

# Effect of a Ceramic and a Non-Ceramic Hydroxyapatite on Cell Growth and Procollagen Synthesis of Cultured Human Gingival Fibroblasts\*

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**Background:** Ceramic hydroxyapatites and non-ceramic hydroxyapatites have been used extensively as alloplastic materials for bone reconstruction. However, different forms of hydroxyapatite induce different types of tissue response.

**Methods:** Human gingival fibroblasts (FMM1 cells) were used to analyze ceramic and non-ceramic hydroxyapatite biocompatibility. The cells were grown on surfaces covered either by collagen (control group), collagen plus ceramic hydroxyapatite, or collagen plus non-ceramic hydroxyapatite. Scanning electron microscopy, growth and cell viability curves, and procollagen immunoprecipitation were obtained. For the growth and viability curves,  $10^4$  cells were seeded on 60 mm dishes. Cells from each group were counted, in triplicate, at 1, 3, 4, 5, and 6 days after seeding using the Trypan blue dye exclusion assay.

**Results:** The cells grew in close contact with both types of hydroxyapatite particles. No differences were found in the amount of procollagen synthesis among any experimental group. The cultures treated with ceramic hydroxyapatite had a growth delay for the first 5 days. There was no difference in cell viability between the control group and the non-ceramic hydroxyapatite group. However, cultures treated with ceramic hydroxyapatite showed significantly lower viability percentages than the other groups.

**Conclusions:** Hydroxyapatite supports cell growth and fibroblast metabolism including collagen production, and hence is biocompatible. Cell viability and structural studies showed that non-ceramic hydroxyapatite has relevant physical and biological properties as an implant material. *J Periodontol 2000;71:540-545.*

## KEY WORDS

Cell culture; periodontal regeneration; biocompatible materials; hydroxyapatite/therapeutic use; comparison studies.

Ceramic (sintered) and non-ceramic (non-sintered) hydroxyapatites have been used extensively as alloplastic materials for bone reconstruction.<sup>1-4</sup> In dentistry, they are used as surface coating of dental implants and as an implant material to treat periodontal bone defects.<sup>5-7</sup> Hydroxyapatite surface coating for dental implants has been introduced to obtain a rapid and complete integration of implant devices to bone tissue. Hydroxyapatite is also used to fill human intra-osseous defects.<sup>8-12</sup> Some studies<sup>9,10</sup> have shown that non-resorbable hydroxyapatite failed to initiate osteoinductive or osteoconductive effects, as proposed by earlier studies.<sup>12,13</sup>

Several studies have demonstrated that changes in physical properties are critical in determining the biological response to either hydroxyapatite or tricalcium phosphate. The most important physical property is their crystalline structure. Variations of particles present in different types of hydroxyapatite may modify resorption level and degradation of material. Assuming that different forms of hydroxyapatite would induce different types of tissue response, we used an in vitro system to assess biological properties of hydroxyapatites. This in vitro system was based on tissue culture techniques, since this method enables a sensitive and fast evaluation of biocompatibility of alloplastic materials.<sup>2,14-16</sup>

The objective of this paper was to compare the in vitro biocompatibility of 2 types of hydroxyapatite: non-ceramic and ceramic. The effect of hydroxyapatite on cultured

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human gingival fibroblasts was analyzed through the study of cell growth and procollagen synthesis.

## MATERIALS AND METHODS

### Cell Culture

A cell line, FMM1, was established from a human fragment of gingiva, obtained after a gingivectomy for prosthetic reasons. Cells were cultured in Dulbecco's modified Eagle medium (DMEM),<sup>†</sup> supplemented by 10% fetal bovine serum<sup>‡</sup> and 1% antibiotic-antimycotic solution.<sup>†</sup> Cells were incubated at 37°C in humidified 5% CO<sub>2</sub> and 95% air atmosphere.

### Hydroxyapatites

We used 2 forms of hydroxyapatite: a pure ceramic type<sup>17</sup> (kindly provided by Dr. Richard van Noort, University of Sheffield, School of Clinical Dentistry, UK) and a non-ceramic, resorbable hydroxyapatite.<sup>§</sup> Both hydroxyapatites were diluted in a sterile collagen I solution<sup>†</sup> in acetic acid in a final concentration of 10 mg/ml. These mixtures were applied on 60 mm Petri dishes. We used this collagen I mixture plus hydroxyapatite to obtain a homogeneous and thin coating. These coated dishes were left inside the laminar flow to allow acetic acid evaporation. After evaporation, these substrates were gently washed with phosphate-buffered saline solution (PBS). Then, culture medium was added to the dishes, followed by cultured gingival fibroblasts (10<sup>4</sup> cells/dish).

