·Basic Research·

Hydroxyapatite modified titanium promotes superior adhesion and proliferation of corneal fibroblast in comparison with pure titanium

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Abstract

• AIM: To determine whether hydroxyapatite modified titanium promotes superior adhesion and proliferation of rabbit corneal fibroblast in comparison with pure titanium.

• METHODS: We used bioactive hydroxyapatite to modify titanium surfaces. Fourth passage fibroblasts of rabbit cornea were seeded on hydroxyapatite modified titanium surfaces, pure titanium and glass surfaces. Cell adhesion, proliferation and morphology were detected at 24, 48, and 72 hours using a acridine orange stain. Further studies of cell morphology were performed using scanning electron microscopy.

• RESULTS: Cell counts were significantly greater on hydroxyapatite modified titanium surfaces at each time point ($P<0.05$). At 24 hours, cell spreading was greater on hydroxyapatite-coated titanium and glass than on the pure titanium. At 72 hours, compared with pure titanium and glass surfaces, the cells on hydroxyapatite modified titanium surfaces had greater spreading area and longer stress fibers.

• CONCLUSION: Hydroxyapatite modified titanium promotes superior adhesion and proliferation of rabbit corneal fibroblast in comparison with pure titanium.

• KEYWORDS: hydroxyapatite; titanium; keratoprosthesis; corneal fibroblasts

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INTRODUCTION

Penetrating keratoplasty is the most successful form of tissue transplantation, and enjoys a success rate of more than 90% in the treatment of corneal diseases [1]. However, the failure rate is very high in patients with severe corneal diseases such as Stevens-Johnson syndrome (SJS), chemical burns, ocular cicatricial pemphigoid(OCP), severe keratoconjunctivitissicca, stem cell deficiencies [2], owing to past or ongoing chronic inflammation [3]. In such severe cases, keratoprosthesis (KPro) surgery may be the final solution.

An ideal Kpro should be made of a truly bio-integrating material which permit the migration of keratocytes and subsequent deposition of extracellular matrix [4]. In the past, the selection of inert materials such as polytetrafluoroethylene(PTFE), polyhydroxyethyl methacrylate (HEMA) and titanium as KPro skirt materials has been based on their record of safe implantation rather than any special merit as an interfacial surface to promote normal tissue differentiation [3-5]. Currently, research to improve bio-integration between the KPro and the host tissue is becoming prominent. Many approaches have used to enhance the keratocytes adhesion and proliferation on the surfaces of KPro materials. Surface modification is one of these related approaches[6-7].

Pure titanium as a skirt material for KPro [8] had been modified with bioactive glass-ceramic [9]. Glass-ceramic modified titanium fastened the KPro to the corneal tissue before the epithelium ingrowth. However, the glass-ceramic coating used was too thick, causing technical difficulties in surgery, and the retention time was short because of resorption [9]. In this study, we used bioactive hydroxyapatite to modify titanium surfaces. Hydroxyapatite, as a highly biocompatible and nonbiodegradable materials, is widely used in ophthalmology as post enucleation ball implants [10]. The goal of this study was to determine whether hydroxyapatite modified titanium enhances the adhesion and proliferation of rabbit corneal fibroblast superiorly in comparison with pure titanium, and thus be used as a better skirt material for KPro.

MATERIALS AND METHODS

Hydroxyapatite Modified Titanium Pure titanium discs of

6-mm diameter and 0.13-mm thickness were provided by Horologe Limited Company of Beijing. Half of these titanium discs were coated with bioactive hydroxyapatite by a two-step chemical treatment. The manufactural method has been described previously [11]. Scanning electron microscope (Hitachi, Japan) was used to determine the surface texture after the treatment. All the materials were sterilized by autoclaving before using them. In our study, hydroxyapatite modified titanium discs and titanium discs without any treatment were used for this experiment.

Cell Culture Two cornea buttons from a one-month New Zealand rabbit (Animal center, General Hospital of PLA, Beijing) were carefully excised, and epithelia and endothelia were removed. The obtained corneal stromas were treated with 2.5g/L trypsin (Sigma Chemicals Co, St Louis, USA) at room temperature for 5 minutes before cutting into small fragments. Then the corneal stromas were digested by 1g/L collagenase at 37℃ for 3 hours. After centrifugation at 1 000r/min for 5 minutes, dispersion keratocytes were collected and seeded on 75cm^2 tissue culture flask containing 3mL Dulbecco's modified eagle's medium (DMEM) plus 150mL/L fetal calf serum (FCS) (Sigma Chemicals Co, St Louis, USA), 50mg/L gentamicin and placed in a 50mL/L CO_2/air incubator at 37℃.

