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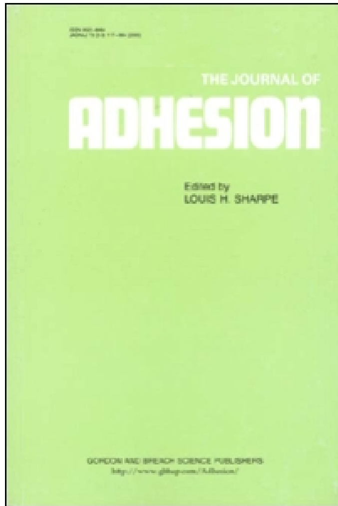


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# The Effects of Sterilization Treatments on Adhesion of Bone Cells to Titanium

J. C. Keller[a]; D. G. Wick[b]; R. A. Draughn[c]; J. P. Wightman[d]
[a] Dows Institute for Dental Research, College of Dentistry, The University of Iowa, Iowa City, Iowa, USA [b] Radiation Research Laboratory, Department of Radiology, The University of Iowa, Iowa City, Iowa, USA [c] Department of Materials Science, Medical University of South Carolina, Charleston, SC, USA [d] Department of Chemistry, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA

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# The Effects of Sterilization Treatments on Adhesion of Bone Cells to Titanium*

J. C. KELLER**

*Dows Institute for Dental Research, College of Dentistry, The University of Iowa, Iowa City, Iowa 52242-1010, USA*

D. G. WICK

*Radiation Research Laboratory, Department of Radiology, The University of Iowa, Iowa City, Iowa 52242, USA*

R. A. DRAUGHN

*Department of Materials Science, Medical University of South Carolina, Charleston, SC 29425, USA*

J. P. WIGHTMAN

*Department of Chemistry, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061-0212, USA*

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The effects of surface preparation and sterilization treatments of cpTi on *in vitro* osteoblast cell attachment were investigated. Surface characterization by XPS and AES and determination of wetting angles indicated that sterilization by exposure to ultraviolet light or gamma irradiation resulted in highly energetic, relatively contaminant-free cpTi surfaces which supported high levels of osteoblast cell attachment. Steam autoclaving, however, resulted in relatively low energy surfaces with high levels of O-C- and N-containing contaminants. Other inorganic contaminants were also detected on the surface of selected samples. As a result, steam-autoclaved surfaces supported significantly lower levels of cell attachment. At 2 hours after initial attachment, the osteoblast cells failed to spread and integrate onto the surfaces sterilized by steam autoclaving. These results confirm previous studies regarding the contamination resulting from steam autoclaving and indicate that non-contaminating sterilization procedures should be employed for the sterilization of commercial metallic implant materials.

KEY WORDS Titanium surfaces; X-ray photoelectron spectroscopy; Auger spectroscopy; bone cells; cell attachment; steam autoclaving; gamma irradiation; ultraviolet light; adhesion.

* One of a Collection of papers honoring James P. Wightman, who received the 13th Adhesive and Sealant Council Award at the ASC's 1993 Fall Convention in St. Louis, Missouri, USA, in October 1993.

** Corresponding author.

## 1. INTRODUCTION

It has been demonstrated repeatedly in recent years that the surface characteristics of biomedical implants can affect both the *in vitro* and *in vivo* biological properties of these materials.[1-12]. Implant surface characteristics which are believed to affect host tissue responses include materials selection,[13,14] surface morphology and roughness[12,15] and surface composition.[9,10,16] In relation to metallic biomedical implants, the composition and structure of metallic oxides are affected by chemical treatments and sterilization procedures.[4-7,9,10,17]

From both an *in vitro* and *in vivo* standpoint, previous research from several investigators has demonstrated that sterilization treatments can also affect tissue integration at implant interfaces.[1-3,8,10,11,16,17] Our laboratory has previously demonstrated that techniques such as steam autoclaving, exposure to ethylene oxide or certain chemical solutions can alter surface characteristics and thus affect initial *in vitro* biological responses such as cell attachment and morphology.[9,10] The detrimental effects of these sterilization treatments were thought to be due, in large part, to the presence of contaminants which remained on the surfaces following the sterilization procedure. Hartman *et al.*[8] were able to demonstrate *in vivo* that both argon radio frequency glow discharge and ultraviolet (UV) chamber treated implants were associated with rapid ingrowth and maturation of new bone when implanted into crania of Yucatan minipigs. Implantation of steam autoclaved surfaces, on the other hand, resulted in the formation of thick collagenous interfaces rather than neo-osteogenesis. In a later study, Budd *et al.*[11] demonstrated that the use of Ti implants which had been sterilized by UV or glow discharge treatments resulted in shortened time intervals required between implant placement and clinical loading situations.

