EFFECT OF THREE-DIMENSIONAL (3D) SIMULATED MICROGRAVITY CULTURE ON MORPHOLOGY AND SECRETORY FUNCTION OF MURINE PITUITARY CELLS

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Summary: Pituitary transplantation is an effective treatment for hypopituitarism but lack of pituitary cells with secretory function has slowed down its progress. The present study explores changes to hormone production and morphology of murine pituitary cells in pseudo-microgravity culture. It aims to improve current concepts of culture methods for pituitary cells that may provide new options for pituitary transplantation in the future. The RCCS-4H simulated microgravity 3D method was used for primary pituitary cell culture. Primary pituitary cells were isolated and cultivated in both 2D and 3D cell culture. Cell morphology was observed by visual light and immunofluorescence microscopy. Hormone production was measured by ELISA. Pituitary cells that were cultured in the 3D pseudo-microgravity system maintained their natural morphology and function. Growth rate and hormone secretion of 3D-cultured pituitary cells were higher from day 5 to day 17 as compared to that of 2D cell culture. These results indicate that pseudo-microgravity culture facilitates murine pituitary cells to maintain a natural morphology, better survival time, and physiological hormone production. This method therefore might be suitable for the cultivation of analogous human pituitary cells that may serve as excellent donor cells for cell transplantation therapy in patients with hypopituitarism.

Keywords: Pituitary Cells, 3D Cell Culture, Simulated Microgravity, Rotary Cell Culture System (RCCS), Rotating Wall Vessel Bioreactor (RWVB)

INTRODUCTION

The pituitary gland, located on the ventral side of the hypothalamus, is the most important endocrine gland in the human body. The anterior pituitary gland is the major producer of systemic hormones such as adrenocorticotrophic hormone (ACTH) and growth hormone (GH) that play a crucial role in growth and homeostasis [7, 13]. Due to the increasing incidence of brain trauma, sella tumors [10] and the wide application of pituitary surgery and radiotherapy, the incidence of pituitary dysfunction is increasing. Pituitary dysfunction affects the secretion of various hormones in the body, resulting in endocrine disorders which can markedly reduce the patients' quality of life. At present, pituitary transplantation is an effective treatment for hypopituitarism [7, 10, 13]. However, the lack of sufficient pituitary cells with secretory function has slowed down the progress of pituitary transplantation.

In the study at hand, we used a cell culture under simulated microgravity in order to cultivate primary pituitary cells and to continuously observe their morphology, hormone secretion and growth *in vitro*. Cells naturally grow in a three--dimensional (3D) environment. The spatial arrangement of cells within this environment affects not only the microenvironment but also cell-cell interaction. These signals in turn affect differentiation, morphology and a range of cellular functions. Consequently, when drug candidates are tested using cell-based assays, the culture condition should mimic the *in vivo* environment. Traditional two-dimensional (2D) culture methods cannot accurately represent the *in vivo* cell growth environment, as it limits the interactions between cells. The most natural, tissue-mimicking method of cell growth for drug discovery applications is therefore 3D [2]. Here, we performed a 3D culture of pituitary cells under simulated microgravity provided by a rotary cell culture system (RCCS) in order to better simulate the growth environment of normal cells in the body.

METHODS AND MATERIALS

EXPERIMENTAL MATERIALS

Forty 4-7 weeks old female C57/BL6 mice obtained from the Guangdong Medical Lab Animal Center were used in this study. Ten mice were randomly selected for pituitary cell culture. The initial experiment was repeated 3 more times. The RCCS bio-reactor (model: RCCS-4H) was obtained from Synthecon Inc. (USA). The effect of gravity on cultured cells was reduced by the rotary motion of the bio-reactor which produces simulated microgravity. The bio-reactor contains a culture vessel and a 4-rotor host machine with 2 motors. Each motor controls 2 culture dishes, which can be placed in a carbon dioxide incubator. The culture vessel is screwed on the rotator, which rotates the chamber slowly around

the horizontal axis [3]. The rotational speed can be adjusted to achieve optimal culture conditions and prevent cells from adhering to the wall of chamber.

