurements were blindly performed. Standardized grids (1175×880 μm) were constructed and laid over sutural histologic specimens under 4× magnification for measuring sutural width. Linear sutural widths were measured by drawing circles with diameters equal to the width of the suture in each grid block. Each circle's diameter was equal to the width of the suture within each grid block. The total number of cells present in the suture, but excluding those lining the sutural bone formation fronts and blood vessel-borne cells, were counted within 6 randomly selected grid units in standardized 110×110 μm grids under 20× magnification. The average cell density was converted to the number of cells per $mm^2$. To quantitatively analyze bone formation and resorption under the mechanical stimuli, we measured the total sutural bone surfaces, cubic-shaped, active osteoblast lining surfaces and multi-nucleated osteoclast lining surfaces on multiple histological sections. The sutural bone surface lined by flat bone-lining cells was excluded in either bone formation surface or bone resorption surface.

### *Data analysis and statistics*

All data were expressed by mean±standard deviation. All numerical data including the average sutural width, sutural cell density and bone formation and resorption surfaces for control and experimental groups were subjected to the analysis of variance (ANOVA) with Bonferroni tests at an $\alpha$ level of 0.05.

## Results

### *Exogenous forces*

Force traces applied to the rabbit maxilla and recorded with a load cell incorporated in the computerized servohydraulic system are demonstrated in Figs. 1B and C. The polarity of tensile forces was positive (Fig. 1B), whereas the polarity of compressive forces was negative by convention (Fig. 1C). Both tensile and compressive forces oscillated at 8 Hz and peaked at 1 N, as shown in the representative 2-s time course (Figs. 1B and C).

### *Sutural width and intramembranous bone adaptation*

Representative photomicrographic images of PMS and NFS are demonstrated in Fig. 2. In comparison with the control PMS and NFS sutures (Figs. 2A and B, respectively), marked sutural widening occurred in response to both exogenous tensile forces (Figs. 2C and D for the PMS and NFS, respectively) or compression forces (Figs. 2E and F for the PMS and NFS, respectively). The PMS showed typical zigzag structures formed by interposing sutural tissue and surrounding sutural intramembranous bone (Fig. 2A). By contrast, the NFS displayed less robust zigzag patterns formed by interposing sutural tissue and surrounding sutural intramembranous bone (Fig. 2B). These qualitative observations of sutural widening are substantiated by the following quantitative data, using standardized grids and circles by computerized histomorphometric analysis. In the PMS, exogenous cyclic compressive forces induced an average sutural width of 163.4±27.7 μm, whereas cyclic tensile forces evoked an average sutural width of 174.9±34.2 μm, both representing significant increases over the control sutural width (69±16.3 μm) ($P<0.01$) (Fig. 4A). By contrast, the average sutural width of the NFS treated with cyclic tension (292.4±73 μm) was significantly greater than sham control (196±64 μm) ($P<0.05$), whereas cyclic compression failed to induce significant increases in NFS sutural width in comparison with control (Fig. 4B). No apparent microcracks or microfracture were observed in sutural histological specimens.

### *Sutural cell density*

Fig. 3 demonstrates representative photomicrographic images of the distribution of sutural cells in sham control