### Scanning Electron Microscopy and Microanalysis

For scanning electron microscopy, cells were fixed in 2% glutaraldehyde in 0.1M phosphate buffer and post fixed in 1% osmium tetroxide in the same buffer. Samples were then dehydrated in ethanol and submitted to chemical drying in hexa methyl disilazane<sup>||</sup> (HMDS). Cells were then sputter coated with gold.<sup>¶</sup> For x-ray microanalysis, specimens were fixed, post-fixed, dehydrated, and dried as described above, after which they were carbon coated.<sup>#</sup>

Scanning electron microscopy and x-ray microanalysis were carried out in a scanning electron microscope\*\* equipped with an x-ray acquisition detector. X-ray data were processed and analyzed using computer software.<sup>††</sup>

### Experimental Groups

Group 1: control: gingival fibroblasts grown on dishes coated only by collagen I; group 2: ceramic hydroxyapatite: gingival fibroblasts grown on dishes coated by a mixture of ceramic hydroxyapatite and collagen I; and group 3: non-ceramic hydroxyapatite: gingival fibroblasts grown on dishes coated by a mixture of non-ceramic hydroxyapatite and collagen I.

### Growth Cell and Viability Curves

Growth curves were carried out as previously described.<sup>18,19</sup> Briefly, the cell number and cell viability

(%) were determined by counting the viable cells in a hemocytometer using the Trypan blue dye exclusion assay. We plated 10<sup>4</sup> cells per 60 mm Petri dishes, a subsaturating number of cells for this binding surface. Three dishes of each group were counted 1, 3, 4, 5, and 6 days after seeding. The number of viable cells harvested from each Petri dish was obtained by the following mathematical equation:

$$\frac{UC \times D \times 10^4}{\#SQ}$$

where UC = unstained cell count (viable cells); D = the dilution of the cell suspension; and #SQ = number of squares of the hemocytometer counted. The percent viability of the cell population of each Petri dish was obtained by the following mathematical equation:

$$\frac{UC}{TC} \times 100$$

where UC = unstained cell count (viable cells) and TC = total cell counting (stained plus unstained cells). The counting provided data for obtaining cell growth and cell viability (%) curves.

### Statistical Analysis

Each data point corresponded to the mean ± standard error (SEM) of either cell numbers or cell viability (%) from 3 dishes. The data were compared by ANOVA,<sup>††</sup> complemented by Tukey's test. The level of significance was 5% ( $P \leq 0.05$ ).

### Immunoprecipitation

For immunoprecipitation we used 3 confluent monolayers of FMM1 cells from each of the 3 groups. Each monolayer was plated on 100 mm coated dishes. Culture media were discarded and cells were rinsed twice with cold PBS before solubilizing total cell protein in lysis buffer (50 mM TRIS, pH 7.2 to 7.8; 1% NP-40 or Triton X-100; 2 mM EDTA, 100 mM NaCl; 1 mM vanadate; 1 mM PMSF; 0.1% Aprotinin). Cells were then scraped from the dishes, vortexed, and centrifuged. Procollagen was immunoprecipitated from the supernatant using an anti-collagen I antibody<sup>20</sup> (kindly provided by Dr. Larry Fisher, NIDCR, NIH, Bethesda, Maryland) with the aid of protein A-sepharose.<sup>†</sup> Samples were then boiled in loading buffer and electrophoresed on 7% SDS-polyacrylamide gel. The gel was stained with Coomassie blue and analyzed in a computer system.<sup>§§</sup>

† Sigma Chemical Company, St. Louis, MO.

‡ Cultilab Ltda, Campinas, SP, Brazil.

§ OsteoGen (HA Resorb), Implants Ltd., Holliswood, NY.

|| Electron Microscopy Sciences, Fort Washington, PA.

¶ Sputtering SCD 020, Bal-Tec, Liechtenstein.