PITUITARY CELL CULTURE

Ten mice were sacrificed by cervical dislocation and sprayed with ethanol. Their heads were soaked in 75% ethanol for disinfection. The whole pituitary gland was removed via craniotomy in a biosafety cabinet, and then placed in a culture dish with DMEM/F12 medium. Penicillin-streptomycin supplemented DMEM/F12 medium was used to rinse the pituitary gland 3 times. It was then cut into small pieces (≤ 1 mm^3), which were filtered with screen mesh followed by FBS-free culture medium washing. Type II collagenase was added to disperse the tissue for 45 minutes. The same amount of culture medium containing serum was added to terminate the digestion. Then centrifugation was performed for 5 min at 1500 rpm to remove supernatants. Centrifugation was repeated twice, and the supernatants removed each time were collected in a culture dish for observation.

This study used the RCCS-4H simulated microgravity 3D method [9] to culture primary pituitary cells. The medium, cells, and tissues were placed in the culture trays or columns, and all air bubbles were removed. The culture trays or columns were installed on the host machine attached to a rotary motor. The internal tissues, cells, or cell masses were in suspension due to the dual influence of a rotational tangential force and gravity. Since there was only small shearing power and no air bubbles in the culture container [1, 12] the cells remained in suspension.

Four milliliters of culture medium containing serum and antibiotics were added for dispersion, followed by culturing in an incubator containing 5% CO_2 at 37°C. Observation was not performed during the first 48 hours in order to allow cells to recover from stress during tissue preparation. Four milliliters of the pituitary cell suspension was resuspended evenly and divided into 2 groups according to the culture conditions. Eight milliliters of complete medium was added to 1 ml of the pituitary cell suspensions, which were then placed in a 75 cm^2 culture flask for culture. Thirty milliliters of complete medium was added to 3 ml of the pituitary cell suspension, which was then placed in a 3D container for culture. The air in the culture container was eliminated in the biosafety cabinet. The 3D rotary container was placed in a constant temperature incubator containing 5% CO_2 and 95% air at 37°C. The initial speed was high so that the cells in the container could be completely mixed, then the speed was lowered as appropriate.

ELISA DETERMINATION OF HORMONES

Beginning the 3rd day, samples were collected every other day, and inverted fluorescence microscope was used to observe the cellular morphology. After obtaining samples, the supernatants were collected and preserved at -20°C. ELISA was used to determine the concentration of follicle-stimulating hormone (FSH).

IMMUNOFLUORESCENCE ASSAY

Pituitary cells were seeded on cover slices and fixing in 4% paraformaldehyde for 1 hour after 13, 17, 22 days. TBS-T solution containing 0.1% Triton×100 was used to wash the smears 3 times. After blocking with blocking solution, an appropriate dilution of primary antibody, mouse monoclonal antibody (Santa Cruz, USA) and rabbit polyclonal antibody (Abcam, USA) was added and the slides were incubated overnight at 4°C. Goat anti-rabbit IgG (H+L) and goat anti-mouse IgG (H+L) (Beijing Zhongshan Jinqiao Biological Technology Co., Ltd., China) were then added (1/500 dilution) and the slides were incubated for 1 hour. Finally, the nuclei were stained, and after washing 3 times an inverted fluorescence microscope was used to observe the slides.

RESULTS

MORPHOLOGICAL OBSERVATION

The 2D cultured cells are shown in figure 1. With extension of culture time, the number of cells decreased, and there were a small number of fibroblasts adhering to the wall. On the 13th day of culture, the cells completely adhered and deviant morphologies could be observed under microscopy. Some cells were elongated, some were spindle shaped and others were round. After subculture by digestion, the cells grew well and completed adhered to the wall.

The 3D cultured cells are shown in figure 2. The cells grew individually, and a small number of undigested cell masses could be observed on the first three days of culture. Under microscopy, the dispersed cells were small with strong refraction. On the 5th day, the number of cells had increased and there were some cells aggregating. On the 9th day, the number of cells had increased further and so had the cell masses. On the 13th day, these cell masses had become large (Fig. 3), there were a few single cells, and the cell masses looked nearly round or oval. Samples of cell masses were obtained for measurement, and the maximum diameter was 1000 μm. On the 17th day, isolated cells almost disappeared and the cell masses developed an oval or irregularly shape. The maximum diameter of the cell masses was 2800 μm. On the 21th day, the cell masses appeared damaged and stopped growing. The basic morphology of the cells was destroyed.

