A SIMPLIFIED METHOD FOR ISOLATION AND CULTURE OF NEURAL STEM CELLS FROM ADULT MICE BRAIN: *IN VITRO*

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ABSTRACT

The present study aimed to develop an uncomplicated method for isolation and culture of mice brain derived-neural stem cells (NSCs) - neurospheres, using a simplified method. In order to obtain single cells suspension, the brain tissue was dissociated using mechanical methods (trituration) instead of enzymatic method. These single cells suspension were *in vitro* cultured in DMEM)/Ham's F12 (1:1) mixture (high glucose) plus 20 ng/ml Epidermal growth factor (rHuEGF) and 20 ng/ml Fibroblast growth factor-basic (FGF-2). The results showed the combined actions of rHuEGF and bFGF-2 was in inducing proliferation of brain derived-NSCs. So, after 3 days culture numerous number of NSCs were observed floated in culture medium. Then within the time of culture the numbers of NSCs were increased. After 7 days culture some of these cells began to aggregate and form small sized neurosphere of undifferentiated cells (early stage) and within 3 weeks culture, large sized mature neurospheres were formed. When these neurospheres were aspirated and re-cultured on the gelatin pre- coated Petri dish, these neurospheres were attached and several nerve cells began to migrate from these neurospheres and occupy the surrounding area. This result confirms the incidence of NSCs derived neurospheres and it is ability to differentiate to nerve cell. In conclusion this method is simple and less expensive, less effort, but take longer time to get NSCs derived-neurosphere, as well as it is possible to use mechanical dissociation of cells (trituration) instead of enzymatic method to obtain single cell suspension.

KEYWORDS: Neural stem cells, Neurosphere, Mice brain, *in vitro*.

INTRODUCTION

A stem cell is a cell that has the ability to divide for indefinite periods, often throughout the life of the organism (Appasani and Appasani, 2011). Neural stem cells (NSCs) are self-renewing, multipotent cells that generate main phenotypes of the central nervous system (CNS) including: neurons, astrocytes, and oligodendrocytes. In 1989 Sally Temple described multipotent self-renewal progenitor and stem cell in the subventricular zone of adult mice brain (Temple, 1989), but the studies of Reynolds, Weiss and their colleagues definitively isolated NSCs for the first time (Reynolds *et al.*, 1992; Reynolds and Weiss, 1992).

The importance of NSC comes from it is ability to differentiate into major cell types of CNS, and the multipotency of these cells shed a light on the possibility of using NSC as a therapeutic tool for various neurological disorders such as: Parkinson's disease, Alzheimer's disease, spinal cord injury, multiple sclerosis, stroke, and epilepsy (Phinney, 2011).

However, the majority of NSC studies requires, isolation and culture of NSCs not only to provide an important source of cells for *in vitro* studies but are also important supply for CNS transplantation studies. On the other hand, neural tissue are composed of both neuronal and non-neuronal cells as well as connective and vascular tissues, when removed from the brain and deposit in culture, the cells loss the physiological connections, anchorages and their microenvironments (niches). Therefore strategies to isolate and culture of NSCs from CNS involved: 1) Isolation of cells adhesive contacts without causing damage to the target cells. 2) Separation of NSCs from other brain cells and connective tissue debris, and 3) providing the appropriate environmental conditions including specific nutrients and growth factors required for the survival and the proliferation of NSCs (Ray *et al.*, 1997).

The most commonly used methods for isolation and culture of NSCs were used serum-free culture media supplemented with various hormones, nutrients, and mitogenic growth factors (Epidermal growth factor "EGF" and/or Fibroblast growth factor " FGF-2") (Alvarez-Buylla and Lois, 1995; Gage *et al.*, 1995; McKay, 1997), therefore, these methods of NSCs isolation and culture have great

requirements and are not free from certain elaborations, thus, in present study we have developed an uncomplicated method for isolation and culture of NSCs, that is by modifying several existing ones as: Reynolds and Wiess, 1992; Ray *et al.*, 1995; Gritti *et al.*, 1999; Marshak *et al.*, 2001; Bouab *et al.*, 2011.

MATERIAL AND METHODS

Adult albino mice obtained from Animal Breeding House/ College of Veterinary Medicine/ University of Mosul. The NSCs isolation and culture was conducted in the Postgraduate Students Research Laboratory \Faculty of Medical Sciences/University of Duhok.

Culture conditions

All the experiments of the present study were conducted under restrict sterile conditions: Laminar air flow hood (Kojair Tech, Finland) with UV light source and air purifier were used. All of glass and plastic wares are sterile. All solutions were sterile by autoclaving while the enzyme and media were sterilized by millipore filter (0.22µm porosity) and prewarmed to 37C◦ before using (Al-Azawwi, 2003).