The results of the above studies clearly indicate that certain sterilization techniques, such as steam autoclaving, can be detrimental to the integration of metallic surfaces by inorganic contaminants on the surface. Other techniques including UV light and radiofrequency glow discharge treatment do not contaminate the surface and appear to promote adequate biological responses.[10,16] A more recent technique by which manufacturers sterilize biomedical devices is the use of gamma ($\gamma$) irradiation, which possesses greater penetrating power than other methods due to the shorter wavelength employed. The effects of this sterilization procedure, however, on the chemical and related biological properties of biomedical devices has not been thoroughly explored.[17-19] The objective of this present study is to report on recent findings concerning the characteristics of Ti implant surfaces following $\gamma$ irradiation sterilization.

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## METHODOLOGY

### Implant Disk Preparation

Disk-shaped specimens which simulated implant surfaces were prepared from cpTi according to previously-published methods.[9,10,16] Briefly, discs were cut from 1.25 cm diameter bar stock and were hand polished through 600 grit SiC metallographic papers. The specimens were subsequently solvent cleaned in methylethyl ketone (5 min.), washed in deionized, distilled water (10 min.), acid passivated in 30% nitric

acid (30 min.) and given a final rinse (15 min.) in deionized, distilled water prior to air drying in a vacuum degiccator. Specimens were then sterilized by one of the three following methods before use in surface characterization or *in vitro* biological response assays. Specimens selected for steam autoclaving were placed in a glass petri dish, sealed and treated at 107°C, 0.14 MPa with a 20-minute pressure cycle using a standard clinical autoclave (American Sterilizer Co., Erie, PA, USA).[10] Additional samples were exposed uncovered to UV light irradiation at 254 nm, 300 $\mu W/cm^2$ for 10 minutes in a laminar flow hood (UVP Corp., San Gabriel, CA, USA). For $\gamma$ irradiated samples, monoenergetic (662 keV) $\gamma$ irradiation was performed on the cpTi discs utilizing a 12,000 Curie $^{137}Cs$ source (J. L. Shepard, San Fernando, CA, USA). The discs were placed in a petri dish with the discs arranged in a row approximately 0.5 cm apart and 13 cm long, parallel to the long axis of the irradiator source. The discs were carefully positioned at the same location during irradiation to ensure reproducibility of the minimal commercial irradiation value of 2.5 M Rads (25 KGy). The exposure time to achieve this level was calculated from the 1340 rads/min dose rate of the $^{137}Cs$ source as verified with a calibrated thimble chamber and r-meter (Victoreen, Cleveland, OH, USA). Treated tissue culture plastic (TCP) was used as a control substratum for the biological response assays.

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### Surface Characterizations

A series of surface analytical techniques was utilized to characterize the prepared surfaces following sterilization. Three samples were chosen for each surface characterization technique. X-ray photoelectron spectroscopy (XPS) was used to determine the overall surface composition. Analyses were performed using an X-ray photoelectron spectrometer (Model 5300, Perkin-Elmer Corp., Instruments Division, Norwalk, CT, USA) employing a Mg anode with a $K_\alpha$ radiation energy of 1253.6 eV. Elements present on the surface as a result of the treatments described were qualitatively evaluated from wide scan spectra. Narrow-scan spectra were subsequently taken to establish the binding energy and photopeak area of semi-quantitative analysis. The binding energies for all photopeaks were corrected by taking the carbon 1s photopeak at 285.0 eV. The atomic percentage of each element on the surface was calculated from the photopeak area and the surface composition was expressed as ratios of the calculated atomic percentages compared with Ti.