On day 1, 3, 5, 7, 9, 13, 17, and 21, cells in the 3D culture were selected for observation. On each examination day, 5 large cell masses under the same field of microscopic view were randomly selected to measure their diameters. The mean values were used to draw a growth curve graph (Fig. 3).

Supernatants were collected to determine FSH secretion (Fig. 4). Until the 5th day of culture, FSH secretion in 2D culture was higher than that in 3D culture.

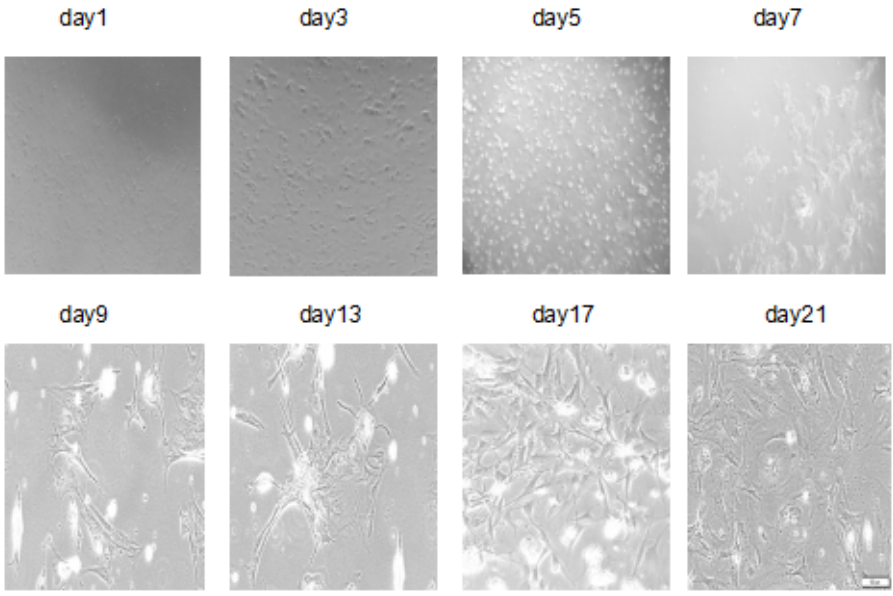

FIGURE 1. Morphological observation of 2D cultured cells

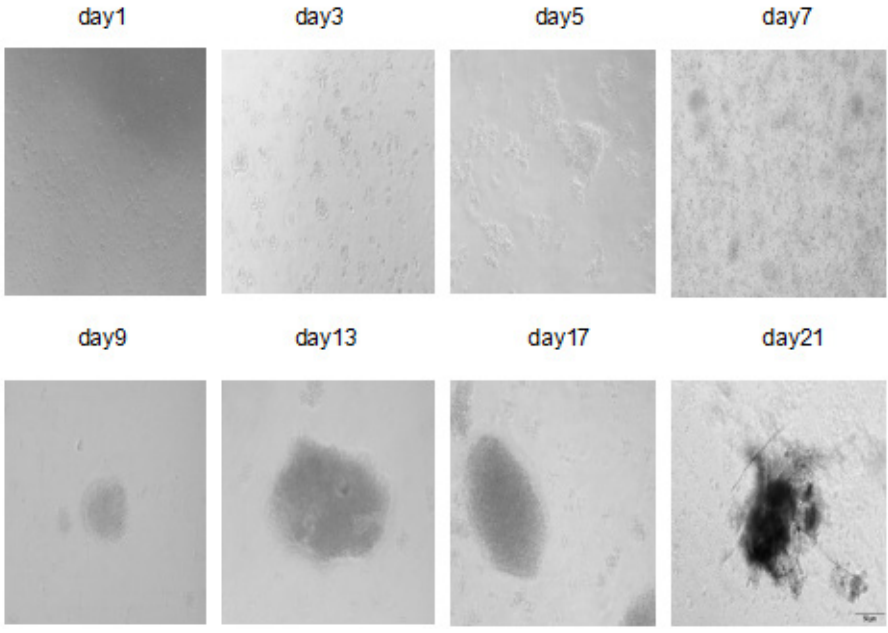

FIGURE 2. Morphological observation of 3D cultured cells

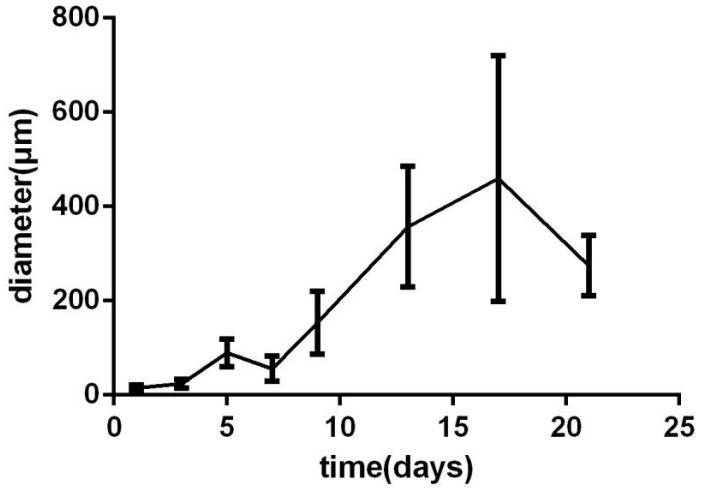

FIGURE 3. Growth curves of cell masses

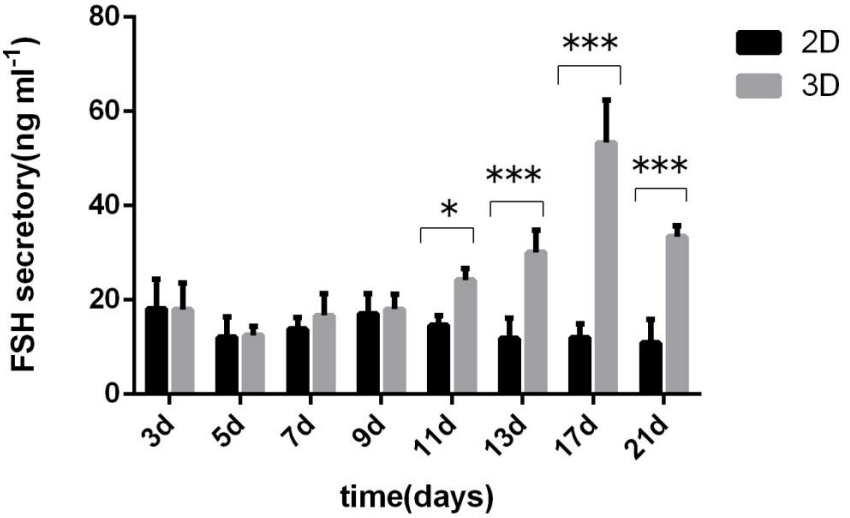

FIGURE 4. Comparison of FSH secretion of the 2D and 3D cultured pituitary cells. * $P < 0.05$; *** $P < 0.001$

This is most probably the case because the solutions were not changed on the first 5 days in the 2D culture. The cellular morphology and function did not change, and the cells continued to grow under the stimulation of culture mediums. Peak FSH secretion was reached on the 9th day, indicating that the 2D cultured pituitary cells maintained a good morphology for the first 9 days. In the 3D culture, after

cell masses visible to the naked-eye appeared on the 10th day, the culture mediums were replaced by half every 4 days to facilitate cell mass growth and proliferation. In the 3D culture, FSH secretion reached a maximum on the 17th day.

DETERMINATION OF CELLULAR HORMONE SECRETION

FSH secretion of the 2D and 3D cultured pituitary cells was determined by ELISA (Fig. 4). FSH reached a maximum concentration on the 9th and 17th day, respectively. There were statistical significant differences between the two groups on day 11, 13, 17, and 21.

IMMUNOFLUORESCENCE ASSAY OF PITUITARY CELLS

The results of the immunofluorescence assay on day 13, 17, and 21 of the 2D and 3D cultures are shown in figure 5, 6, and 7, respectively. The fluorescence intensity of ACTH and GH is shown in Figure 8 and 9, respectively. With extension of culture time, the protein expression of ACTH and GH in the pituitary cells decreased significantly. The levels of ACTH and GH in the 2D cultured pituitary cells reached a maximum on day 13. The pituitary cell masses in the 3D culture grew well between day 13 and 17. Thereafter, the growth of the cell masses slowed down.