The cells were passed by standard trypsin dispersion. In the cultural condition, cells developed a myofibroblast phenotype [12], and we called it fibroblast of corneal stroma. The fourth passage cells were used for experiments.

Cell Adhesion and Proliferation 6mm diameter discs of each test material were placed in the wells of a standard 12 well plate. The fourth passage fibroblasts of corneal stroma (6×10^4 in 2mL DMEM containing 100mL/L FCS) were seeded onto hydroxyapatite modified titanium discs and titanium discs without any treatment before further incubation at 37℃ in 50mL/L CO_2/air. Glass coverslips were used as positive control. After 24, 48, and 72 hours, culture medium was carefully removed from the wells, adherent cells in situ were fixed in 950mL/L alcohol for 15 minutes at room temperature.

Acridine orange stain was used to observe cell adhesion and proliferation on each of the test material. Fixed cells on the test materials were treated with 10mL/L acetic acid for 30 seconds before staining with 1×10^{-4} acridine orange for one minute, then treated with 0.1mol/L $CaCl_2$ for one minute. After washing with phosphate buffer solution (PBS), stained cells were then viewed by fluorescence microscopy (Olympus IX70, Japan). Sequential illumination at wavelengths of 592nm was used to highlight stained cells. A total of seventy-two specimens were examined in this experiment. Cell counts in five preselected fields per specimen were performed at a magnification of ×100.

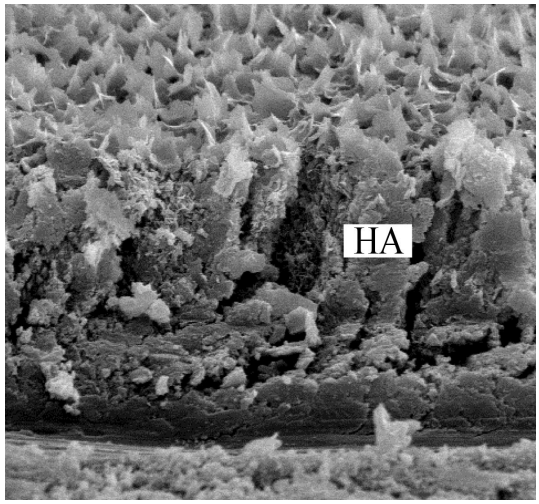

Figure 1 The thickness of the HA was seen about 20μm after the deposition on the new-cut side face (NC). Arrows indicate the interface of the surface HA/.Ti substrate

Electron Microscopy Cells inoculated and cultured on test materials for 72 hours as described above were fixed in 20mL/L glutaraldehyde for 1 hour and stored at 4℃. Twenty-four specimens were treated with 10mL/L osmium at 4℃ for 1 hour before dehydration in ethanol, substitution by isoamyl acetate, and drying by hexamethyldisiloxane (HMDS). After fixation, the discs were mounted on stubs using conductive silver paint and sputter coated with gold. Specimens were then examined in a Hitachi S450 (Hitachi, Japan) scanning electron microscope.

Statistical Analysis Data were analyzed using the software program SPSS. Statistical comparison between the surfaces of test materials and the controls was determined by using the analysis of variance. $P<0.05$ was considered statistically significant.

RESULTS

Surface Texture of Hydroxyapatite Modidied Titanium The hydroxyapatite (HA) deposited on the two-step treated pure titanium surface were scratched to varying degrees using a scalpel in order to investigate the internal structures of the hydroxyapatite (HA) and their interfacial bonding to the metallic substrates. The HA deposited on the new-cut side faces (derived from the cutting procedure) of several samples were removed to observe the thickness of the HA. The HA was composed of two sublayers, an outside loose crystal sublayer and an inside dense sublayer. The thickness of hydroxyapatite layer was estimated to be 20μm (Figure 1).

Primary Fibroblast of Corneal Stroma Keratocytes outgrew in DMEM with 150mL/L FCS 3 days after primary seeding. After passage fibroblastoid type, cells rapidly overgrew the culture (Figure 2). The period of primary cornea fibroblast culture was about 2 weeks.

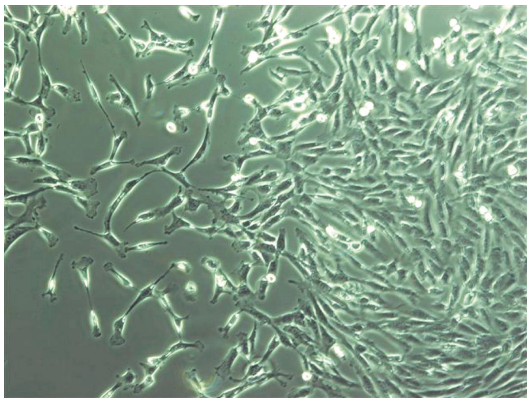

Figure 2 Primary fibroblasts of corneal stroma (magnification×100)

