Turk Neurosurg, 2024

Original Investigation

DOI: 10.5137/1019-5149.JTN.35812-21.7

Received: 22.07.2021
Accepted: 17.02.2024

Published Online: 23.10.2024

Experimental Feasibility Study of TRAIL Gene Transfected into Neural Stem Cells

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ABSTRACT

AIM: To investigate the feasibility of transfecting the TNF-related apoptosis-inducing ligand (TRAIL) gene into neural stem cells (NSCs) in vitro, and explore whether NSCs retain their proliferative and differentiated activities after transfection.

MATERIAL and METHODS: NSCs were obtained from fetal mouse brains, cultured in serum-free medium and identified by immunofluorescence staining. Lentivirus vector solution containing green fluorescent protein (GFP) gene was added to the NSCs based on the multiplicity of infection (MOI). The transfection efficiency of GFP was observed using a fluorescence microscope and detected by flow cytometry. NSCs were transfected with GFP-TRAIL fusion genes mediated by the optimized MOI lentivirus solution. The expression of TRAIL proteins in NSCs was detected by immunofluorescence and Western blot analysis. The differentiation of NSCs were induced and identified by immunofluorescence staining.

RESULTS: The optimal MOI value of virus transfection was 10, resulting in a transfection rate was higher than 90%. GFP fluorescence could be observed at 24 hours after transfecting GFP-TRAIL genes into NSCs with an MOI of 10, and reached the maximum value at 72 hours. Immunofluorescence and Western-blot assays confirmed that GFP-TRAIL fusion proteins could be continuously expressed stably. Transfected NSCs could differentiate into neurons and glial cells without any statistically significant difference compared to the non-transfected group.

CONCLUSION: Neural stem cells retained their proliferative and differentiated potential after being transfected with the TRAIL gene while sustainably expressing TRAIL protein.

KEYWORDS: Neural stem cells, TRAIL, Transfection, Glioma, Gene therapy

ABBREVIATIONS: TRAIL: TNF-related apoptosis-inducing ligand, **NSCs:** Neural stem cells, **GFP:** Green fluorescent protein, **MOI:** Multiplicity of infection, **LV:** Lentivirus

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INTRODUCTION

Glioma, the most prevalent malignant tumor within brain, is characterized by its high degree of malignancy, challenging treatment options, and poor prognosis (16). The quest to extend the survival time of glioma patients has long perplexed neurosurgeons. However, recent advancements in gene therapy have opened up possibilities for glioma treatment. Neural stem cells (NSCs) possess remarkable abilities such as self-proliferation, migration towards diseased regions, and differentiation into neurons and glial cells. Numerous fundamental studies have demonstrated the anti-tumor effects of NSCs carrying tumor suppressor genes. NSCs are tumor-tropic cells that can traverse normal organs easily, localize to tumor foci throughout the body, and penetrate through the blood-brain barrier to reach the brain tumors. Transfecting antioncogenes into NSCs has shown potential in inhibiting tumor growth (14). Tumor necrosis factor related apoptosis inducing ligand (TRAIL), a member of TNF family, selectively induces apoptosis in tumor cells without harming normal cells (11). In our study, we transfected TRAIL-loaded lentivirus into NSCs to investigate both target gene expression stability and biological characteristics alterations post-transfection for subsequent glioma gene therapy experiments.

MATERIAL and METHODS

All protocols were approved by the Ethics Committee of Southwest Medical University (NO. 2012-59, 2012-10-18).

Culture and Identification of NSCs

The brains of fetal rats from Sprague Dawley rats, pregnant for 12 to 14 days, were dissected using sterile blades. Their tissues were then mixed with appropriate NSCs culture medium, and the cells were adjusted to a concentration of 5×10^5 /ml in culture flasks. Subsequently, they were cultured at 37 °C in a humidified incubator with a CO_2 level of 5%. The cell passage was conducted when the central region of cellular spheres exhibited a brown coloration after approximately 5 to 7 days. Morphological characteristics of NSCs and cell spheres were observed using an inverted microscope. The subsequent experiments utilized the 3rd generation of NSCs.