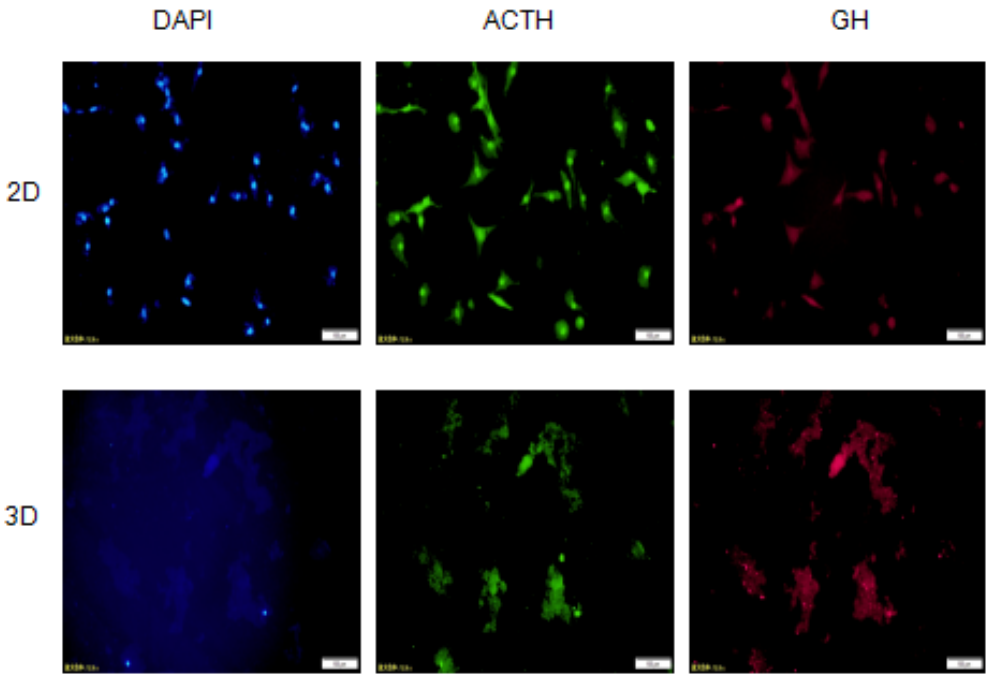

FIGURE 5. Immunofluorescence assay of 2D and 3D cultured pituitary cells on day 13

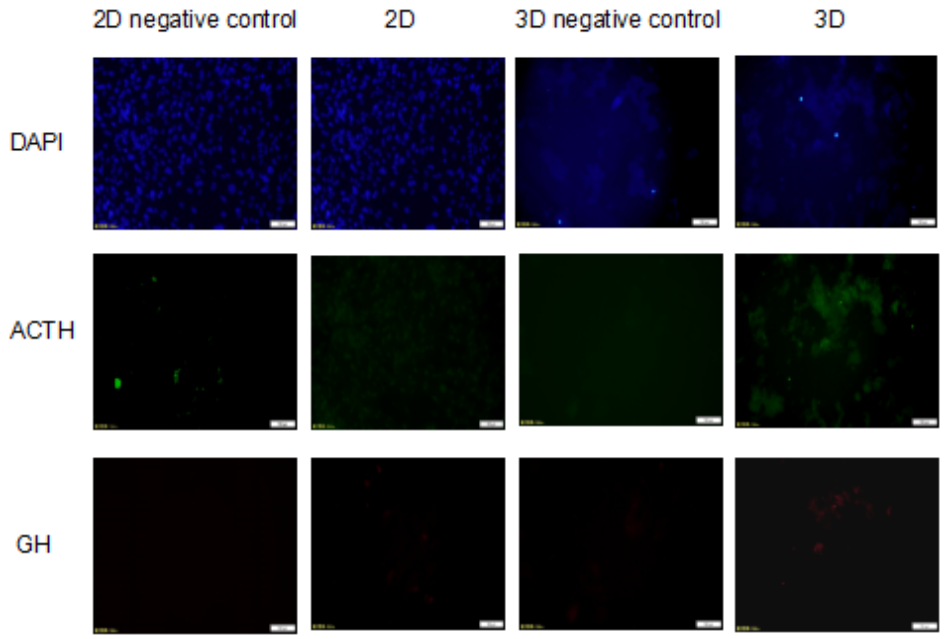

FIGURE 6. Immunofluorescence assay of 2D and 3D cultured pituitary cells on day 17

