EFFECT OF LIPOPOLYSACCHARIDE CONTAMINATION ON THE ATTACHMENT OF OSTEOBLAST-LIKE CELLS TO TITANIUM AND TITANIUM ALLOY IN VITRO

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KEY WORDS

Adherence
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Failing implants with loss of alveolar bone are associated with gram-negative bacteria that carry lipopolysaccharide (LPS) in the bacterial cell wall. Bony regeneration around these implants is still an unpredictable procedure due to the many clinical factors involved. One important factor is the presence of contaminants such as LPS on the implant surface. The effect of implant-associated LPS on the attachment of bone cells to the implant surface is unknown. This project investigated the effect of LPS on the attachment of osteoblast-like cells (MC3T3-E1) to titanium and titanium alloy surfaces in vitro. We hypothesized that LPS would inhibit bone cell attachment either through loss of cellular attachment sites or alteration of cellular function. Three experimental approaches were used. First, alloy surfaces were exposed to LPS (100 μg/mL) before the cells were allowed to attach. In the second approach, the cells were exposed to the LPS before they were allowed to attach. Last, the cells were allowed to attach before exposure to LPS. Cellular attachment to implant materials was measured by using a histochemical stain (MTT). The results indicated that LPS presence did not significantly ($P > .05$) alter osteoblast attachment to titanium or titanium alloy surfaces whether the exposure occurred before or after cellular adherence. It was concluded that LPS did not directly effect the attachment of the MC3T3-E1 osteoblasts to these implant surfaces in vitro. Further research is needed to define the clinical liabilities of LPS during implant placement and maintenance.

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INTRODUCTION

Dental implants are a commonly used treatment option for edentulous areas of the mouth. Studies have confirmed the long-term success of dental implants.[1] Unfortunately, treatment options for failing implants are limited and are still under investigation.[2] Failing implants in partially edentulous jaws are associated with bacteria similar to those found around natural

teeth affected with periodontitis,[3] ie, gram-negative bacteria. The inflammatory disease process around teeth and implants may not be identical but always results in bone loss.[4] Bone resorption is thought to be induced, in part, by the presence of gram-negative bacteria and their by-products, like lipopolysaccharide (LPS).

Lipopolysaccharide is associated with the outer membrane layer of gram-negative bacteria and has been implicated in hard and soft tissue destruction in periodontal disease.[5] It is a potent bone resorbing promoter in vitro. It induces the release of potent inflammatory mediators from several cells,[6] it influences neutrophil migration,[7] and it is heat stable and requires special treatments to destroy its structure.[8]

Studies have shown that LPS is present in high amounts on the root surfaces of teeth affected with periodontitis compared with teeth that have not been exposed to the oral environment.[9] Other studies have shown that LPS adheres tenaciously to several materials.[10,11] Knoernschild and coworkers[10] found that radiolabeled LPS from *Escherichia coli* adhered to dental casting alloys and only 10% of the initially adhered amount came off over 96 hours of elution into LPS-free water. Nelson et al[11] confirmed Knoernschild's results using titanium and titanium alloys. Nelson and his group also compared LPS derived from *E. coli* and *P. gingivalis* and concluded that both forms of LPS behaved similarly in adherence to the metals used. Interestingly, Nakib et al[12] found that *E. coli* LPS on tooth surfaces can be effectively removed by tooth brushing for 1 minute.

The effect of adhered LPS on the attachment of osteoblasts to dental implants is still unknown. We hypothesized that the presence of LPS on a dental implant surface may inhibit the attachment of osteoblasts either by acting as a physical barrier to attachment sites or by affecting the osteoblastic function. This interference with osteoblast attachment might compromise the results of any regenerative treatment around a dental implant.

TABLE 1

Commercially pure titanium (CPT) composition* (wt%)

Iron	0.09
Oxygen	0.14
Nitrogen	0.011
Carbon	0.02
Hydrogen	<0.001
Residual elements total	<0.4
Titanium	Remainder

*As given by manufacturer.

TABLE 2

Titanium alloy (TiAlV) composition* (wt%)

Aluminum	6.5
Vanadium	3.94
Iron	0.26
Oxygen	0.17
Nitrogen	0.013
Carbon	0.012
Hydrogen	<0.005
Yttrium	<0.005
Residual elements total	<0.4
Titanium	Remainder

*As given by manufacturer.