The NSCs suspension (500 µL) was inoculated for 4 hours and fixed with 4% paraformaldehyde for 15 minutes. After being washed with PBS, the blocking buffer (containing 10% sheep serum and 0.3% Triton) was used for incubation at 37°C for 1 hour. 150 µL of Nestin monoclonal antibody solution (Santa cruz, 1:200) was added to cover the coverslips. PBS solution was used as the negative control. The suspension was then incubated at 37°C for an additional period of two hours. After being washed, RBITC-labeled IgG secondary antibody was added and incubated in the dark for one hour. Following another wash with PBS, fluorescent anti-quenching agents (20 µL) were added to the slide before covering it with the coverslips and observing under a fluorescence microscope.

Lentivirus and LV/GFP-TRAIL Transfected into NSCs

Purified NSCs were individually added to lentivirus-GFP (LV/GFP) diluent (Genechem) at different multiplicity of infection (MOI) values: 0, 1, 5, 10 and 20. Polybrene solution was added to each sample. The experiment consisted of five groups with six wells in each group. The MOI 0 group served as the control group without adding virus diluent. After incubation for 10 hours, half of the culture medium were replaced with fresh medium. Following a total incubation period of 48 hours, each well received an additional 250 µL of NSCs culture medium. After a total incubation period of 72 hours, the expression of GFP was observed using fluorescence microscope. Twelve views were examined under microscope at a magnification of ×100 (neurospheres containing fewer than ten cells were not counted). Single-cell suspensions derived from NSCs were analyzed by flow cytometry to determine the transfection rate of GFP gene.

LV/GFP-TRAIL virus diluent (Genechem) was introduced to NSCs at the optimal MOI value (determined by the above experimental procedure). After 48 hours of incubation, the number of neurospheres was quantified and compared with that of the control group without transfection under identical culture conditions. The TRAIL transfection was visualized using a fluorescence microscope.

Detection of TRAIL Expression in NSCs

The NSCs were divided into three groups: the experimental group, the GFP-transfected group, and the control group. In the experimental group, NSCs were transfected with GFP-TRAIL fusion gene; in the GFP-transfected group, NSCs were transfected with GFP gene; and in the control group, NSCs were cultured without any special treatments. Western blot assay was performed to detect the expression of TRAIL protein in each group. Transfected NSCs were seeded onto coverslips in a 24-well plate and subjected to immunofluorescence staining after 4 hours of continuous culture.

Differentiation and Identification of NSCs after Transfection

Immunofluorescence was used to detect expressions of Nestin in NSCs after transfection, aiming to assess the impact of transfection on NSCs. The transfected NSCs were inoculated in the 24-well plates after 5 days of transfection, while non-transfected NSCs served as the control group. Each group was further divided into Group A and Group B. Subsequently, NSCs were induced to differentiate by supplementing with medium containing 10% fetal bovine serum and DMEM/F12, and cultured in an incubator with 5% CO_2 at 37°C. Half of the solution were replaced every 3 days, and neuronal and glial cell morphology were observed under a microscope.

The differentiation of NSCs was detected by immunofluorescence staining. NSE staining was used to identify the expression of neurons in Group A, and GFAP staining was used to identify the expression of glial cells in Group B. Neuronal counts were conducted across 30 randomly selected fields in Group A, while glial cells were counted in Group B.

Statistical Analysis

Data was expressed as mean ± SD. The differences between groups were evaluated by Student's *t*-value, one way ANOVA with post hoc comparison Tukey's test. A significance level of $p<0.05$ was considered statistically significant.

RESULTS

Culture and Identification of NSCs

NSCs were observed under an inverted microscope. After 5-7 days of incubation, the number of cellular spheres exhibited a significant increase, displaying a round or oval morphology with varying sizes, predominantly exceeding a diameter of 200 pm (Figure 1).

The Nestin-RBITC fluorescence labeling demonstrated strong positivity in the NSCs, characterized by intense red fluorescence signal (Figure 2).

