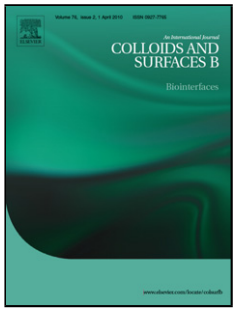

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Concentration effect of gold nanoparticles on proliferation of keratinocytes

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Abstract

"34 nm gold nanoparticles with good stability were synthesized and characterized and their effect (as a function of concentration) on the proliferation of keratinocytes was evaluated by means of MTT and nucleolar organizer region (AgNOR) count (silver staining). The cell morphology was observed by scanning electron microscopy (SEM) and transmission electron microscopy (TEM).The results demonstrate that a low concentration of gold nanoparticles enhances the proliferation of keratinocytes. Specifically, a concentration of 5.0 ppm gold nanoparticle has the best effect on the promotion of cell growth. In the experiment group, the AgNOR-positive areas and AgNOR area/nuclear area ratios of keratinocytes co-cultured with 5.0 ppm gold nanoparticles were greater than those in the control group ($p<0.01$). At a level greater than10.0 ppm, gold nanoparticles were found to have a cytotoxic effect on keratinocytes. It is concluded that a low concentration of gold nanoparticles may be used as a biomedical material in skin tissue engineering."

Keywords: Gold nanoparticles, Proliferation, Keratinocyte, Concentration

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1. Introduction

Patients with severe burn injuries have a higher mortality rate related to ineffective or delayed wound coverage [1]. Therefore, it is essential to cover the wound with dressings immediately. Autografts is the first line treatment for cutaneous deficiency. However, the limited availability of autologous skin is a major disadvantage. To provide an available alternative of coverage, various skin substitutes have been developed. Keratinocytes transplantation and cultured epithelial autografts (CEAs) are definitely major and important progress [2]. But their major disadvantages include fragile handling characteristics, the length of time required to expand the donor cells under culture conditions, and limited ability to expand the cells enough for use in large body burns [3].

Nanoscience, which emerged in the twentieth century, is promising to solve these problems. People found the unique surface properies of nanophase materials, such as a higher number of atoms at the surface compared to bulk, greater areas of surface defects and larger proportions of surface electron delocalization [4] could influence cell adhesion and proliferation. For example, nanophase ceramics can enhance the adhesion of osteblast [5-6]. Hydroxyapatite with a particle size of 20nm can promote the proliferation of osteoblast[7]. PLA/HA nanofiber composites allow the adhesion and proliferation of pre-osteoblasts[8]. It also found that keratinocyte could adhere fast to the gold nanoparticles/chitosan film scaffold [9].

In the present work, gold nanoparticles of 34nm were synthesized and characterized with transmission electron microscopy (TEM) and ultraviolet spectrophotometer (UV-vis), and the research target of this paper is the effect of different concentration of gold

nanoparticles on the proliferation of keratinocytes in Keratinocyte serum-free medium.

2. Materials and methods

2.1. Materials

Keratinocyte serum-free medium (K-SFM) was obtained from Invitrogen, USA. Dulbeccos' Modified Eagles Medium (DMEM) was from Gibco, USA. Fetal bovine serum was from Hyclone, USA. Trypsin was from Amresco, USA. MTT was obtained from Beyotime Boitechnology, China. $AuCl_3 \cdot HCl \cdot 4H_2O$ (Au>48%) was obtained from the Shanghai No. 1 Reagent Factory, China. All Other chemicals were of analytical grade.

2.2. Preparation and characterization of gold nanoparticles

First, gold nanoparticles were prepared according to the literature [10]. The formation of gold nanoparticles can be observed by a change in color since gold nanoparticles are red. Second, the final volume of gold nanoparticles was adjusted with de-ionized water, and the concentration of gold nanoparticles is 100 ppm. Finally, the diameters of the gold nanoparticles were measured by transmission electron microscopy (JEM-200CX, JEOL Co., Japan) and Ultraviolet spectrophotometer (2450, Shimadzu Co., Japan). In addition, the particle size was analyzed by software Image-pro plus 6.0.

Different concentration media were prepared by adding different volumes of 100 ppm gold nanoparticles to K-SFM medium.