The aim of the present investigation was to evaluate the effects of LPS on the attachment of osteoblasts to the surfaces of commercially pure titanium (CPT) and titanium alloy (Ti6Al4V). More specifically, the following aims were evaluated: (1) the effect of implant-adhered LPS on the attachment behavior of osteoblasts, (2) the effect of suspended LPS on osteoblasts preattached to implants, and finally, (3) the effect of exposing osteoblasts to suspended LPS before allowing the osteoblasts to attach.

MATERIALS AND METHODS

Six discs (12.7 mm in diameter, 1 mm thick) made from CPT and Ti6Al4V (Tables 1 and 2; Tico Titanium, Farmington, Mich) were prepared using a diamond saw from a stock rod. The discs were then treated to create a surface as close as possible to the surface of implants used clinically.[13] The discs were polished to a 600-grit surface by hand polishing with sandpaper and were subjected to solvent cleaning in methyl-ethyl-ketone (Fisher Scientific, Fairlawn, NJ) for 15 minutes followed by triple-distilled washing in LPS-free water. The discs were then placed in nitric acid (17.5%, Fisher Scientific) for 15 minutes and triple-washed in LPS-free water. This was followed by exposure to 1.7% hydrofluoric acid (HF) for 30 seconds, triple washing in LPS-free water, and then the discs were dried at 60°C and stored aseptically in a biologic incubator (37°C, 5% CO_2) until used in the experiment. Using the Limulus Amebocyte Lysate (LAL) assay kit (Biowhittaker, Walkersville, Md), pilot studies testing the presence of LPS showed that, at this step, the discs were free of any detectable LPS.

E. coli LPS (serotype 026:B6, Sigma Chemical, St Louis, Mo) was used to contaminate the surface of the discs. Although it may have been more relevant to use LPS from a bacterium associated with periodontal or peri-implant disease (such as *P. gingivalis*), E. coli LPS shares many characteristics with the *P. gingivalis* LPS. For example, both have been found to adhere tenaciously to implant materials,[11] and they both induce the release of several cytokines from human monocytes.[14] Also, *E. coli* LPS was commercially available in sufficient amounts to conduct all the necessary experiments whereas *P. gingivalis* LPS was not.

Cell cultures and experimental protocols

A MC3T3-E1 mouse osteoblastic cell line was used in this project. This cell line has a well-characterized osteoblastic phenotype that is stable and under the correct conditions will form mineralized matrix.[15] MC3T3-E1 osteoblasts are responsive to *E. coli* LPS,[16] and they were available in sufficient numbers to conduct all necessary experiments. These cells were generously provided by Dr Tom Hanks from the University of Michigan and were maintained in α-MEM medium with 10% fetal bovine serum (Life Technologies, Grand Island, NY).

Protocol 1 (Discs + LPS, Then Cells)

A suspension of 1 mL of a 100 μg/mL *E. coli* LPS was added to each disc (n = 6) in a 24-well plate (Costar, Cambridge, Mass) and placed in a biologic incubator (37°C, 5% CO_2) for 24 hours. This high concentration of LPS was used as a worst case scenario. Discs were moved to a new 24-well plate and 1 mL of LPS-free water was added to each disc and left for 24 hours. The water change was repeated every 24 hours for 120 hours to allow any labile LPS to come off the surface of the discs. The eluted LPS was monitored using the LAL assay kit. Discs were then dried in a 37°C incubator for 24 hours. MC3T3-E1 cells (100 000 cells/well) were seeded onto each disc in a 24-well plate and left to adhere for 24 hours.

It was important in this approach to confirm the presence of residual LPS on the disc surface after the elution period and that this residual adhered LPS had biological activity. To test for residual LPS, LPS-exposed then eluted discs were placed in LPS-free water in a 24-well plate. This plate was subjected to ultrasonic treatment for 4 minutes and the resultant suspension was tested for the presence of LPS using the LAL assay.

Another set of discs that went through the LPS exposure and elution were used to test for the biological activity of the adhered LPS on the disc surface. THP-1 human monocytes were incubated (250 000 cells/disc) onto these discs for 24 hours. THP-1 human monocytes (ATCC TIB202) are known to produce TNF-alpha in response to LPS stimulation.[17] TNF-alpha production after the 24-hour incubation period was measured using an enzyme-linked immunosorbant assay (EIA) (Quantikine, R&D Systems, Minneapolis, Minn).