Culture medium

A mixture of liquid Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 (1:1) (with high glucose) containing: 15 mM HEPES, L-Glutamine, D-Glucose, sodium bicarbonate (Caisson Labs, USA) was used as a culture media (Eagle, 1955). This medium was supplemented by addition the following: Epidermal growth factor (rHuEGF) (bio world, USA) as 20 ng/ml of culture media, fibroblast growth factor-basic (FGF-2) (bio world, USA) as 20 ng/ml of culture media, antibiotics: Penicillin (as benzyl penicillin) 100000 units/L of culture media and streptomycin 50 mg/L of culture media, antifungal: Amphotericin B as 1000 µg/L of culture media. The final pH of this media was adjusted to be 7.3 by 1N HCL and 1N NaOH, then the media is dispense into sterile container and kept at 4C$^{\circ}$ until use.

Isolation and culture of mice brain derived neuronal stem cells - neurospheres

The procedure of NSC isolation was done according to the (Weiner, 2008) as follows: mouse was scarified by cervical dislocation followed by washing the incision area by tap water, then sterilized by 70 % ethanol and Povidone. Dorsal longitudinal midline incision in scalp and skull done by scalpel, skull bones were cut dorsolaterally in both directions. The brain was removed carefully to avoid brain laceration and bleeding. Removed brain transferred immediately to prewarmed (37 $^{\circ}$C), CO_2 bubbled artificial cerebrospinal fluid (aCSF-A) for dissection (aCSF-A composed of: 124 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM D-glucose, and 1:25 Penicillin-streptomycin solution), pH of this solution was adjusted to 7.3 (Weiss *et al.,* 1996). Then the brain was cut into small pieces about 1mm^3 by fine scissor. These pieces were transferred into aCSF-B (composed of: 124 mM NaCl, 5 mM KCl, 3.2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM D-glucose, and 1:25 Penicillin-streptomycin solution), the pH of solution was adjusted to 7.3 (Weiss *et al.,* 1996), and mechanical dissociation (trituration) of brain cells were performed by fire polished Pasteur pipette to get a single cell suspension.

After centrifugation (NF-415, Nuve, Turkey) (500 xg) for 5 min, the cell pellet was washed by prewarmed at (37 $^{\circ}$C) PBS. Then Centrifuged again (500 xg) for 5 min too, the pellet was resuspended in prewarmed (37 $^{\circ}$C) culture media (DMEM/Ham's F12, plus rHuEGF, FGF-2, penicillin, streptomycin, and amphotericin B).

Cells in culture media (about 5ml) transferred into culture flask (35 ml capacity) (Falcon, USA) closed firmly and put in CO_2 incubator (Labtech, Korea) at 5 % CO_2 and 37 C$^{\circ}$ temperature. The culture was examined approximately daily by inverted microscope (XDS-1B, China) for observation of NSCs state and neurosphere formation.

One ml fresh culture media (prewarmed to 37 C$^{\circ}$) was added two times weekly to culture flask.

Histological study of NSCs- neurospheres

Small Petri dish (30 mm) covered by gelatin (0.1 % solution of gelatin was prepared in Distal Deionized H_2O (w/v), let to dry in laminar air flow hood.

Under dissecting microscope and by using a finely drawn out (fire polished) Pasteur pipette mouth-controlled tube the Neurospheres were carefully aspirated from the culture plate and transferred into gelatin coated Petri dishes, and return into incubator (37 $^{\circ}$C and 5% CO_2).

After 2-3 days, media was discharged and the Petri dish was washed by phosphate buffer saline (PBS), fixed by buffered neutral formalin (BNF) solution (for 1 hr), covered and kept in -20 $^{\circ}$C until staining.

For staining, after thawing of Petri dish, washed by PBS and stained with Harris hematoxylin for 5 min. then washed with PBS, stained by eosin stain for 3 min.
Washed with PBS, then mounted by aqueous mounted (DPX), covered by coverslip and examined under light microscope (Yarnell and Schnebli, 1974).

RESULTS AND DISCUSSION

In the present study the numbers of supplements that's commonly used in most previous works during NSCs isolation and culture (Weiner, 2008), were reduced and this has advantage economically as well as reduce steps of manipulation without interruption with NSC fate.