Auger electron spectroscopy (AES) in the depth-profiling mode was used to determine the oxide thickness. AES was performed using a scanning Auger microprobe (PHI 610, Perkin-Elmer Corp.) with an electron beam voltage of 3–5 kV and a beam current of 0.05 $\mu A$. Measurements of oxide thickness were made by depth profiling using argon sputtering with an ion beam current of 0.2 $\mu A$. The oxide thickness was calculated by multiplying the crossover time obtained from the Auger depth profile by the sputtering rate. The crossover time was the time corresponding to equal intensities of the titanium and oxygen Auger signals. The sputtering rate of 5 nm/min was obtained from sputtering a 100 nm film of tantalum oxide under the same sputtering conditions as used on the titanium samples.

Equilibrium wetting angles were measured with water using the sessile drop method to assess the wettability of the prepared surfaces. Three water drops were placed on

each disc using a microsyringe and the drops were photographed 30 seconds after placement. The contact angles were then manually measured from projected images of the drops. Repeated measurements of the same drop demonstrated a precision of 0.5 degrees. All wetting angle measurements were conducted in a laboratory atmosphere controlled at 23 °C and 70% relative humidity.

### *In Vitro* Cell Attachment Assays

The cells used in this work were osteoblast-like cells isolated from explants of the calvarial parietal plates of three-day-old Sprague-Dawley rat pups. The specific techniques of isolation and preparation are reported elsewhere.[18–20] In general, the cells were maintained in CMRL 1066 tissue culture media supplemented with 10% fetal bovine serum for 14–21 days. This heterogenous cell population has been used in other studies to demonstrate the expression of the osteoblast phenotype, including alkaline phosphatase, osteocalcin, osteopontin, osteonectin, and mineralization.[21–23]

The cells were harvested from the culture flasks and 50,000 cells were incubated onto the prepared surfaces for 15, 30, 60 and 120 minutes. Quantitative determinations of cell attachment were made after unattached cells were collected and counted using previously described techniques.[9,10] Six samples of each type were prepared for the four evaluation periods. A two way ANOVA and Duncan's multiple range tests were performed for identification of significant differences in the levels of attachment between different experimental groups at individual times ($\alpha = 0.05$).

Following the cell attachment assays, randomly-selected specimens were prepared for scanning electron microscopy (SEM) in order to evaluate alterations in cellular morphology on the different surfaces. Surfaces with attached cells were fixed in 2.5% glutaraldehyde/paraformaldehyde fixatives, dehydrated in an ascending series of alcohols, critical point dried and sputter coated with AuPd prior to SEM evaluation using an Amray scanning electron microscope (Model 1820, Bedford, MA, USA).

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## RESULTS

The prepared and sterilized cpTi discs were evaluated by SEM in order to study whether the preparation and/or sterilization treatments resulted in morphological alterations on the surface. A grooved defect pattern remained on all specimens following polishing through 600 grit SiC paper; however, these evaluations failed to demonstrate any discernable morphological alterations on the surfaces of the discs as a result of surface preparation and sterilization (data not shown).

XPS studies of the prepared surfaces revealed the presence of O-C-and N-containing contaminants in varying concentrations on all prepared surfaces (Table I). The most significant levels of contaminants were observed on the steam-autoclaved surfaces. In addition to the highest levels of O, C, and N contaminants, Fe, Na, Cl and Si were also observed on an inconsistent basis. Relatively clean Ti surfaces, free of inorganic contaminants, were observed as a result of UV light or $\gamma$ irradiation sterilization treatments.

The levels of carbon contamination were reflected somewhat by the high equilibrium contact angles formed by pure water on stream autoclaved surfaces ($70 \pm 16°$), and

TABLE I
Surface Characterizations of Sterilized cpTi

| XPS Atomic Ratios to Ti | UV | γ Irradiated | Steam Autoclaved |
|---|---|---|---|
| C | 0.73 ± 0.01 | 1.66 ± 0.3 | 2.68 ± 1.67 |
| O | 2.06 ± 0.05 | 2.73 ± 0.05 | 3.8 ± 2.6 |
| N | 0.03 ± 0.01 | 0.06 ± 0.03 | 0.1 ± 0.09 |
| Si | – | – | (0.54) |
| Fe | – | – | (0.1) |
| Na | – | – | (0.04) |
| Cl | – | – | (0.1) |
| Oxide Thickness (Å) | 26 ± 6 | 46 ± 7 | 35 ± 6 |
| Wetting Angles (°) | 52 ± 3 | 40 ± 2 | 70 ± 16 |

n = 3 samples per group; mean ± standard deviation
( ) = detected in only one sample.