2.3. Isolation and culture of keratinocytes from newborn mice skin

Newborn mice (Sprague Dawley rats, 1 day old) were provided by the Experimental Animal Center of Nantong University. The research protocol was in compliance with Chinese guidelines for experimental animals. Fresh newborn mouse skin was obtained

according to the literature [11]. The mice were sacrificed and submerged them in 75% ethanol for 5 min and then were rinsed with 2 changes of sterile PBS without Ca^{2+} and Mg^{2+}. The excess connective tissue was trimmed off and the remaining skin was cut into 4～6 mm wide stripes with scissors and then was placed them on a sterile dish covered with sterile gauze containing 0.25% trypsin, dermis facing down. After being incubated at 4°C overnight, epidermis was separated from dermis and placed into DMEM supplemented with 10% fetal bovine serum to stop digestion and then was pipetted vigorously to make single cell suspension. Subsequently, the cell suspension was filtered through a stainless steel mesh (200 mesh). The cells were collected by centrifuge at 1000rpm for 5 min, and rinsed twice with PBS by centrifuge. Finally, keratinocytes were harvested.

2.4. Cell proliferation assay

Freshly isolated mouse keratinocytes were seeded into 96-well plates at a density of 1 × 10^4 cells/well in the presence of 0.0, 1.0 3.0, 5.0, 7.0, 10.0 and 20.0ppm of gold nanoparticles. The culture medium containing different concentration of gold nanoparticles was changed every 2 days.

The viability of keratinocytes after culture was measured at 6, 24, 48, 96 and 120h by MTT assay. Briefly, 20μl of 5 mg/ml MTT in PBS was added to the test wells and left for 4 h, and then 150μl dimethyl sulfoxide was added to each well to solubilize formazan dye. After 15 min, the absorbance was measured at 490 nm in a microplate reader. The background absorbance was measured in K-SFM solution without the presence of cells.

2.5. Silver-staining nucleolar organizer region (AgNOR) staining and image analysis

AgNOR histochemical staining was performed according to the literature [12]. In brief,

after 4 days' incubation, slides with keratinocytes were fixed in 95% ethanol for 50-60min and then hydrated in distilled water. The silver staining was freshly prepared by dissolving 2% gelatin in 50% aqueous silver nitrate solution in a ratio of 1:2. Slides were incubated for 60 min at room temperature in the dark with this solution. After being stained, slides were rinsed with distilled water, dehydrated with graded ethanol, cleared in xylene and coverslipped.

The evaluation procedure consisted of quantification of number and area of the AgNOR dots per nucleus in the keratinocyte cells. We evaluated 100 cells per-slide. The dots were counted visually, and their areas were measured with the digital subtraction tool. The software Image-pro plus 6.0 was used for the analysis according to the manufacturer's instructions.

2.6. Morphological study of keratinocytes

Transmission electron microscopy. After being co-cultured with gold nanoparticles for 4 days, the cells were harvested by scraping and fixed in 2.5% (v/v) glutaraldehyde solution, and post fixed in aqueous osmium tetroxide. The samples were then dehydrated in a graded series of ethanol, block stained in uranyl acetate, and embedded in Epon. Ultrathin sections (100 nm) were contrasted with lead citrate and imaged by TEM (JEM-1230, JEOL Co., Japan) at an accelerating voltage of 80 kV.

Scanning electron microscopy. Keratinocytes were washed with PBS and fixed with 2.5% (w/v) glutaraldehyde solution at 4°C for 48h after co-culturing with gold nanoparticles for 2 days. After being washed with PBS to remove the remaining glutaraldehyde, the cells were dehydrated in a graded series of ethanol. Then, the cells were

further dehydrated with acetone and then treated with isoamyl acetate. After dried by the critical point dry method, the cells were coated with an ultrathin gold layer and observed under SEM (S-3400N, Hitachi Co., Japan).

2.7. Statistics

All experiments were performed three times. The results were presented as mean±SD and analyzed using SPSS 13.0. The one-way analysis of variance (ANOVA) was used for statistical analysis. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Characterization of gold nanoparticles

It showed a typical TEM image of gold nanoparticles and the histogram of particle size distribution in Fig. 1. TEM observations (Fig. 1(a)) clearly revealed that the average diameter of the spherical gold nanoparticles was 34±6 nm and the size distribution of the gold nanoparticles was narrow.

It illustrated the absorptance of K-SFM medium consisting of different concentration of gold nanoparticles and the dispersion of particles in media in Fig. 2. The gold nanoparticles were stable and did not precipitate in the medium, which could be confirmed by the absorption peak position of gold nanoparticles and the TEM image. And as the gold nanoparticle was wine red, the color of medium was dependent on the concentration of gold nanoparticles.