Protocol 2 (Cells + LPS, Then Discs)

MC3T3-E1 cells were exposed to *E. coli* LPS at a concentration of 100 μg/mL. The cells were subjected to gentle shaking with the LPS for 20 minutes.

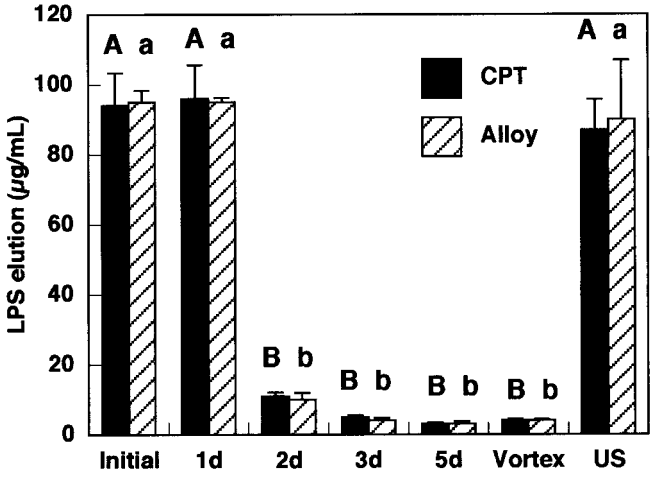


FIGURE 1. Passive LPS elution from TiAlV and CPT discs over 120 hours (5 days) and after using vortex and ultrasonic (US) postelution. The eluting solution (LPS-free water) was changed every 24 hours. Uppercase letters compare CPT groups, lowercase letters compare TiAlV groups. Groups A and a had statistically significant differences from groups B and b, respectively (n = 4; α = .05, ANOVA, Tukey).

The cells were then centrifuged and suspended in LPS-free medium, seeded onto LPS-free discs, and allowed to adhere for 24 hours at a density of 100 000 cells/well.

Protocol 3 (Cells + Discs, Then LPS)

MC3T3-E1 cells were seeded (100 000 cells/well) onto LPS-free discs and allowed to attach for 24 hours. Then *E. coli* LPS was added at 100 μg/mL for 24 hours.

Succinic dehydrogenase activity

Twenty-four hours after the cells were allowed to attach onto the discs (protocols 1 and 2) or 24 hours after the LPS challenge (protocol 3), the discs were triple washed using LPS-free phosphate buffered saline (PBS) in an attempt to remove any loosely attached or nonviable cells. The succinic dehydrogenase (SDH) activity of the cells remaining on the discs was evaluated using a histochemical stain, MTT (Sigma Chemical). 3-[4,5-Dimethylthiazoyl-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) is converted by active SDH to a colored formazan insoluble in aqueous solution. The staining of cells was therefore an indicator of their viability. Pilot studies showed that none of the materials alone caused the conversion of MTT to its formazan. Furthermore, since SDH is active only in the mitochondria of viable cells, nonviable cells cannot convert MTT to its insoluble formazan. After the cells were exposed to MTT, they were fixed (4% formalin in 0.2 M Tris) and the insoluble formazan form of MTT was dissolved in a dimethylsulfoxide (DMSO) solution (6.2% vol/vol 0.1 N NaOH in dimethylsulfoxide). The optical density of the resulting solution was measured using a spectrophotometer at 562 nm (Molecular Devices, Menlo Park, Calif). This technique has been shown to be simple, accurate, inexpensive, and reproducible.[18]

Polystyrene (PS) discs (Fisher Scientific, Newark, Del) were used as positive attachment controls (n = 6), and polyvinylsyloxane (PVS) dental impression material discs were used as negative attachment controls (n = 6).