Lentiviruses and LV/GFP-TRAIL Transfected into NSCs

The addition of LV/GFP virus diluent did not affect the growth of NSCs. After 72 hours, the expression of GFP was evident in all groups except the control group (Figure 3). The distribution of green fluorescence was uneven in each group. In the MOI 10 group, nearly all NSCs exhibited positive GFP expression.

Flow cytometry analysis also revealed that the lever of GFP expression in NSCs increased with higher virus titer when MOI value ranged from 0 to 10. However, there was no further increase in GFP expression when the MOI value reached 20 ($p>0.05$ vs MOI 10 group, Table I, Figure 3). Therefore, an MOI value of 10 was selected for subsequent experiments.

Aggregation of neural stem cells were observed at 24 hours after transfection with LV/GFP-TRAIL. After 72 hours, green fluorescence were observed in all wells (Figure 4). The green fluorescence remained visible even after subculture. There were no significant differences in neurospheres numbers between the transfection and non-transfection groups ($p>0.05$, Figure 5).

Identification of TRAIL Expression in NSCs

The expression of TRAIL protein in NSCs was identified at 72 hours post-transfection with LV/GFP-TRAIL using Western blotting assay (Figure 6). A distinct band near 58kd, corresponding to the fusion protein transcribed by the TRAIL gene, was observed in the transfection group. No detectable protein expression was found in the control group.

Immunofluorescence staining experiment utilizing GFP as an indicator of expression was conducted to assess TRAIL protein expression in LV/GFP-TRAIL transfected NSCs. The expression pattern of TRAIL closely resembled that of Nestin (Figure 7).

Induced Differentiation and Identification of Transfected NSCs

At 24 hours of inoculation, transfected NSCs gradually adherent to the wall.One day later, radial short and thick protuberances emerged from the neurospheres. Two days later, round cells in the center of the neural spheres continued to migrate along these protuberances. Under high magnification, it was observed that cell morphology gradually transitioned from

Table I: Comparison of Virus Transfection Efficiency with Different MOI Values and Neurospheres Generation

	MOI Values	Number	Transfection Efficiency (%)	Number of NSC Spheres
Control	0	6	0	390 ± 17
Group 1	1	6	7.5 ± 1.5	385 ± 15
Group 2	5	6	42.4 ± 2.1	372 ± 14
Group 3	10	6	90.6 ± 1.6	358 ± 14
Group 4	20	6	91.2 ± 1.0*	275 ± 12#

**p=0.464 compared with Group 3. #p<0.001 compared with Group 3.*

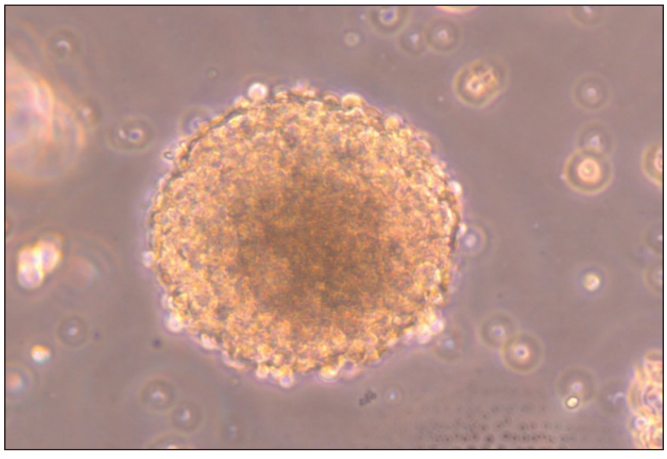

Figure 1: Primary neurospheres (400×).

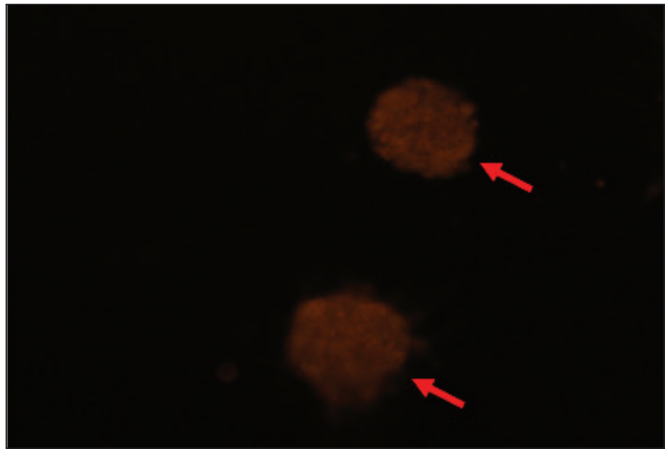

Figure 2: Nestin-labeled neural stem cells (200×).