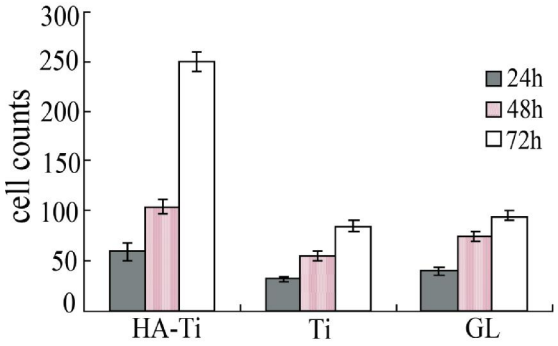

Figure 3 Cell counts on test materials hydroxyapatite modified titanium (HA-Ti), pure titanium (Ti), glass (GL) at 24, 48, and 72 hours

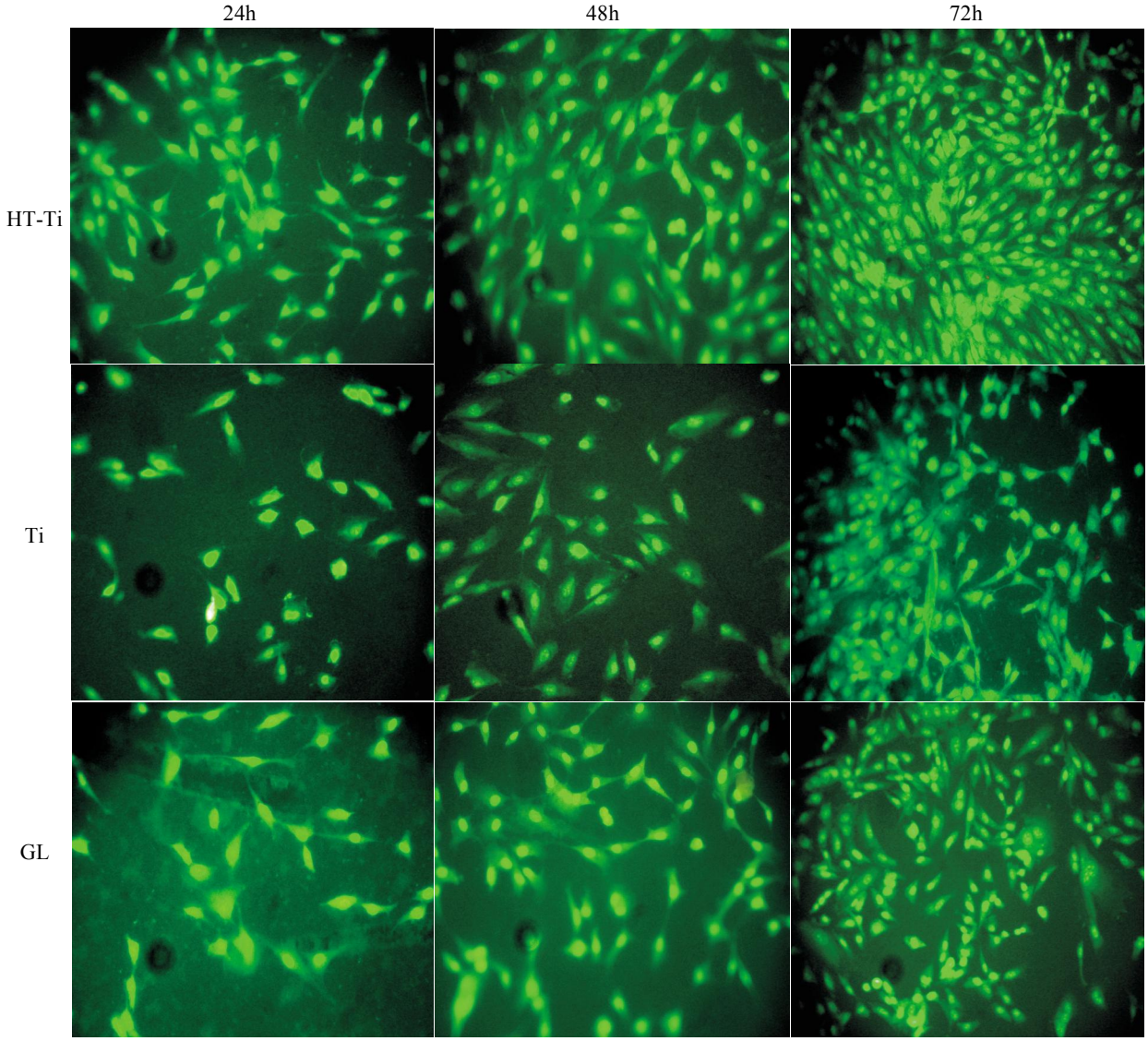

Figure 4 Fluorescence microscopy showing acridine orange stained cell morphology and adhesion at different time points (24, 48, and 72 hours) on hydroxyapatite modified titanium(HA-Ti), titanium(Ti), and glass(GL)