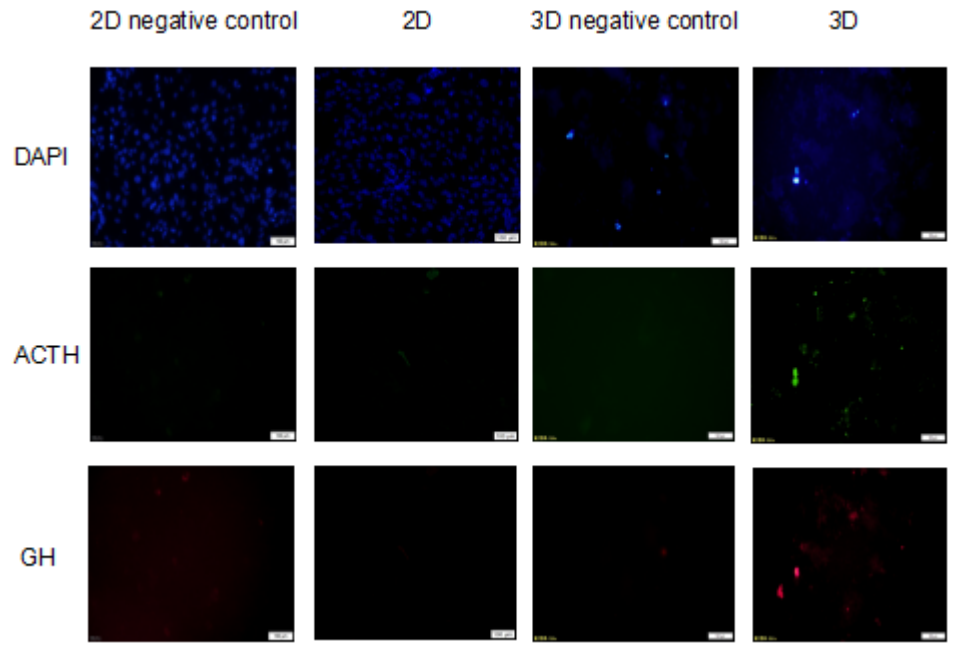

FIGURE 7. Immunofluorescence assay of 2D and 3D cultured pituitary cells on day 21