3.2. Morphology and viability of keratinocytes

The wall-attached kerationcytes first adhered to the surface of the tissue culture plate within 24h and then presented an elongated morphology. And the morphology of cells

co-cultured with gold nanoparticles was similar to that of the control group. As time went by, the cells grew to form a monolayer except those of 10.0 and 20.0 ppm groups. The keratinocytes co-cultured with 10.0 and 20.0 ppm gold nanoparticles became round and small on day 3 and 2, respectively. They appeared loosely packed, although still attached on the surface of the culture plate.

Viability of keratinocytes was evaluated by MTT assay. As shown in Fig.3, the keratinocytes co-cultured with gold nanoparticles adhered to the surface faster than the control group (6h) and the cell co-cultured with a low concentration of gold nanoparticles presented a high proliferation rate throughout the culture time. Furthermore, cell viability was significantly higher with 5.0ppm as compared to the other concentration, and the confluence of keratinocytes with 5.0ppm was 1-2 days earlier than the other groups. But the keratinocyte co-cultured with 10.0 and 20.0ppm gold nanoparticles proliferated at first, and then began to be at a standstill.

3.4. AgNORs results

After being stained by a silver impregnation method, the borders of AgNOR and the nucleus were clearly visible. The nuclei stained pale yellow whereas the AgNOR dots or aggregates stained dark. As illustrated in Fig. 4, most of the neurons contained one or two AgNORs in 0.0, 3.0 and 5.0 ppm gold nanoparticles. And a high number of AgNOR proteins were observed in 5.0 ppm. However, the keratinocyte co-cultured with 10.0 ppm gold nanoparticles was smaller than the other groups and there were fewer AgNOR dots or aggregates.

The image analysis of AgNORs was detailed in Table 1. The nuclear areas of

keratinocyte without the presence of gold nanoparticles were larger than the other groups. The AgNOR area/nuclear area ratios of keratinocyte co-cultured with 3.0 and 5.0ppm gold nanoparticles were greater than the control group. And the AgNOR-positive areas and AgNOR area/nuclear area ratio of keratinocyte cells with 5.0ppm of gold nanoparticles were significantly greater than the other concentration. When the concentration of gold nanoparticles reached to 10.0 ppm, the image analysis parameters of AgNOR were significantly lower than the other groups.

3.5. Cell morphology observed by SEM and TEM

The cell morphology was further subjected to SEM observation (Fig. 5). The cells co-cultured with 3.0 and 5.0 ppm gold particles showed very similar morphology as compared to that in the control group, where abundant filopodia were observed. By contrast, the cells co-cultured with 10.0ppm gold particles had less filopodia.

In TEM photographs, keratinocytes co-cultured with 3.0 and 5.0ppm gold nanoparticles displayed slight cytoplasmic changes compared to the control group, such as more vesicles, and swollen endoplasmic reticulum. More vesicles might be the endosomes or traffic vesicles, which should be generated during the cell uptake or the later intracellular transport [13]. And the swollen endoplasmic reticulum may be related to the activity of protein synthesis.

But for keratinocytes co-cultured with 10.0 ppm gold nanoparticles, visible morphological changes, such as reduced cell size, distorted nucleus and chromatin condensation, were observed.

In addition, we found some nanoparticles residing in the cytoplasm (Fig. 6(e)),

organelles (Fig. 6(f)), nuclera envelope (Fig. 6(g)) and even in the nucleus (Fig. 6(h)), implying that the particles can enter the nucleus.

4. Discussion

Gold nanoparticles possess unique properties of nanoparticles such as volume, surface, macro quantum tunneling effect, optical property, and so on. It has been used not only in chemistry and physics but also extensively in biomedical filed. It can be used as a matrix and cytochemical label for the immobilization and study of macromolecules and cells [14-20]. The gold nanospheres are also used for cancer cell imaging [21-22] and cancer cells detection [23]. And, gold nanoshells and Au/TiO_2 nanocomposite has been demonstrated to selectively kill cancer cells [24-25]. It was shown previously that hepatocytes immobilizing on 24nm-sized gold nanoparticles could proliferate and maintain their biological activity well [26].

In this study, the effect of different-sized gold nanoparticles（16,24,31,42 and 51nm） on the proliferation of keratinocytes was investigated. It was found that 34nm-sized gold group was better than the other groups, but there was no statistically difference. Hence, 34nm gold nanoparticle was used to investigate the effect of different concentration of gold nanoparticles on the proliferation of keratinocytes in K-SFM medium.