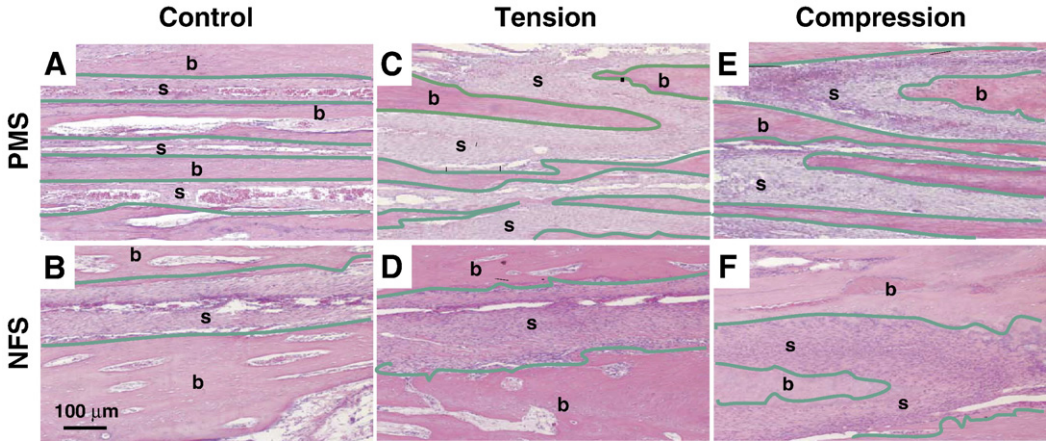


Fig. 2. Representative photomicrographic images of the geometric widths of control and mechanically stimulated cranial sutures. (A) Representative sham control premaxillomaxillary suture (PMS) under native growth; (B) representative sham control nasofrontal suture (NFS) under native growth; (C) PMS upon tensile loading; (D) NFS upon tensile loading; (E) PMS upon compressive loading; (F) NFS upon compressive loading. Green lines were manually drawn to indicate sutural edge between sutural connective tissue (s) and mineralizing bone (b). H&E stain; scale bar: 100 μm.

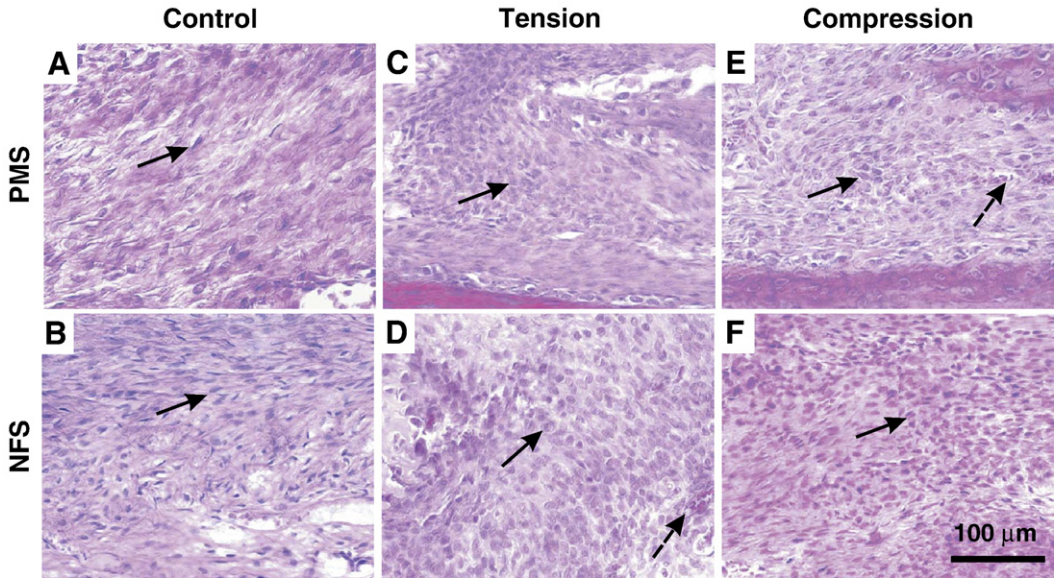


Fig. 3. Representative photomicrographic images of suture cell density of control and mechanically stimulated cranial sutures. (A) Sham control premaxillomaxillary suture (PMS) under native growth; (B) sham control nasofrontal suture (NFS) under native growth; (C) PMS upon tensile loading; (D) NFS upon tensile loading; (E) PMS upon compressive loading; (F) NFS upon compressive loading. Cell density was marked in both the PMS and NFS upon either cyclic tension (C and D) or cyclic compression (E and F). H&E stain; scale bar: 100 μm.