Scanning electron microscopy

Since the discs were opaque to light microscopy, a scanning electron microscope was used to confirm the presence of attached cells to the discs. Preparation for the scanning electron microscopy included the standard techniques of fixing the specimens in 1% to 2% glutaraldehyde (overnight), dehy-

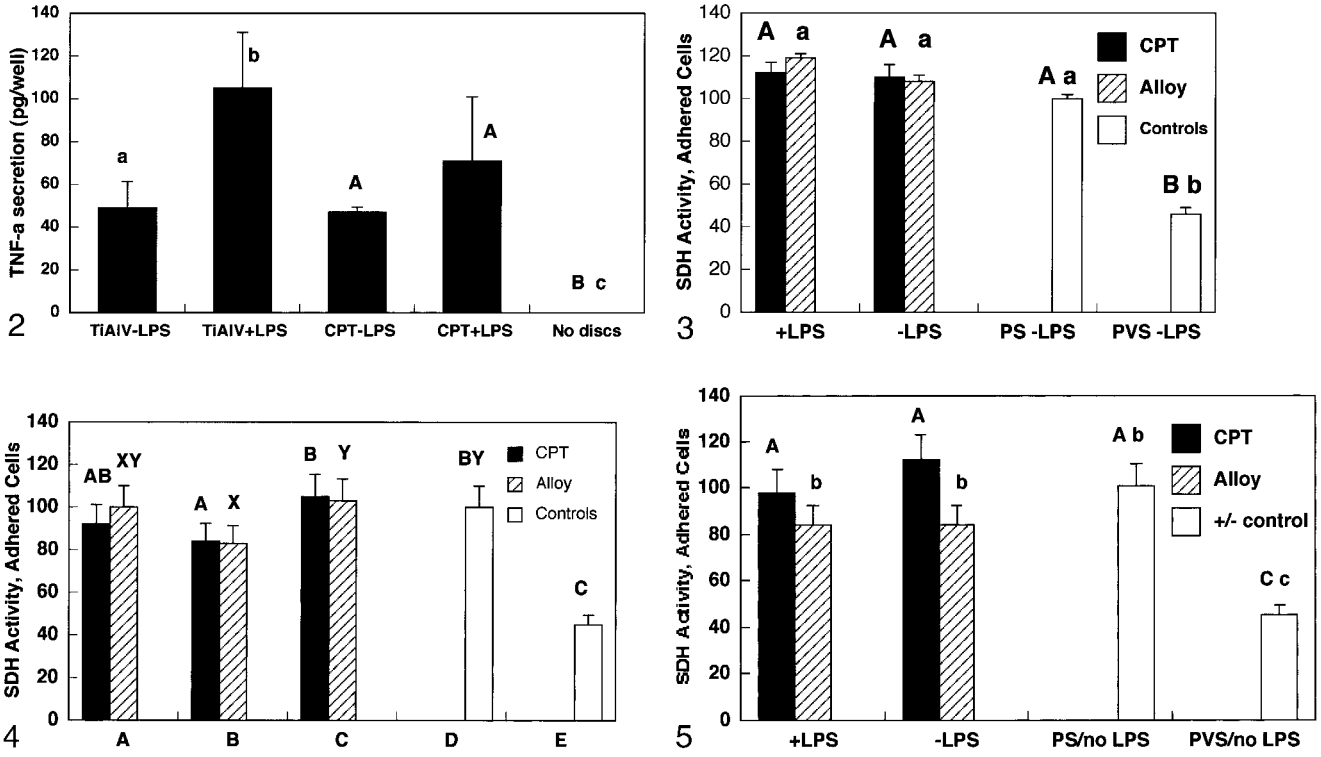


FIGURE 2. Response of monocytes (THP-1 cells) to LPS adhered to TiAlV and CPT discs. Control discs were CPT and TiAlV without LPS exposure. Uppercase letters compare CPT ± LPS. Lowercase letters compare TiAlV. Different letters indicate statistical differences (n = 4; α = .05, ANOVA, Tukey).

FIGURE 3. MC3T3-E1 cellular attachment behavior on TiAlV and CPT discs. Discs were exposed to *E. coli* LPS for 24 hours and were eluted for 120 hours. Uppercase letters compare CPT ± LPS, PS, and PVS. Different letters indicate a statistical difference (n = 6; α = .05, ANOVA, Tukey). Lowercase letters compare TiAlV. The LPS-exposed groups did not differ from the LPS-free groups, and both of these groups did not differ from the positive control (PS + cells). They were, however, different from the negative control group (PVS + cells).