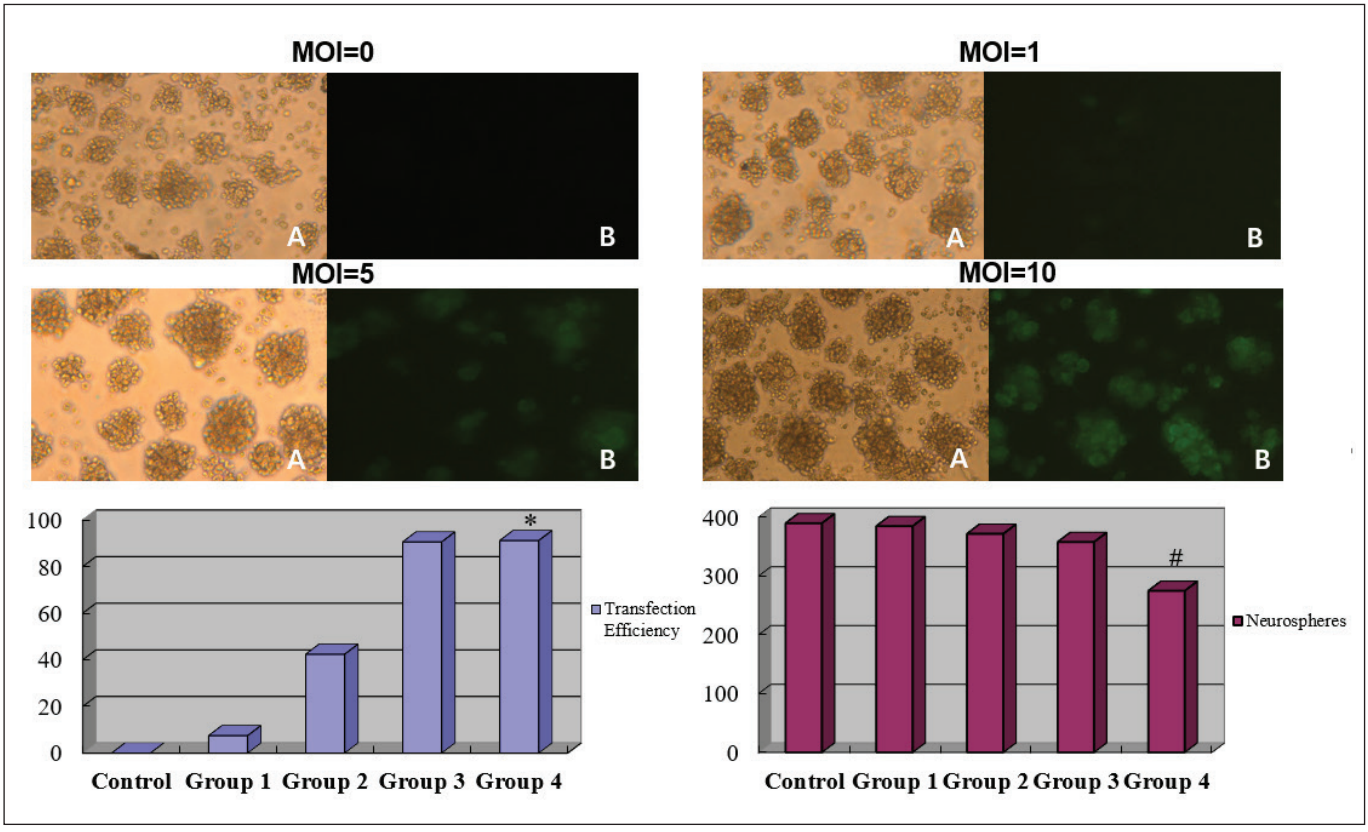

Figure 3: Comparison of GFP transfection efficiency and the number of neurospheres in different MOI groups (*p>0.05 vs Group 3, #p<0.01 vs Group 3) (**A:** Inverted microscope field, **B:** Fluorescence microscope field).