(Figs. 3A and B for the PMS and NFS, respectively), exogenous tensile forces (Figs. 3C and D for the PMS and NFS, respectively) or compressive forces (Figs. 3E and F for the PMS and NFS, respectively). Qualitatively, cell morphology of control specimens of both the PMS and NFS sutures (Figs. 3A and B) apparently differed from cell morphology of mechanical loaded sutures in either tension (Figs. 3C and D for PMS and NFS, respectively) and compression (Figs. 3E and F for PMS and NFS, respectively). Sutural cells appeared to be somewhat spindle-like in control specimens (Figs. 3A and B), whereas mechanically loaded cells seemed more rounded (Figs. 3C–F). The average sutural cell density of the PMS under both tension (9835±913 cells/mm$^2$) and compression (9917±846 cells/mm$^2$) was significantly higher than the average cell density of the sham control PMS (3471±376 cells/mm$^2$) ($P<0.01$) (Fig. 3C). The NFS treated with exogenous tension (12066±844 cells/mm2) or compression (10992±713 cells/mm$^2$) showed significantly greater average sutural cell density than sham control (3802±398 cells/mm$^2$) ($P<0.01$) (Fig. 3D).

*Sutural bone formation and resorption surfaces*

Quantification of osteoblast and osteoclast surfaces of PMS and NFS revealed interesting patterns. The average osteoblast surface of the PMS suture loaded under tension at 52.13±15.03% (center solid bar in Fig. 4E) was significantly higher than the average osteoblast surface of sham control at 31.25±9.91% (left solid bar in Fig. 4E) in the PMS suture ($P<0.05$), suggesting that tension induced significantly more osteoblast surface. Interestingly, tensile loading significantly reduced the average osteoclast surface at 4.63±7.35%, in comparison to the control at 13.75±5.77% (left and central open bars in Fig. 4E) in the PMS suture. For the NFS suture, tensile loading significantly increased the average osteoblast surface to 68.56±8.64%, in comparison with the average osteoblast surface of the sham control at 37.42±15.08% (left and central solid bars in Fig. 4F) ($P<0.05$). Also for the NFS suture, compression significantly reduced the average sutural osteoclast surface from sham control at 20.46±9.31% to 3.88±4.67% (left and right open bars in Fig. 4F) ($P<0.05$). No significant difference was observed between either osteoblast or osteoclast surfaces under tension or compression (Figs. 4E and F). The osteoblast surface of either PMS or NFS, with or without mechanical loading, was significantly higher than the corresponding osteoclast surface in the same suture (Figs. 4E and F).

## Discussion

The present work is a continuation of our previous study [16] that demonstrated anabolic responses of cranial sutures to cyclic compressive forces at 5 N and 1 Hz, but has adopted an 8-fold increase in force frequency (8 Hz) and 5-fold reduction in force amplitude (1 N). The present frequency of 8 Hz slightly more than doubles the natural chewing frequency of New Zealand White rabbits at about 3.5 Hz [17]. The rationale of doubling the natural functional frequency is that bone cells may more readily respond to mechanical signals that they do not customarily experience [12,31]. The present study also distinguishes from our previous work [16] in that both tension and compression have been applied, both leading to modeling and growth sutural responses. However, due to the structural complexity of cranial sutures as evidenced from the present data, either tensile or compressive forces likely are transmitted as tissue-borne shear stresses and flow-induced mechanical strain on cell membrane [12,15], consistent with several models of fluid-flow mechanics in osteoblasts [18–23].