# CED 030 Carbon Evaporator, Bal-Tec.

\*\* Leo Ltd., Cambridge, UK.

†† Oxford-Isis, Cambridge, UK.

‡‡ Systat 5.2.1 for the Macintosh, SPSS Inc., Chicago, IL.

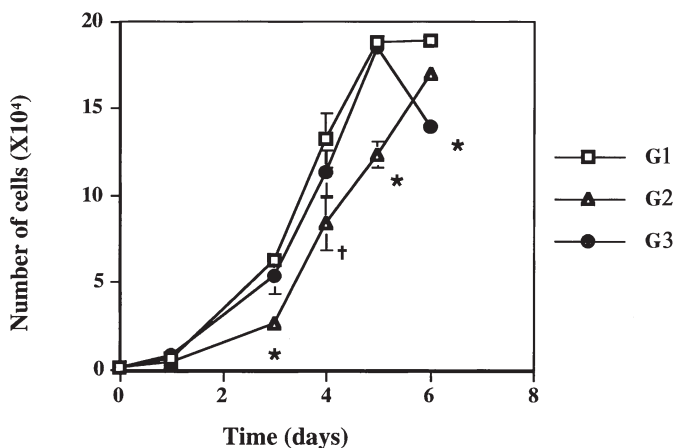
§§ Macintosh 9600 MP, Apple Computer Co., Cupertino, CA.

Comparative densitometry of procollagen bands was obtained by the software NIH IMAGE 1.62.

## RESULTS

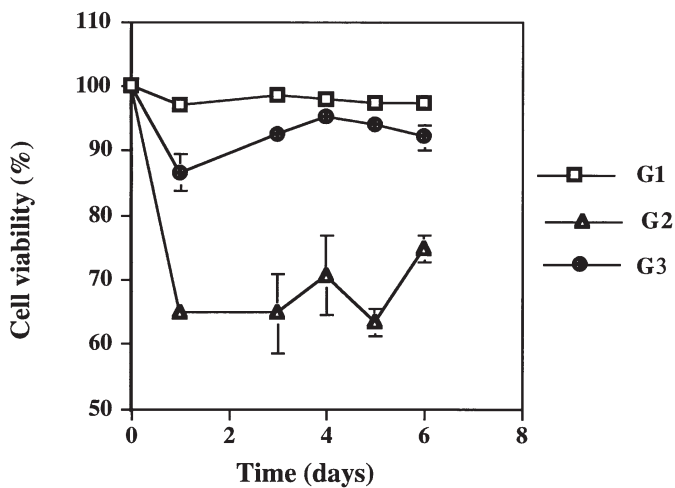
### Growth and Cell Viability Curves

We observed that all cell cultures treated with hydroxyapatite (groups 2 and 3) and control (group 1), grew from the first until the last day after seeding (Fig. 1).



**Figure 1.**

Growth curves of cells grown on hydroxyapatite (groups 2 and 3), compared to control (group 1). All groups grew from first to last day after seeding. Group 1 (control) and 3 (non-ceramic hydroxyapatite) showed no statistical differences, until the fifth day. The growth curve of group 2 (ceramic hydroxyapatite) presented a 24-hour delay until the fifth day, when compared to the other 2 groups (\*significantly different from G1 ( $P \leq 0.05$ ); †significantly different from G3 ( $P \leq 0.05$ )).



**Figure 2.**

Cell viability of groups 1 and 3 ranged from 85% to 100%, with no statistical differences. For group 2 the cell viability ranged from 60% to 75% and was significantly smaller than the other 2 groups ( $P \leq 0.001$ ).

Growth curves from group 1 (control) and 3 (non-ceramic hydroxyapatite) did not show any statistical difference until the fifth day. Cultures of group 2 (ceramic hydroxyapatite) had a growth day until day 5 when compared to the other 2 groups.

Cell viability percentages are shown in Figure 2. This percentage ranged from 60% to 100%. From the first day after seeding, viability percentages of group 2 (ceramic hydroxyapatite) were significantly smaller than those of both group 1 (control,  $P < 0.001$ ) and group 3 (non-ceramic hydroxyapatite,  $P < 0.001$ ). Group 3 (non-ceramic hydroxyapatite) showed no significant differences in cell viability when compared to the control group.