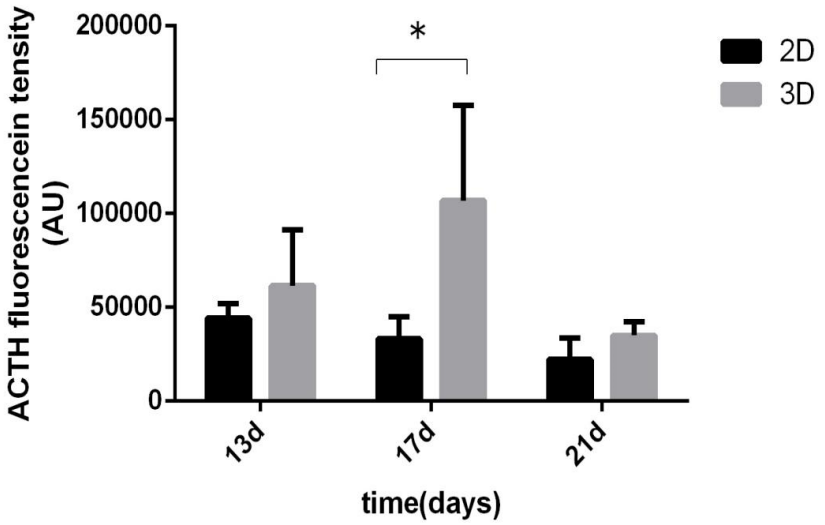

FIGURE 8. Fluorescence intensity of ACTH. * $P < 0.05$

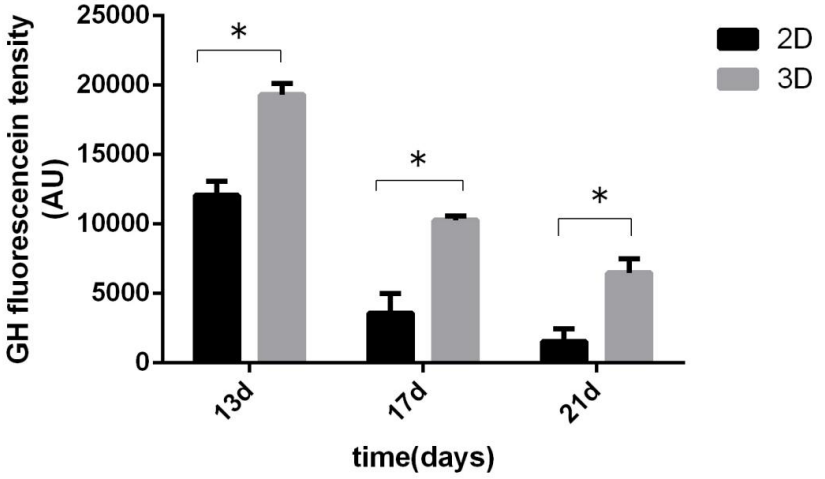

FIGURE 9. Fluorescence intensity of GH. * $P < 0.05$

With the extension of culture time, the expression of ACTH and GH in the 2D and 3D cultured pituitary cells were both decreased (Fig. 5-7). However, hormone secretion in the 3D culture was higher than that in the 2D culture on every day of measurement. The bar graphs of fluorescence intensity (Fig. 8, 9) showed that the 2D cultured cells had stopped secreting hormones on the 17th day in contrast to the 3D cultured cell masses. After the 2D cultured cells completely adhered to

the wall, the pituitary cells differentiated into various different cells. However, there were still some ACTH secreting pituitary cells left. On the 17th day in the 3D culture, the cell masses still secreted hormones, but grew slowly. On the 21st day, the cell masses ruptured and stopped growing. The secretion of all hormones further decreased accordingly.

DISCUSSION

The morphological observation of simulated microgravity cultured pituitary cells indicated that the pituitary cells could survive for some time, but without the addition of nerve growth factor or stimulation from the hypothalamus, the cells would stop growing and their morphology and function could only be maintained for a certain time. This is in accordance with the so called "neurotrophic hypothesis", which posits that the survival of neurons depends on the continuous retrograde supply of trophic factors from target organs [5, 6]. As both neural activity and neurotrophic factors have been experimentally demonstrated to be neuroprotective for the magnocellular neurons (MCNs) in response to axonal injury [8], the "neurotrophic hypothesis" can be regarded as correct with a high level of evidence. As far as the exact factors involved in this regulatory process are concerned, Vutskits et al. 1998 [11] were able to demonstrate *in vitro* that the programmed neuronal cell death is attributable to the loss of ciliary neurotrophic factor (CNTF) from the neurohypophysis. Additionally, an increase in intracellular cAMP is sufficient to promote and maintain neuronal survival by recruiting the neurotrophin receptor TrkB to the plasma membrane by translocation from intracellular stores [4]. None of these neuroprotective processes is typically active in a classical 2D cell culture.

In the presented microgravity 3D culture, the cells inoculated in the container remained in suspension under conditions with small shearing power, no air bubbles in the culture container and efficient oxygenation [1, 12]. As the presented results demonstrate, simulated microgravity 3D culture has certain advantages over traditional 2D cell culture. The culture time of the pituitary cells *in vitro* was prolonged. Cellular morphology could also be easily observed. This finding indicates that the cellular morphology in the 3D culture was maintained well for the first 17 days; an appropriate time for pituitary transplantation.

The comparisons of hormone secretion and protein expression showed that simulated microgravity cultured pituitary cells had better morphology and secreted a greater amount of hormones. In simulated microgravity, cultured pituitary cells grew well on the 7th and 13th day. This finding could help identify an optimal time for pituitary transplantation and benefit the development of further new cell culture methods. Since pituitary cells contain a variety of sub-types, this study may also provide a clinical reference for inducing pluripotent stem cells into pi-

tuitary cells. In addition, this study may be a reference for further quantitative and qualitative studies of the secretion of various hormones in pituitary cells, which may be helpful for pituitary transplantation.

CONCLUSION

The present study suggests that primary pituitary cells can survive and produce hormones *in vitro* in 3D culture for a period of time. This hormone production is both higher and last longer than in conventional 2D culture. This technique therefore offers advantages for pituitary cell transplantation.

COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest.

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Use of animals in the present research project was approved (No. 0122017) on March the 16th 2017 by the animal ethical committee of the First Affiliated Hospital of Guangdong Pharmaceutics University.

The current research project does not contain any studies with human participants performed by any of the authors and does therefore not require informed consent.

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