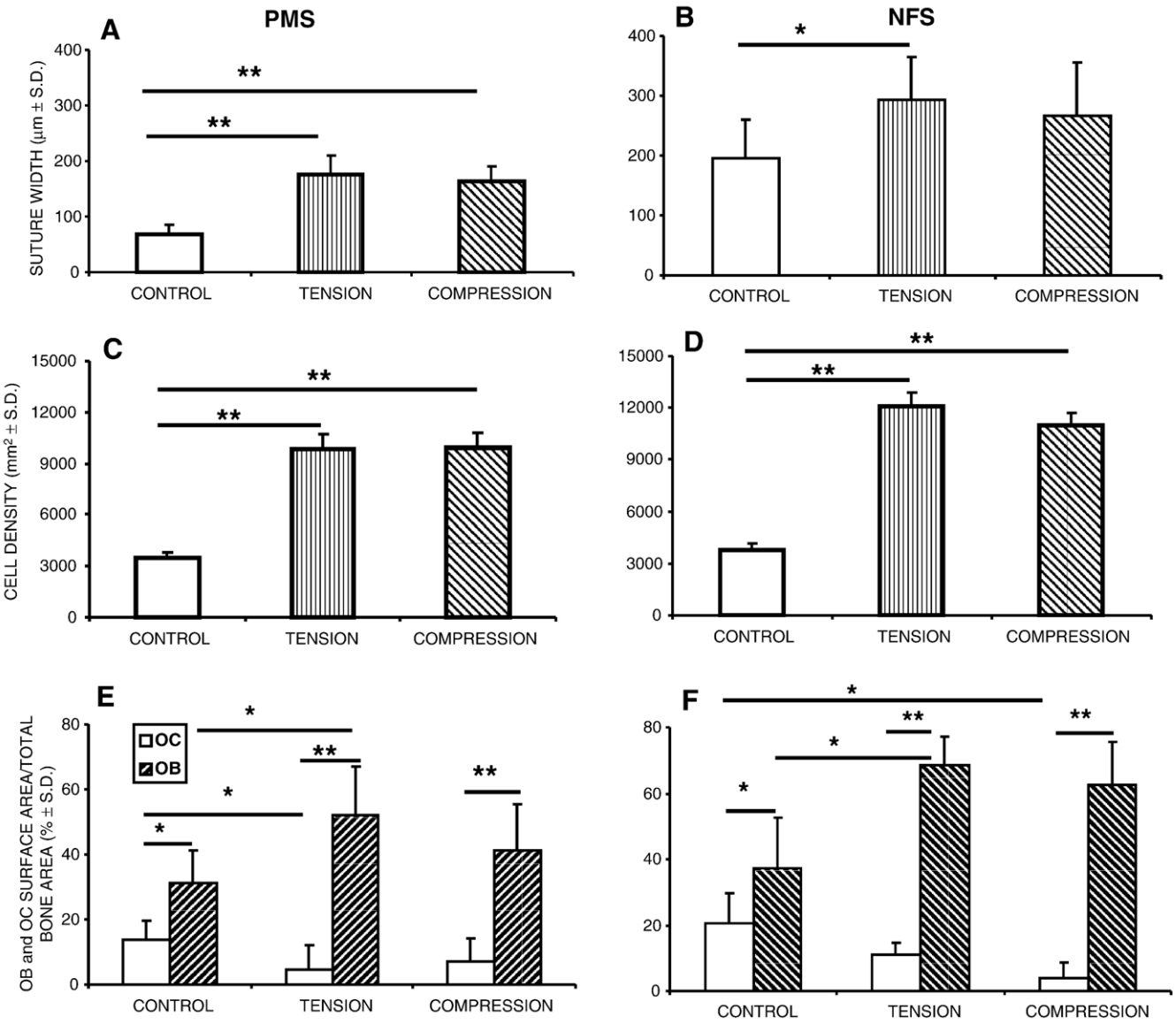


Fig. 4. Quantification of suture widths, cell density, and osteoblast and osteoclast surfaces. Suture width was quantified by fitting circles with the diameter of each circle equal to the width of the suture in the center of each standardized grid block. Suture cell density was quantified by counting all cells in the suture, excluding blood vessel-borne cells and cells lining sutural bone formation fronts, within standardized grids. (A and B) Suture widths of the premaxillomaxillary suture (PMS) and nasofrontal suture (NFS), respectively. (C and D) Suture cell density of the PMS and NFS, respectively. (E and F) The average osteoblast and osteoclast surfaces of the PMS and NFS, respectively. Compression and tension at 1 N and 8 Hz both induced significantly greater suture width, suture cell density, and suture osteoblast surface than sham controls ($P<0.05$ or 0.01).