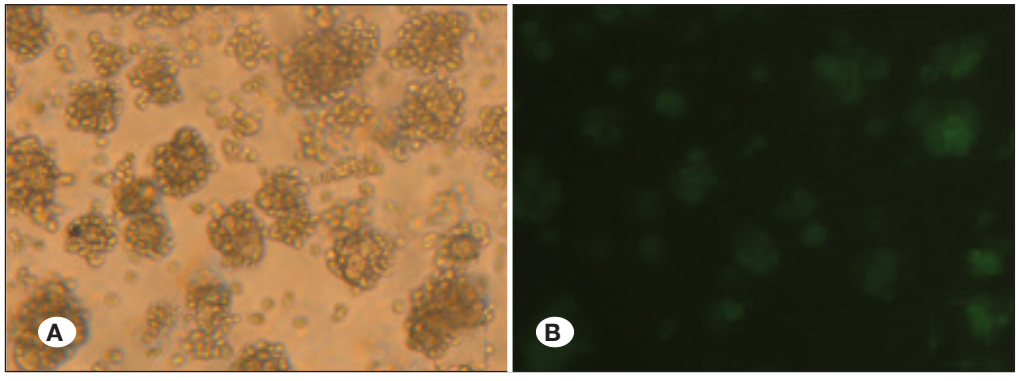

Figure 4: GFP expression at 72h after transfection with LV/GFP-TRAIL (**A:** Inverted microscope field, **B:** Fluorescence microscope field).

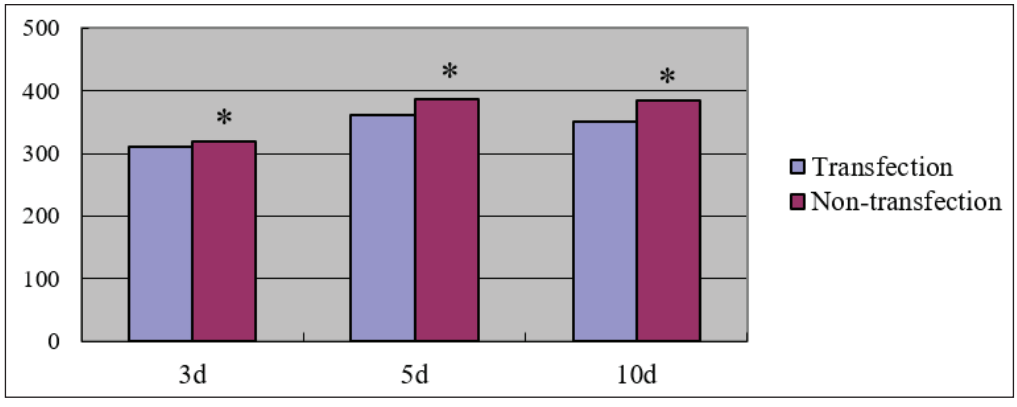

Figure 5: The number of NSCs generated in different times (*p>0.05 vs transfection group).

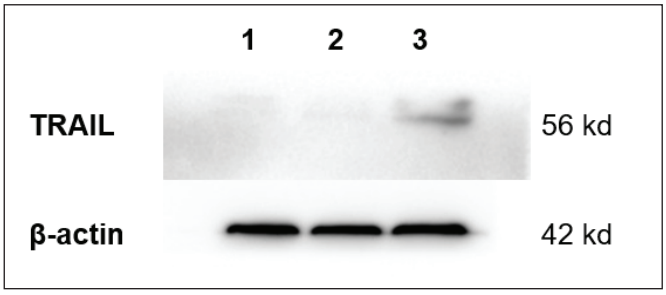

Figure 6: The expression of TRAIL protein in NSCs after transfected (1. Primary NSCs, 2. LV/GFP transfected NSCs, 3. LV/GFP-TRAIL transfected NSCs).