The results suggested that a low concentration of gold nanoparticles could stimulate keratinocytes proliferation. From the SEM, we found that cell adhesion and spreading co-cultured with low concentration gold nanoparticles followed a similar pattern, compared to that found on standard tissue culture. In addition, keratinocyte co-cultured with gold nanoparticles adhered to the surface faster than that without gold nanoparticles, which was

consistent with our pervious study [9]. When the concentration exceeded 10.0 ppm, the keratinocyte exhibited marked changes, including loss of swollen mitochondria and condensed chromatin. This means that a high concentration of gold nanoparticles is toxic to keratinocytes.

Nucleolar organizer (NOR) is a chromosomal region, in which most of ribosomal synthesis occurs. It was reported that the amount of AgNORs is related to cell proliferation activity and the interphase quantitative changes are related to the cell proliferation rate [27]. From the analysis of the AgNORs results, the AgNORs dot or aggregates and AgNOR total area in 5.0ppm gold nanoparticles was significantly larger than the other groups, indicating that the cellular protein synthetic activity in 5.0 ppm was stronger than other groups. This may be due to that the small mount of gold nanoparticles could enter the cell and enhance DNA synthesis. This was supported by the TEM results: some nanoparticles resided in the cytoplasm, organelles, nuclera envelope and even in the nucleus. The proliferation and differentiation of cells may be associated with Ca^{2+}[7], or phosphorus and sulfur[28]. Ca^{2+} serves as a second messenger for the activation of a broad group of calcium/calmodulin dependent kinased, and promotes the keratinocytes proliferation and differentiation. But, as is known, the increase in calcium is not only associated with cell proliferation but also with apoptosis. Some studies have shown that a sharp increase in Ca^{2+} is involved in the apoptosis [29-30]. Therefore, when the gold nanoparticles exceeded 10 ppm, the cytoviability was significantly decreased.

Some literatures have reported that the uptake of the small particles might occur via endocytosis, chathrin-coated vesicles, caveolae or their independent reporters [31]. In this

study, it was found an interesting phenomenon that the gold nanoparticles were embedded by the invagination of the nuclear membrane (Fig. 6(h)), whereas in another TEM image gold nanoparticles was found to be located on the nuclear envelope (Fig. 6(g)), implying that the gold nanoparticles can enter the nucleus through many pathways. But the actual pathways need further elucidation.

5. Conclusion

In this study, gold nanoparticle of 34nm was synthesized and their concentration effect on keratinocytes was evaluated. The results demonstrate that a low concentration of gold nanoparticles can enhance the growth of keratinocyte. A concentration of 5.0 ppm gold nanoparticle was the most effective at promoting cell growth. Therefore, the low concentration of gold nanoparticles may be used as a biomedical material in skin tissue engineering.

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Caption of figure

Figure1: Characteristics of gold nanoparticle (a)TEM image, (b)Histogram of particle size distribution.

Figure2: Characteristics of gold nanoparticle in keratinocyte serum-free medium (K-SFM) media (a)Absorption spectra of different concentration of gold nanoparticle in the media *Inset*, absorption spectra of gold nanoparticles, (b) TEM image.

Figure3: Viability of keratinocytes incubated with different concentration of gold nanoparticle for 6, 24, 48, 72, 96 and 120h. n=6; *significantly different from 0.0ppm group, $p<0.05$. **significantly different from 0.0ppm group, $p<0.01$.

Figure4: AgNOR straining in nucleoli of keratinocytes cultured with different concentration of gold nanoparticle. (a)0.0 ppm, (b)3.0 ppm , (c)5.0 ppm, (d)10.0 ppm. Original magnification ×1000.

Figure5: SEM photographs of keratinocyte co-cultured with different concentration of gold nanoparticle for 48 h (a) 0.0ppm, (b) 3.0ppm, (c)5.0 ppm and (d)10.0 ppm.

Figure6: TEM photographs of keratinocyte co-cultured with different concentration of gold nanoparticle for 4 days. (a) 0.0ppm, (b) 3.0ppm,(c)5.0 ppm and (d)10.0 ppm. The photographs in (e-h) illustrate the distribution of gold nanoparticle inside the cell as indicated by the arrows (e) in the cytoplasm (f) in the organellse (g) nuclera envelope (h) in nucleus.