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significantly lower wetting angles observed on UV (40 ± 2°) and γ irradiated surfaces (52 ± 3°), respectively. The levels of contamination did not appear to be related to the oxide thickness, as slight, but not significant, differences in oxide thickness were noted between experimental groups (26–46 Å).

In regards to the *in vitro* biological response assays, in general, the percent of cell attachment on the control surface increased with time until a plateau phase was reached at 1 hour (Figure 1). The highest percentage of cell attachment was observed on TCP control surfaces for each of the time periods investigated. Of the experimental surfaces, UV and γ irradiated surfaces supported the highest levels of cell attachment.

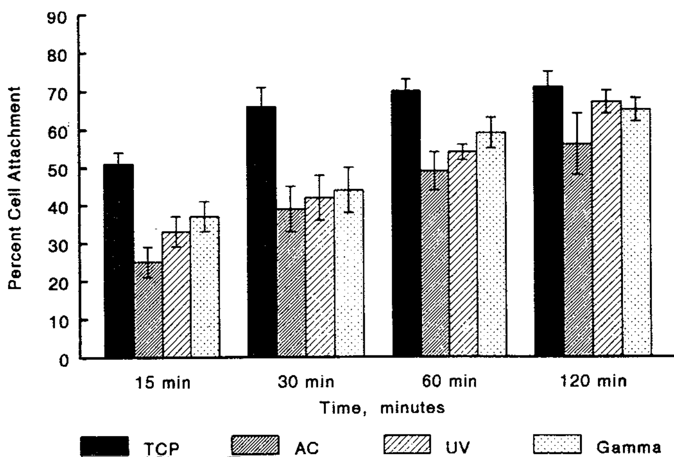


FIGURE 1 Graph of percent osteoblast cell attachment as a function of time for the control and sterilized cp Ti surfaces.

At 2 hours, the percentage of cell attachment for UV light and $\gamma$ irradiation treated specimens did not differ statistically from the TCP control. The lowest levels of cell attachment were observed with steam-autoclaved surfaces at each of the time intervals investigated in this work.

The results of the SEM evaluation of cellular morphology are shown in Figures 2a-c. In general, at 2 hours, the osteoblast-like cells had undergone significant attachment and spreading on surfaces sterilized by UV light and $\gamma$ irradiation. The intimate nature of the cell on the respective surfaces is demonstrated by the fact that the flattened cell morphology closely mimics the underlying morphology of the discs and there is substantial filopodial development. However, by 2 hours, the cells on the steam-autoclaved surfaces did not display significant spreading and integration on the prepared surfaces. This is depicted by the few cellular filopodial extensions observed on these surfaces and a general rounded appearance of the cell body.

## DISCUSSION

The data obtained from this investigation reinforced the need to understand the physical and chemical characteristics of the implant surface before undertaking *in vitro*