Table II: The Number of Neurons and Astrocytes Were Compared Between the Transfection Group and the Control Group (n=10)

	Transfection group	Non-transfection group
Number of neurons	12 ± 2	11 ± 3*
Number of astrocytes	35 ± 5	37 ± 7#

**p=0.512 compared with transfection group. *p=0.305 compared with transfection group.*

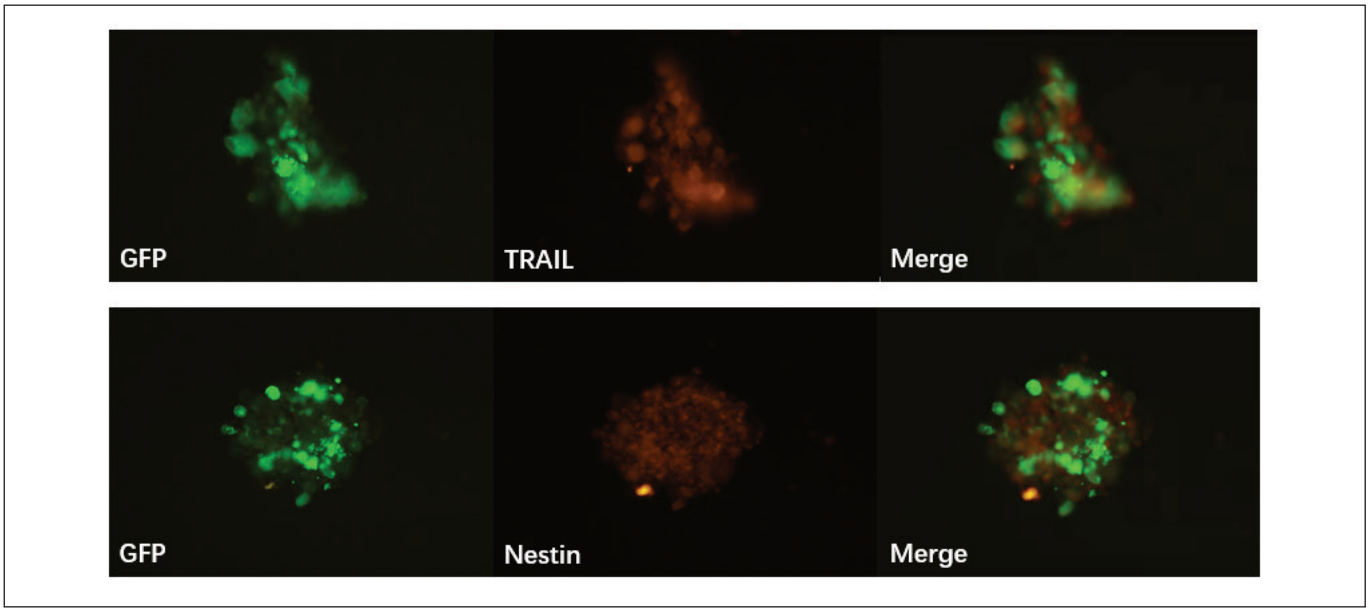

Figure 7: The expression position of TRAIL in NSCs after transfected by LV/GFP-TRAIL.

round to polygonal shape. The cellular protrusions increased in length and number over time. After 3-5 days of induction, the neural spheres flattened out, meanwhile the protuberances continued to elongate further. Some undifferentiated cells still remained in the center of the neurospheres (Figure 8).

At 5 days after differentiation, neurons and astrocytes were identified using GFAP and NSE markers respectively through Immunofluorescence staining. The results showed that LV/TRAIL-transfected NSCs could successfully differentiate into bipolar neurons with rounded cyton and protrusions at both ends, as well as flat astrocytes with multiple protrusions (Figure 9).

Meanwhile, counts were conducted for neurons and glial cells in both transfection group and control group (Table II), revealing no statistically significant difference between TRAIL gene-transfected NSCs and non-transfected group regarding neuronal or glial cell differentiation ($p>0.05$ both).