### Scanning Electron Microscopy and X-Ray Microanalysis

Scanning electron microscopy of the FMM1 cell showed either a spindle-shaped bipolar or dendritic morphology, which is typical of fibroblasts (Figs. 3A and B). These morphological features were present in both hydroxyapatite groups. FMM1 cells were observed in proximity to hydroxyapatite particles in both groups. X-ray microanalysis revealed that both ceramic and non-ceramic hydroxyapatites were composed only of calcium, phosphorus, and oxygen. These data show a high purity for both types of hydroxyapatites, since no contaminants were observed.

The particles of both forms of hydroxyapatite were arranged as clusters. The clusters of ceramic hydroxyapatite were mostly spherical and homogeneous in size (Fig. 4A). Non-ceramic hydroxyapatite formed round clusters, irregular in size, most of them bigger than those of ceramic hydroxyapatite (Fig. 4C). Figures 4B and 4D show a schematic representation of the spatial arrangement of ceramic (Fig. 4B) and non-ceramic (Fig. 4D) hydroxyapatites. The clusters of ceramic hydroxyapatite, due to their small size, left small areas of the Petri dishes exposed, while the bigger clusters of non-ceramic hydroxyapatite allowed large uncovered areas on the Petri dish.

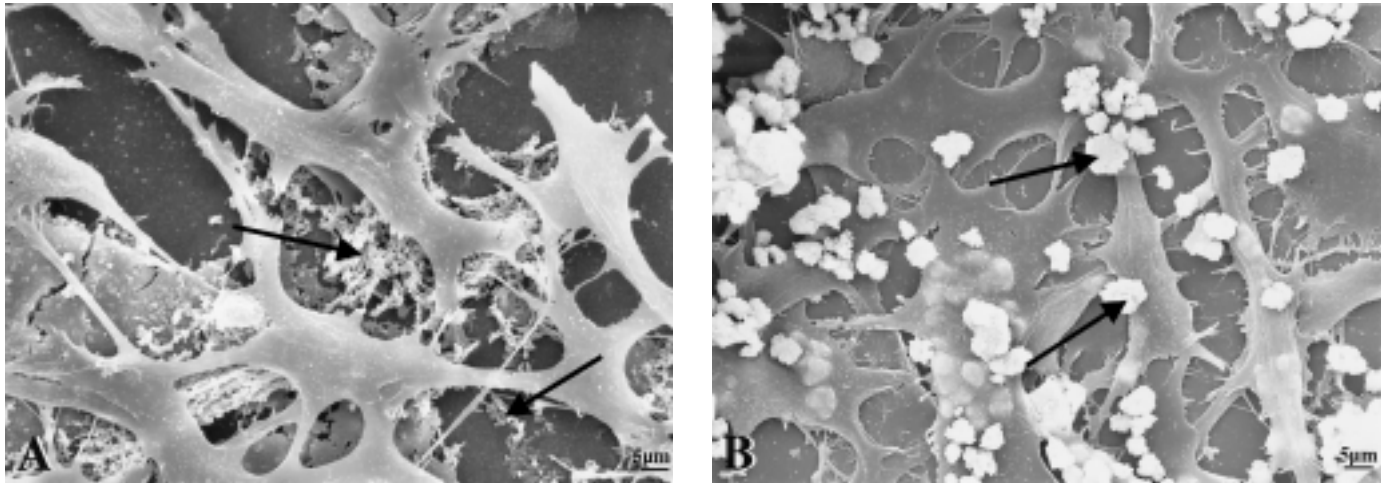
### Immunoprecipitation

Figure 5A shows a gel with bands of procollagen of FMM1 cells from all 3 groups. Figure 5B graphically demonstrates the densitometric results of these procollagen bands. These results yielded comparative analysis of the amount of protein, in this case procollagen, synthesized by cells of each group. No differences were found in the amount of procollagen among all groups.