Hydroxyapatite Modified Titanium Promoted Superior Adhension and Proliferation of Corneal Fibroblast in Comparison with Pure Titanium and Glass Cell counts were significantly greater for hydroxyapatite modified titanium specimens at each time point than for the titanium and glass (P<0.05 at 24, 48, and 72 hours; Figure 3). Cell counts increased between 24 hours and 72 hours on hydroxyapatite modified titanium, pure titanium, and glass, indicating that cells proliferated on all these materials and these materials had no cytotoxicity. At 24 hours, cell

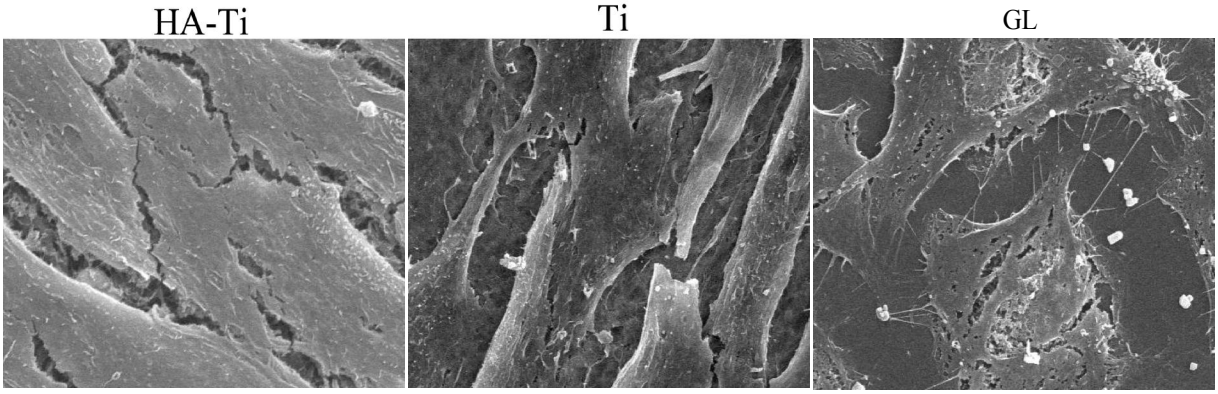

Figure 5 Scanning electron microscopy showing cell spreading area on hydroxyapatite modified titanium(HA-Ti), titanium(Ti), and glass(GL)

spreading was greater on hydroxyapatite modified titanium and glass than on the titanium (Figure 4). At 72 hours there was a mass of cells on the glass, hydroxyapatite modified titanium, and pure titanium.

Cell Spreading Area and Stress Fibers Detected by Electron Microscopy After 72 hours, compared with titanium and glass surface, the cells on hydroxyapatite modified titanium surfaces had greater spreading area and longer stress fibers (Figure 5).

DISCUSSION

Hydroxyapatite is similar to the principal mineral constituent of bone and tooth, and is thought to promote integration between hard and soft tissues[13]. Therefore, it is well used in ossicular replacement, periodontal bone regeneration, and orbital floor repair. As described above, bioactive glass-ceramics fastened the KPro to the corneal tissue, owing to the formation of a thin layer of hydroxylapatite on their surfaces in contact with body fluid[9].

As such an excellent biocompatible materials, hydroxyapatite had already been used as KPro coral skirt element [14]. However, a rigid hydroxyapatite skirt could potentially lead to problems with device fracture, and mechanical stress concentrations at the skirt/sclera interface would cause corneal ulceration [3]. The ideal keratoprosthesis would have an elasticity similar to the eye wall [3]. In our study, we used hydroxyapatite to modify titanium surface which has the advantage of hydroxyapatite and the elasticity needed for KPro. Basic studies of cell adhesion and proliferation in our study suggest that, as an initial adhesion environment for fibroblasts of corneal stroma, hydroxyapatite modified titanium surface is superior to pure titanium surface. The hydroxyapatite deposited on the two-step treated pure titanium surface was thinner and more stable than that of glass-ceramics coating described above. The hydroxyapatite coating was about 20μ m thickness, and composed of two sublayers. The outside part could be easily removed by lightly scratching, while it was difficult to peel the inside part off [11]. However, whether hydroxyapatite modified titanium is better than pure titanium for KPro materials *in vivo* needs further studies.

After modifying with hydroxyapatite, the surface roughness increased. The surface texture exerted an important influence on cell behavior [15], which could not be controlled in this study. This limitation needs further works.

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