DISCUSSION

Neural stem cell (NSC) lines serve as a versatile model for exploration in the fields of stem cell and developmental biology, regenerative medicine and neuroscience (6). NSCs possess several distinctive characteristics including rapid division and differentiation, selective tumor aggregation, and efficient traversal through organs and the blood-brain barrier (13). Moreover, NSCs exhibit genetic stability, clonal expandability, and easy transfectability - experimental attributes compatible with targeted genetic manipulations (4,6). Numerous studies on cancer treatment have focused on the potential utilization of NSCs as carriers to selectively deliver anticancer genes to tumor sites (13). Consequently, NSCs have been proposed as promising therapeutic agents for glioma treatment.

Malignant glioma responds poorly to currently available therapies. Some of this resistance can be attributed to the tumor cells failed to undergo apoptosis upon anticancer treatment (8). Recently, considerable researches focused on apoptosis triggered by chemotherapy and radiation therapy-induced resistance in cancer cells. The binding of death ligands, mem-

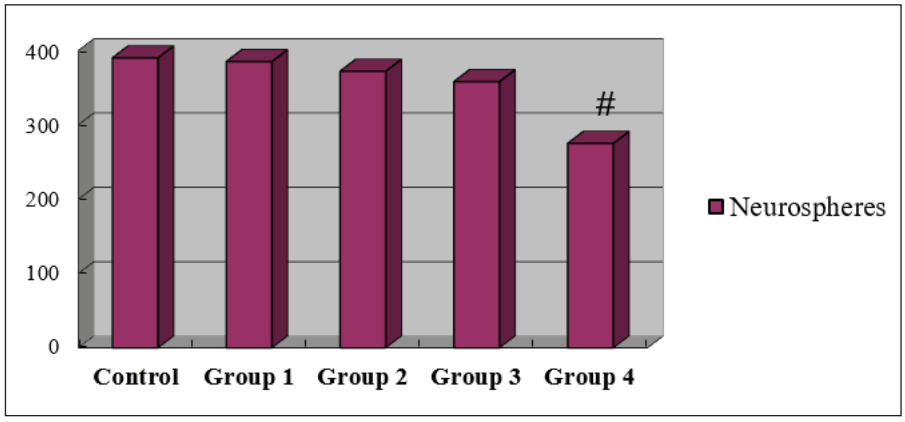

Figure 8: Induced differentiation of transfected neurospheres after 5 days (×100).

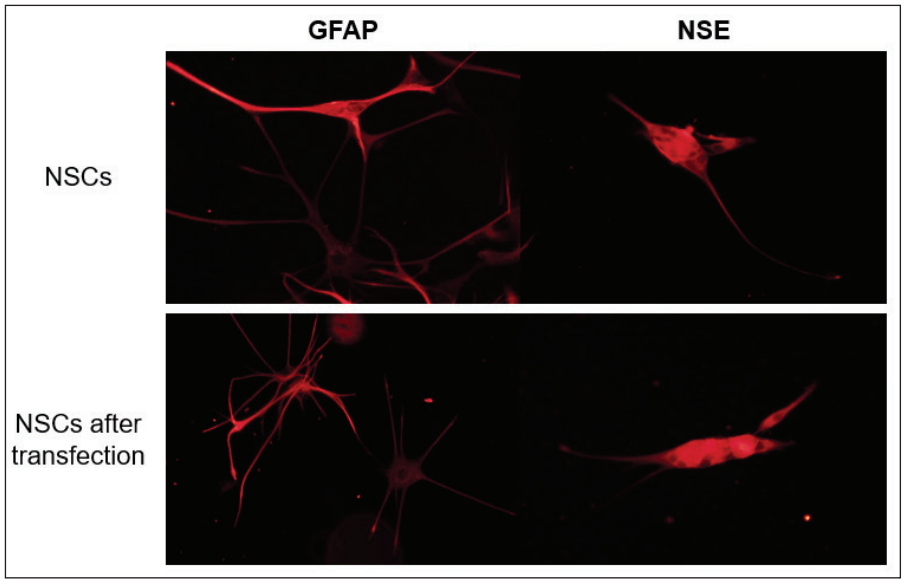

Figure 9: The positive expressions of GFAP and NSE after differentiation of NSCs (×400).