The results of this study also demonstrate that using mechanical dissociation (trituration) of brain cells instead of using enzymes such as trypsin enzyme, can obtained healthy cell suspension, because enzymatically dissociated remain critical step in cells suspension preparation, due to it is harmful effect on cell viability and activity especially to brain cells. Enzymatically dissociated require confirm manipulation and restrict timing, else the bulk reduction in cell viability will occur; therefore trituration was prefer over using enzyme to get single cells suspension.

The results of the present study indicate the combined actions of rHuEGF and bFGF-2 in inducing proliferation of NSCs. So, after 3 days from brain cells cultured in the presence of these growth factors, numerous number of NSCs were observed floated in medium culture without any attachment, these cells appeared with a high ratio of nucleus to the cytoplasm (Fig. 1). Then within the time of culture the number of NSCs were increased so, after 7 days culture some of these cells began to aggregate and form small sized neurosphere of undifferentiated cells (early stage) (Fig. 2). At day 15 of culture, these neurospheres were increased in size due to the cells proliferation (Fig. 3), and within 3 weeks culture, the formation of large sized mature neurospheres were recorded (Fig. 4). These results also were observed and recorded by (Reynolds and Weiss, 1992; Morshead *et al*., 1994), they found that EGF induced the proliferation of multipotent, self-renewing, and expandable stem cells that were isolated from the adult subependymal cell layer of the forebrain and these cells formed spheres of undifferentiated cells that could generate neurons and glia. Weiss *et al.,* (1996) also found when adult thoracic spinal cord was dissected, enzymatically dissociated, and plated in the presence of EGF (20 ng/ml) or bFGF (20 ng/ml). After 8 days *in vitro*, cells cultured in the presence of EGF showed no evidence of the characteristic spheres of proliferating NSCs , also they observed that in the presence of bFGF, very small clusters of cells were found; however, these clusters could not renew (only15% produced one secondary sphere) or expand (none produced more than one (secondary sphere). But they mentioned when EGF and bFGF were combined, large self-renewing and expandable spheres were generated.

While the results of the Marshak *et al*., (2001) and Bouab *et al*., (2011) showed that by using routine procedure, within 5-10 days mature neurospheres were formed in culture.

When these neurospheres were aspirated and re-cultured on the gelatin pre- coated Petri dish, after 3 days from the re-culturing these neurospheres were attached and several nerve cells began to migrate from these neurospheres and occupy the surrounding area. This result confirm the incidence of NSCs and it is ability to differentiate to nerve cell (Fig. 5,6).

From this work we concluded that this modified method is simple and less expensive, less effort, but take longer time to get NSCs derived-neurosphere, as well as it is possible to use mechanical dissociation of cells (trituration) instead of enzymatic method to obtain single cell suspension.

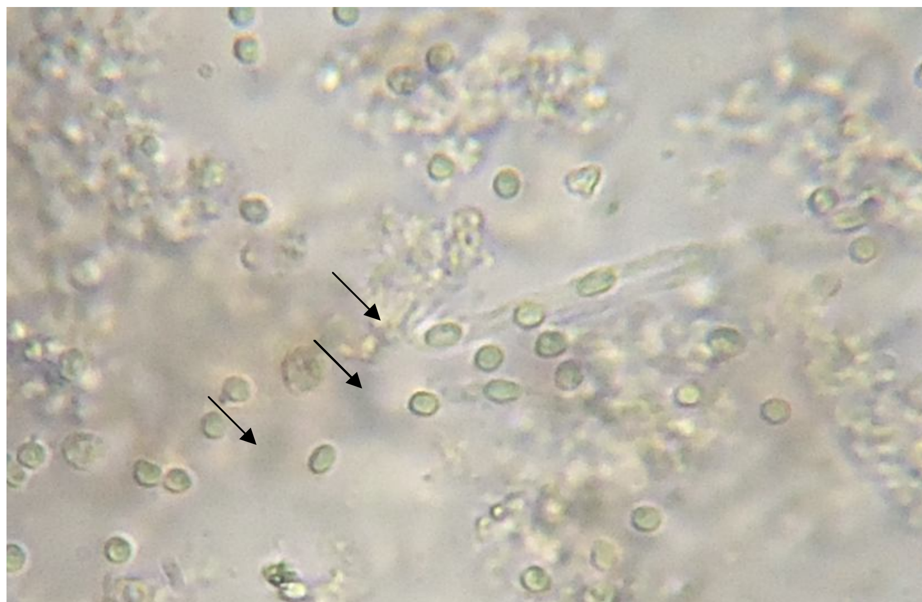

Figure (1): Single neural stem cells (arrows) after 3 days culture. Living tissue (160X)