## DISCUSSION

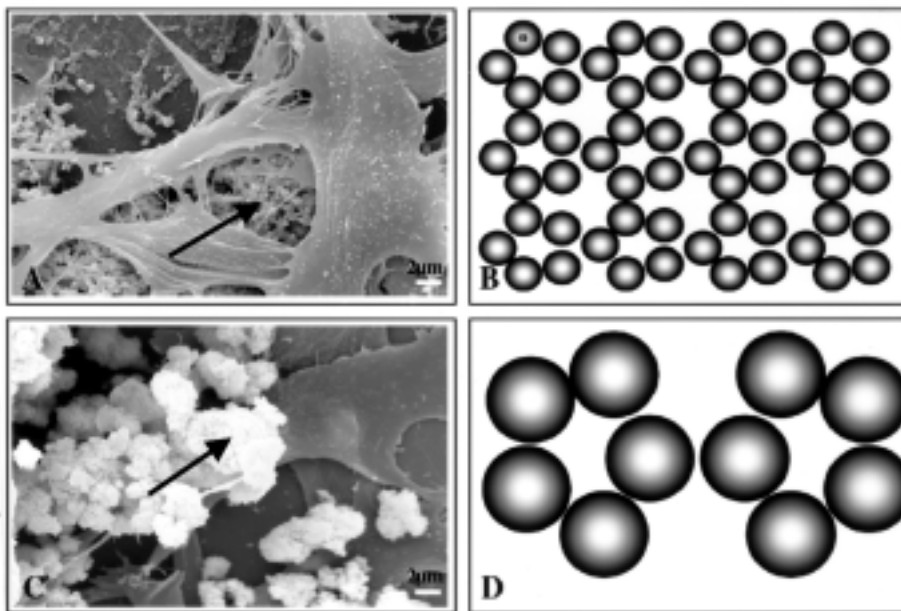
We observed that both ceramic and non-ceramic types of hydroxyapatite are biocompatible. Cell cultures treated with both types of hydroxyapatite showed slight differences on their growth properties. However, the percent-





### Figure 3.

**A.** Scanning electron microscopy of FMMI cells treated with ceramic hydroxyapatite. Cells are either spindle-shaped or dendritic, establishing contacts among them. Hydroxyapatite clusters (arrows) are distributed throughout the surface of the Petri dishes in proximity to the FMMI cells. **B.** Scanning electron microscopy of FMMI cells treated with non-ceramic hydroxyapatite. Cells are either spindle-shaped or dendritic, establishing contacts among them. Hydroxyapatite clusters (arrows) are in proximity to FMMI cells.



### Figure 4.

Particles of ceramic hydroxyapatite are small and spherical (**A**, arrow), while non-ceramic hydroxyapatite forms irregular clusters of particles (**C**). **B.** Diagram of the distribution of ceramic hydroxyapatite clusters. These small clusters are distributed throughout the Petri dish, leaving sparse exposed surfaces of the dish to fibroblast attachment. **D.** Diagram of the distribution of non-ceramic hydroxyapatite clusters. These large and irregular clusters are distributed throughout the Petri dish, leaving large exposed areas of the dish to fibroblast attachment.

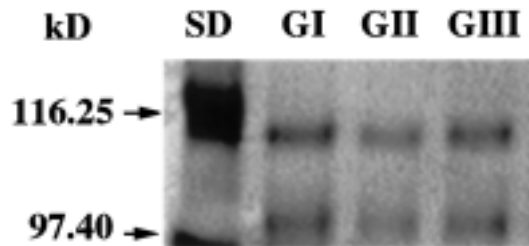
ages of viable cells were significantly smaller for cultures grown in contact with ceramic hydroxyapatite when compared to the other groups. Regarding fibroblast metabolic activity, procollagen synthesis was not affected in cells treated by either ceramic or non-ceramic type.

In dentistry, hydroxyapatites have been used in corrections of bone defects in different anatomical sites, such as periodontal tissue, alveolar process, and maxillary sinus.<sup>4,6,12</sup> However, there are only a few in vitro studies on biological properties of hydroxyapatite.<sup>20-24</sup> Thus, we decided to compare the biological properties of non-ceramic hydroxyapatite to the ceramic type.