bers of the tumor necrosis factor-alpha (TNF-alpha) family, to their receptors on the cell surface initiates this pathway (8). Death ligands can kill some cancer cells that are resistant to the apoptotic pathway induced by conventional anticancer treatments (7-9). TNF-related apoptosis-inducing ligand (TRAIL), which shares sequence homology with tumor necrosis factor, is a transmembrane protein processed by cysteine protease and generate a soluble ligand that binds to death receptors, activating the extrinsic apoptotic pathway (10,12). TRAIL has emerged as a promising anticancer agent due to its ability to selectively induce apoptosis in various malignant cells while sparing normal cells (3,12).

NSCs are primarily distributed in cerebral cortex, subependymal region, striatum, hippocampus dentate gyrus, and other regions (15). In the study, NSCs were isolated from the brains of fetal rats at gestational age of 12 to 14 days. Additionally, NSCs could also be isolated and cultured from neonatal rats' brains. Maintaining an optimal cell density is crucial for promoting NSC growth during the culture process. For this experiment, a density of 5×10^5 cells/mL was utilized. It is important to carefully consider the timing and method of passage when culturing NSCs. If nerve spheres grow excessively large, central cells may perish due to inadequate nutrition. Therefore, timely passage should be conducted when brown discoloration appears in the centers of nerve spheres.

The significance of gene therapy lies in the introduction of exogenous genes into NSCs. The vector plays a crucial role in the process. An ideal vector should possess high transfection efficiency, stable expression ability for exogenous genes, and low cytotoxicity. Lentivirus exhibits characteristics such as reduced immune response and large vector capacity, enabling effective integration of exogenous genes into target cells and long-term stable expression of target genes within the host (1,5). The combination of lentiviral vector with GFP reporter gene is commonly employed as a transfection medium due to its stability and efficiency (2). Since the GFP gene does not interfere with the expression of target genes, the transfection efficiency can be observed by fusing GFP with the target gene (2). In this study, lentivirus was used as a vector to successfully transfer the GFP gene into NSCs, resulting in positive GFP expression that confirms efficient expression of exogenous genes in transfected NSCs.

In this study, NSCs were transfected with GFP-TRAIL fusion genes. The transfected NSCs retained their proliferative ability and could be subcultured, confirming the steady and continuous expression of exogenous TRAIL proteins without affecting NSCs proliferation and differentiation.

However, further investigation is required to fully understand the potential of using NSCs as carriers for glioma therapy. In our study, we successfully cultured and identified NSCs which were then transfected with the TRAIL gene. These transfected cells differentiated into neurons and astrocytes without any negative impact from lentivirus transfection. The established culture protocol is stable and can serve as a foundation for future transfection experiments both in vitro and in vivo.

CONCLUSION

Our preliminary study suggest that it may be possible to effectively target tumor cells by transfecting the TRAIL gene into NSCs followed by their stable differentiation. In the following studies, we aim to validate its anti-tumor effects through in vivo experiments, potentially paving the way for clinical treatment of glioma in the future.

Declarations

Funding: The study is supported by the top-notch talent funding of Chongqing Traditional Chinese Medicine Hospital, the special projects of incentives and guidance for scientific research institutions in Chongqing (cstc2019jxjl130020), Chongqing natural science foundation (cstc2020jcyj-msxmX0116, CSTB2022NSCQ-MSX0628).

Availability of data and materials: The datasets generated and/or analyzed during the current study are available from the corresponding author by reasonable request.

Disclosure: The authors declare that the article content was composed in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHORSHIP CONTRIBUTION

Study conception and design: LX, ZJ

Data collection: LX, JM, XP, CL

Analysis and interpretation of results: LX, JM, ZT

Draft manuscript preparation: ZT, JM

Critical revision of the article: ZT

Other (study supervision, fundings, materials, etc...): ZT, GW, YH, LW

All authors (LX, JM, YH, LW, CL, XP, GW, ZJ, ZT) reviewed the results and approved the final version of the manuscript.

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