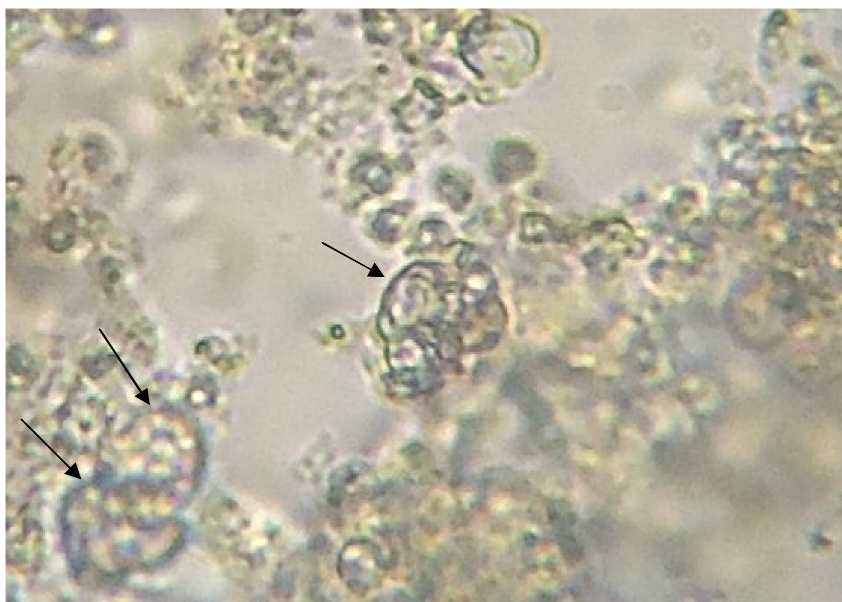

Figure (2): After 7 day's culture, the formation of early stage neurospheres (arrows). Living tissue (160 X).

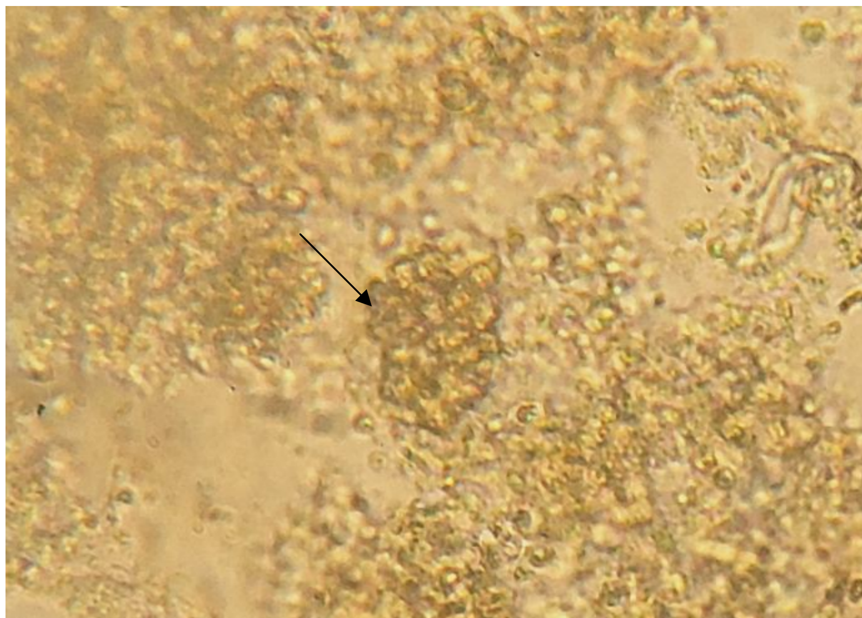

Figure (3): After 15 day's culture neurosphere formation (arrow). Living tissue (160 X).