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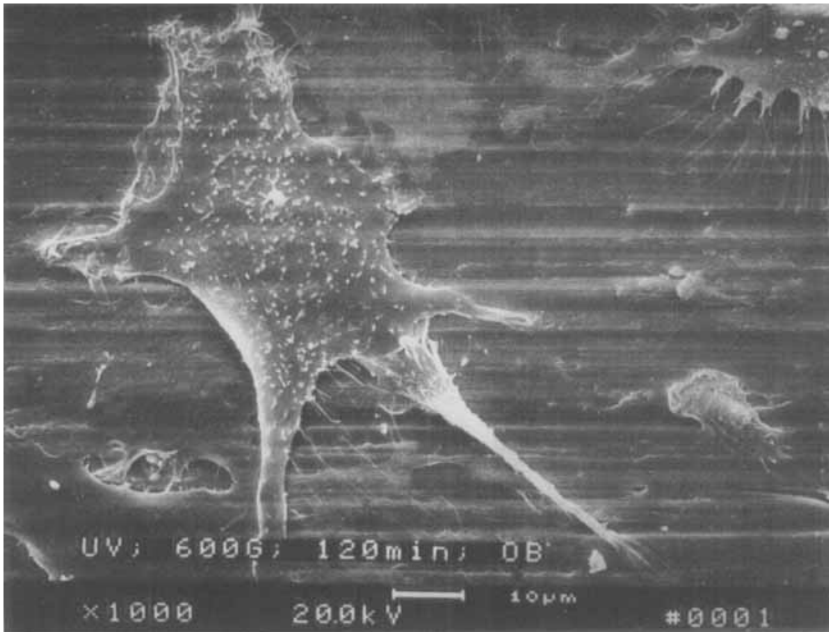
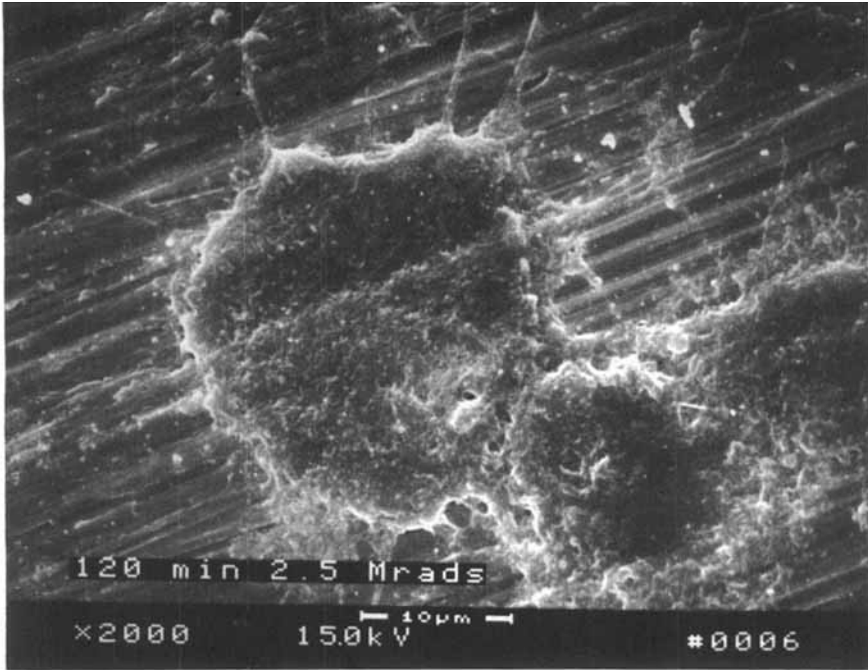
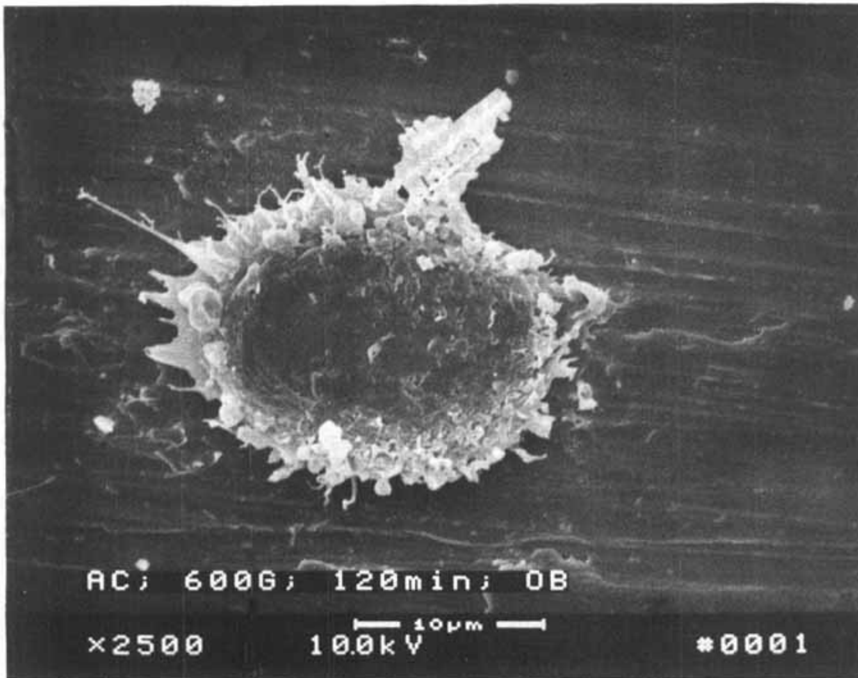



(a)

FIGURE 2 a) Scanning electron micrograph of osteoblast cell spreading on ultraviolet light sterilized cpTi surface; 120 minutes following initial cell attachment.
b) Scanning electorn micrograph of osteoblast cell spreading on $\gamma$ irradiated cpTi surface; 120 minutes. Note extensive cell flattening and development of filopodial extensions in a and b.
c) Scanning electron micrograph of osteoblast cell spreading on steam autoclaved cpTi surface; 120 minutes. The cell body is rounded, and lacks significant development of filopodial extensions.