FIGURE 4. MC3T3-E1 cells were exposed to *E.coli* LPS for 20 minutes (gentle shaking), then resuspended in LPS-free medium and plated on TiAlV, CPT, PS, and PVS discs for 24 hours. Groups: A, cells + LPS + shake (TiAlV, CPT); B, cells − LPS + shake (TiAlV, CPT); C, cells − LPS − shake (TiAlV, CPT); D, cells − LPS − shake (PS); E, cells − LPS − shake (PVS). Group C is a control group for the shaking effect on cells. Letters above bars represent statistical analysis (n = 6; α = .05, ANOVA, Tukey). Uppercase letters compare CPT, whereas lowercase letters compare TiAlV. Different letters indicate statistically significant differences.

FIGURE 5. MC3T3-E1 attachment behavior on TiAlV and CPT discs with LPS exposure occurring after 24 hours of attachment. LPS exposure lasted 24 hours. Positive controls were PS discs with cells, negative controls were PVS discs with cells. Uppercase letters compare CPT ± LPS, PS, and PVS groups. Lowercase letters compare TiAlV ± LPS, PS, and PVS groups. Different letters above bars indicate statistically significant differences between groups (n = 6; α = .05, ANOVA, Tukey)

dration in alcohol, critical point drying, mounting the specimens, and then gold coating before viewing.

Statistical analysis

The optical densities for SDH staining on the PS discs were defined as 100%, and all other results were expressed as percentage of PS. Experimental conditions for each protocol were compared using one-way ANOVA with Tukey multiple comparison intervals (α = .05).

RESULTS

Figure 1 shows the data from the experiment that assessed elution of LPS from discs after 24 hours of LPS exposure. The results indicate that at 120 hours (5 days) of elution, there were minimal amounts (<25 ng/mL) of LPS released from the discs. However, ultrasonic treatment of the discs after elution released abundant amounts of LPS, confirming the presence of LPS on the discs after the elution period. Figure 2 shows that adherent LPS retained at least some biological activity. LPS on TiAlV had a stronger effect on the surrounding THP cells than the LPS on CPT and reached statistical significance when compared with the alloy discs without the LPS (n = 3; α = .05, ANOVA, Tukey). On the other hand, no statistical significance can be detected when comparing the CPT-LPS with the CPT+LPS group. However, there was some indication of increased TNF-alpha on LPS-treated CPT discs as well.

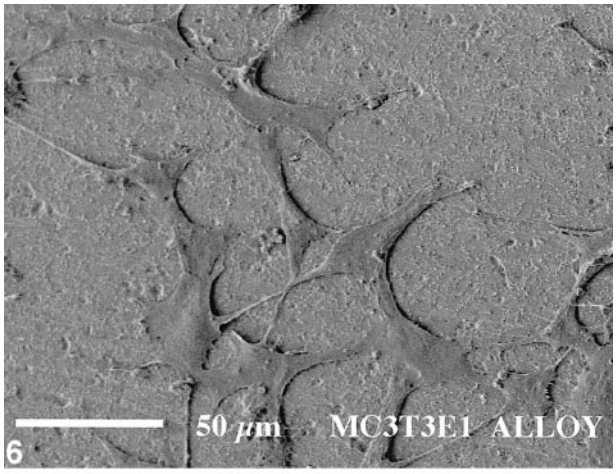
Figure 3 shows the cellular attachment behavior on CPT, TiAlV, CPT+LPS, and TiAlV+LPS discs (protocol 1). Cells attached to CPT and TiAlV discs without LPS at levels statistically equivalent to PS. Surprisingly, cellular attachment to LPS-contaminated discs did not differ from the attachment to noncontaminated discs (α =

.05) for either CPT or TiAlV. The negative control group (PVS) behaved as expected; there was a statistically significant reduction in cellular attachment.

Figure 4 shows that cellular exposure to LPS before the cells were allowed to attach did not effect their attachment to the discs (protocol 2). When evaluating the effect of shaking on the cellular attachment, it was apparent that there was a tendency to have a lower response from the cells that were exposed to shaking alone.

Figure 5 shows that, once the cells attached to the discs (protocol 3), LPS exposure for 24 hours did not significantly reduce the number of viable cells attached to the discs.

Figure 6 is a scanning electron microscope (SEM) micrograph of typical cellular attachment on TiAlV discs not exposed to LPS, verifying that cellular attachment occurred to the TiAlV. Figure 7 shows an SEM of cellular attachment on LPS-contaminated TiAlV discs. No apparent change in cellular morphology was detected, and cells appear to spread well on surfaces even in the presence of LPS.