We also analyzed chemical composition of hydroxyapatite. The ceramic hydroxyapatite we used is a highly purified type.<sup>17</sup> However, the composition of the non-ceramic hydroxyapatite is not as well understood as that of the ceramic type. We then compared the chemical compositions of the ceramic and non-ceramic hydroxyapatites, to detect possible contaminants. Microanalysis revealed that both types are highly purified consisting of only calcium, phosphorus, and oxygen. Therefore, the different reactions of fibroblasts to these

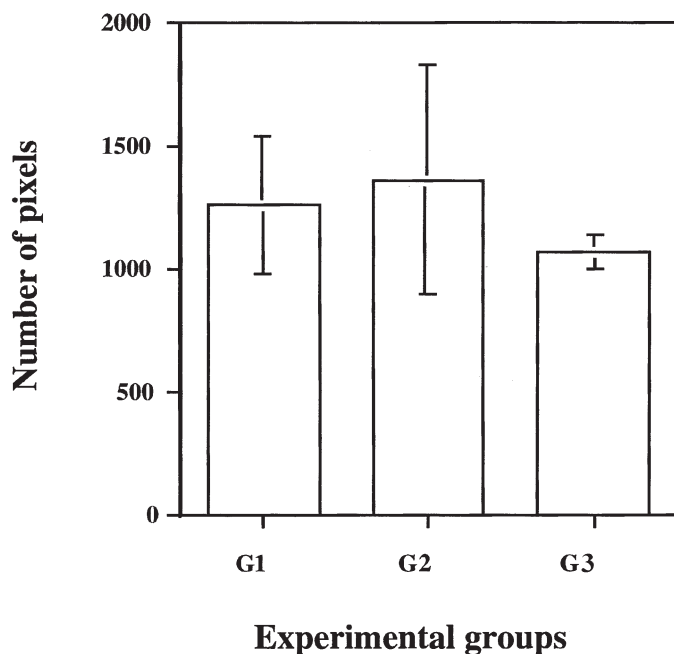
hydroxyapatites will reside only in their physical properties.

This first result was related to the cell growth of fibroblasts in Petri dishes coated with either ceramic or non-ceramic hydroxyapatite. Growth of fibroblasts



**Figure 5A.**

Digital image of polyacrylamide gel, showing standard protein bands and bands obtained from procollagen I immunoprecipitates of all 3 groups. The 3 bands correspond to the a and b fragments of collagen molecule. Standard proteins correspond to b galactosidasis (116, 25 kD) and phosphorilasis (97.4 kD).



**Figure 5B.**

Optical densities comparative graph of procollagen bands from polyacrylamide gel. The number of pixels corresponds to mean  $\pm$  SEM from densitometry pixel areas from procollagen bands of each group.

treated by ceramic hydroxyapatite was delayed 24 hours, when compared to the other groups. This delay may be explained by fibroblast adhesion properties and by the physical structure of hydroxyapatite.

Fibroblasts are anchorage-dependent cells; thus, they need to establish contact with a given substrate to initiate cell division and proliferation. The cells adhere to both plastic surfaces and clusters of hydroxyapatite. However, the adhesion is enhanced in plastic Petri dishes treated for tissue culture.<sup>25</sup> Thus, in larger exposed areas of dishes, a higher number of cells will promptly adhere. Arrangement of the large particles of non-ceramic hydroxyapatite creates large, exposed

plastic surfaces in between these particles that would facilitate fibroblast adhesion and initial proliferation (Fig. 4).

The arrangement of the clusters of hydroxyapatite particles also has in vivo implications. Higashi and Okamoto<sup>24</sup> suggested that larger particles could be better absorbed, thereby facilitating osteogenesis more rapidly than smaller ones, because small particles allow smaller spaces between them, which may not be sufficient to allow blood and bone cell migration and growth.

Biochemical analysis of procollagen synthesis showed no differences among the groups. From this, we infer that procollagen synthesis, the most important physiological property of the fibroblast, is not impaired by any of the hydroxyapatites tested. This is a strong result, providing molecular evidence for biocompatibility of both hydroxyapatites.

Our results showed that both hydroxyapatites are biocompatible, even at the molecular level, since they did not impair either cellular proliferation or procollagen synthesis. Cells treated by non-ceramic hydroxyapatite showed a growth pattern and viability similar to those of the control group. This may suggest that non-ceramic hydroxyapatite is more suitable as an implant material, since fewer fibroblasts were induced to necrosis. Furthermore, three-dimensional configuration of non-ceramic hydroxyapatite leaves more space between particles, when compared to the ceramic type. In vivo, these spaces would facilitate cellular and tissue proliferation within the implanted material, probably allowing a faster osteointegration. However, we must recall that in vitro observations evaluate biocompatibility in a controlled environment, different than that in vivo.

#### ACKNOWLEDGMENTS

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