Sutural growth is characterized by increases in cell proliferation, differentiation and matrix synthesis of mesenchymal, fibroblastic and osteoblastic cell lineages [12]. Sutural cell proliferation and differentiation likely accommodate two primary sutural functions to transmit mechanical stresses and to enable longitudinal growth of cranial bones. Stress transmission is a typical function of all joints, and likely 'tunes' sutural cells to mechanical stresses commonly experienced in native functions. The present increases in cell density upon loading at a frequency that is approximately twice the native chewing frequency likely represent increasing proliferation and/or decreasing apoptosis of sutural cells in response to uncommonly experienced mechanical signals. *In vitro*, cells isolated from cranial sutures, both fibroblastic and osteoblastic cells, are sensitive to mechanical stimuli [24–26,31]. Suture's other function to enable longitudinal growth necessitates the proliferation and differentiation of mesenchymal cells [12,31]. The attribution of some of presently observed increases in sutural cell density to mesenchymal cells is supported by the responses of mesenchymal stem cells to mechanical stresses [27]. It is unlikely that a substantial number of inflammatory cells contribute to sutural cell density due to short doses of daily loading for 20 min/day, representing only 0.1% of total daily time. Although we have not observed any marked inflammatory signs in the present study, we aim to continue to determine whether chronic inflammation may have occurred in long-term loaded cranial sutures in our ongoing studies.

Increase in sutural width identified in the present study can probably be attributed to a number of processes such as increased sutural matrix synthesis, as we previously discussed [15,16,31]. Hypothetical osteoclastogenesis and bone resorption induced by cyclic strain in the suture could have induced greater sutural width. The present osteoclast surface count lends little support of a major contribution by osteoclasts to the increased suture width. Bone resorption can occur radially at a rate of 12 μm/day during remodeling process [28–30]. Whether bone resorption is a major factor in mechanically induced sutural modeling or remodeling is not well understood. The present data suggest that osteoclast populated surface decreased somewhat in some, but not all, situations. This is at variation with our previous work [32] showing that osteoclast activity was apparently enhanced by cyclic loading, albeit in a different animal model with a different age. To what degree bone resorption is involved in sutural growth, with or without the interference of mechanical stress, is poorly understood but warrants additional studies using osteoclast markers such as RANKL. It appears that sutures can be viewed as a "confined chamber" containing fibroblastic cells in the center, osteogenic cells in the periphery between sutural bone edges, and covered by dense fibrous connective tissue periosteum [16]. This "confined chamber" appears to constitute an environment sensitive to mechanical stimulation. It is probable that mechanically induced fibrogenesis, including differentiation of sutural mesenchymal cells into fibroblast-like cells, sutural fibroblast proliferation, accounts for at least some of the increased sutural width and is indirectly supported by the present increases in sutural cell density upon cyclic strain and the observation that the increased sutural space is occupied by cells and their extracellular matrices [31]. In addition, the contribution of sutural osteoclasts to this proposed model of 'confined chamber' needs to be elucidated. The general qualitative impression of a change in cell morphology, from spindle-like cells in control sutures, to more rounded cells in mechanically loaded sutures, serves as additional indication that there may have been a change in sutural cell lineages. An intrinsic deficiency of the present study is that potentially different sutural cell lineages were not distinguished. Cell labeling, immunohistochemistry and other genetic approaches are necessary for differentiating among various sutural cell lineages.

The present work assumed the pattern of sutural bone strain to be the similar to that measured in our previous work [16] utilizing exogenous compressive forces from 1 to 5 N. Interestingly, application of exogenous compressive forces against the rabbit maxilla induces compressive strain in the PMS, but tensile strain in the NFS [16], and yet modeling and growth responses have taken place in both the PMS and NFS as observed in the present study. These sutural bone strain data further substantiate the observation that exogenous tensile and compressive forces both induce anabolic sutural growth responses.

Besides the interest in studying the responses of intramembranous bones and sutures to mechanical stress, an additional motivation of the present study is to determine whether cyclic forces with higher frequencies induce effective sutural growth, there may be ground for designing new clinical devices. Current devices in dentofacial orthopedics rely on the application of static forces. Converging data from our previous work and the present study suggest that cyclic forces induce more effective bone growth than static forces. The present data represent a rare study of the effects of tensile cyclic loading on the growth of cranial sutures, with compressive loading as a comparison. Taken together, the present data may have eventual implications in craniofacial orthopedics in that brief doses of cyclic forces in either tension or compression, may effectively stimulate sutural growth.

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