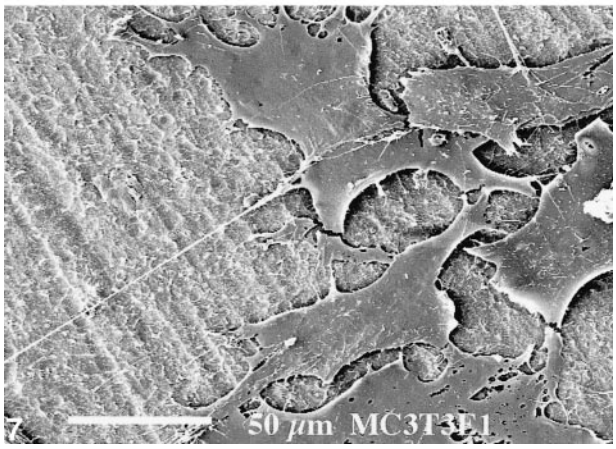


FIGURE 6. SEM micrograph of MC3T3-E1 cells attached on LPS-free TiAlV discs for 24 hours (50,000 cells/cm^2, ×490).

FIGURE 7. SEM micrograph of MC3T3-E1 cells attached on TiAlV disc that was preexposed to *E. coli* LPS for 24 hours with no LPS elution (50,000 cells/cm^2, ×451).

DISCUSSION

Experiments that measured LPS elution from the metal discs (Figure 1) gave results that corresponded well with those reported by Knoernschild et al[10] and Nelson et al.[11] On the other hand, experiments that measured attachment of MC3T3-E1 cells to LPS preexposed discs (Figure 3) gave unexpected results since our hypothesis predicted reduced cellular attachment in the presence of LPS. Zoller et al[19] have reported that fibroblast attachment was decreased on titanium surfaces treated with saliva. They confirmed their results with SEM micrographs. Our results using the MC3T3-E1 osteoblasts and LPS as a surface contaminant showed a contrasting result.

In an attempt to explain why cellular attachment was not affected in the presence of LPS (protocol 1), one can argue that LPS was not uniformly distributed over the disc surface. In other words, the distribution of LPS on the disc surface may have been patchy in nature and did not totally cover the surface. Subsequently, the osteoblasts could have attached to the LPS-free spots on the surface. This possible explanation is supported by Williams and Williams,[20] who found that albumin adsorbed onto the surfaces of Ag, Au, Ti, and sintered Ag 10%-Ti and Ag 10%-Ta in a nonuniform manner across the heterogeneous surface of these materials. Despite the difference between LPS and albumin, one can speculate that a similar phenomenon could have occurred in the current experiments.

Nonuniform LPS attachment could also explain why there was a difference between the THP response to the adhered LPS on TiAlV discs compared with CPT discs. Since the composition of each alloy was different and the surfaces were certainly different, LPS adherence could have been different for each material. The amount of LPS adhered to the discs or even the position of the LPS molecule on the disc may also have been different. Thus, the response of THP-1 human monocytes (Figure 2) to LPS attached to TiAlV or CPT discs might have varied as well.

When considering the results from all 3 protocols combined (Figures 3 through 5), it can be concluded that *E. coli* LPS exposure, whether it occurs before or after cellular attachment or is contaminating the attachment surface, does not directly effect the attachment

behavior of osteoblasts. However, one should keep in mind that, in the current project, only cellular attachment was evaluated and was evaluated indirectly by evaluating the vitality of the cells that survived a washing procedure. It is possible that LPS could have affected other cellular functions not measured. For example, osteoblasts attached to the implant surface may no longer have been capable of producing a mineralized matrix on that surface. These functions should be evaluated in more detail in future experiments.

In vivo, cells other than osteoblasts are usually in contact with LPS adhered to implants. These other cells will produce several cytokines in response to an LPS challenge.[6,14] Cytokines could effect the behavior of adjacent cells, like osteoblasts, and hence effect osteoblast function and behavior. Again, further study is necessary to answer these questions.

A complete understanding of the interaction between the contaminants present on the surface of a failing implant and the cells present in the surrounding environment is of paramount importance to scientifically design a treatment approach with predictable results. Although the results of the current in vitro study indicate the LPS was not a factor in initial cellular attachment of osteoblasts to CPT and Ti alloy, conclusions about clinical strategies to manage LPS should be reserved until more information can be obtained.

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