(b)




(c)

**FIGURE 2** ***(Continued)***

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or *in vivo* biological response assays. The series of surface characterization techniques used in this study were designed to highlight differences in the surface chemistry following preparation and sterilization of cpTi surfaces. The short-term *in vitro* cell attachment assays used in this investigation were designed to address the initial responses of cells at the interface following surface preparation and sterilization treatments.

The series of surface characterization techniques used in this study demonstrated that implant surfaces were significantly altered by steam autoclaving procedures.[1–8,10] Although macroscopic particulate deposits were not observed on the surface of steam-sterilized Ti discs, changes in the nature of the oxide surface were detected by XPS and by measurements of wetting angles. Following steam autoclaving, higher levels of O, N, and C were observed on the Ti surfaces, probably as a result of adventitious contaminants. Other inorganic contaminants, such as Cl, Na, Si and Fe were also observed but in an inconsistent manner. This probably resulted from the water used for preparing the steam. It has been suggested by other investigations that the purity of the water used to prepare the steam for this sterilization technique can be quite variable.[5,8] Steam autoclaving also resulted in surfaces that demonstrated increased wetting angles which are associated with poor tissue adherence and integration.[1–3,5,10] This result was confirmed in the present study, in which an inhibiton of osteoblast-like cell attachment and poor cellular adaptation on the prepared implant surfaces was observed. The adverse effects of steam autoclaving on the ability of the osteoblast cells to undergo mineralization is also of concern and raises serious questions as to the ability of this sterilization treatment to support clinical osseointegration.[8] For these reasons, clinical sterilization by steam autoclaving is highly questionable when optimization of host tissue response is required.

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On the other hand, exposure of the prepared Ti surfaces to either UV light or $\gamma$ radiation produced relatively non-contaminated oxide surfaces. Although C-and N-containing contaminants were detected on these surfaces, the levels of the adventitious contaminants were low in comparison with other studies.[10] The O/Ti ratios for both UV and $\gamma$ irradiated surfaces approximated the theoretical value of 2.0 for $TiO_2$. These factors are indications that the contamination present on these materials was probably due to ubiquitous contaminants in the environment, rather than to the preparation and sterilization procedures themselves. The low wetting angles determined for the UV and $\gamma$ irradiation sterilized surfaces relative to those that had been steam autoclaved, were another indicator that these techniques produced relatively contaminant-free surfaces. As determined by the biological response assays used in this work, these energetic surfaces supported high levels of *in vitro* cell attachment and intimate cellular adaptation with the topography of the prepared surface.

These results are in general agreement with the earlier work by Baier *et al.*[1–3] and Keller *et al.*[9,10] in which highly energetic surfaces, free of organic and inorganic contaminants, support cell and tissue attachment. The fact that there were significant differences in cell attachment with relatively poor morphological integration of the cells on the steam-autoclaved surfaces indicated that the contaminants imparted to the surface from these treatments adversely affected osteoblast cell attachment and morphology. The negative influence of steam-autoclaving treatment on cell and tissue response confirm previous investigations conducted in our laboratory[10] and by

others.[1–8] It should be mentioned that preliminary investigations into the effects of steam-autoclave sterilization treatment on longer term cell responses involving expression of extracellular matrix proteins and mineralization responses indicate a detrimental effect on these cellular processes as well.[8,23]

## SUMMARY

The results of this study are in agreement with the hypothesis that surface preparation and sterilization procedures which result in highly energetic surfaces will afford and optimized biological response. The results of this work demonstrated that sterilization procedures such as UV light and γ irradiation reduced the level of contamination on the Ti surfaces. The continued use of these sterilization regimens for clinical applications warrants further investigation. The *in vitro* data provided here indicated that short-term cellular responses such as attachment and morphology are not adversely affected by such treatments. However, longer-term *in vitro* assays involving studies of phenotypic expression including mineralization in addition to *in vivo* experiments should be conducted to confirm these results. Based upon the work presented here, the practice of using steam-autoclaving procedures for implants requiring biological integration for fixation is highly questionable.

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