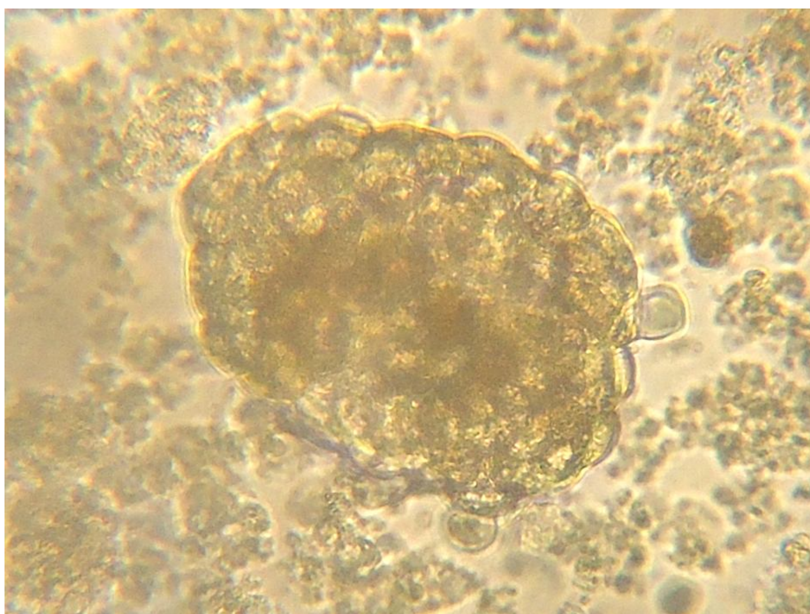

Figure (4): Mature neurosphere after 21 days culture. Living tissue. (320X)

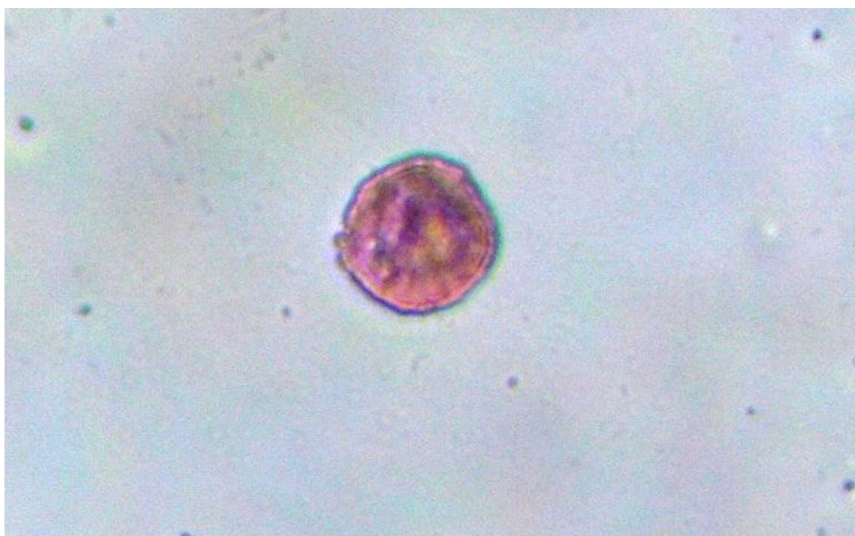

Figure (5): This figure showed the attachment of mature neurosphere after 3 days from recultured on gelatin coated Petri dish. H & E staining, (100 X).

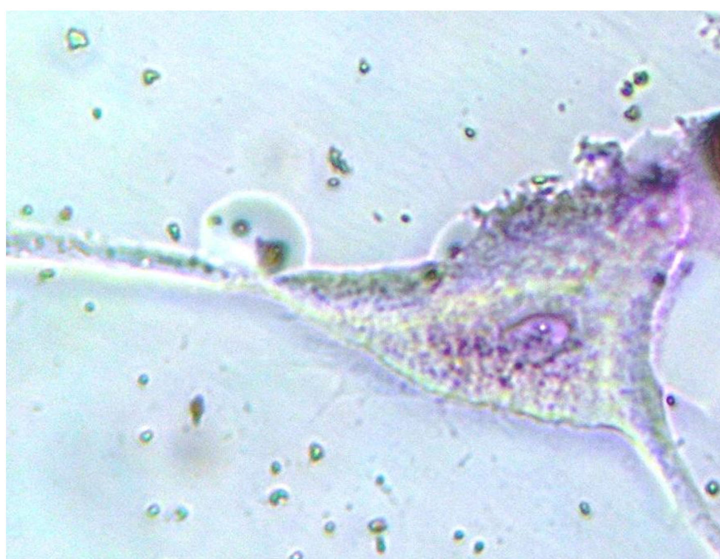

Figure (6): This figure showed the formation of nerve cell after 3 days from recultured neurosphere. H & E staining, (400 X)

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طريقة مبسطة لعزل وزراعة الخلايا الجذعية العصبية من دماغ الفئران البالغة : خارج الجسم الحي

الخلاصة

هدفت الدراسة الحالية تطوير طريقة عزل وزراعة Neurosphere المشتقة من الخلايا الجذعية العصبية المشتقة من أدمغة الفئران وذلك باستعمال طريقة مبسطة. لذا و لغرض الحصول على معلق خلوي فقد تم تفتيت نسيج الدماغ باستخدام الطريقة الميكانيكية بدلا من الطريقة الإنزيمية. ومن ثم زراعة هذا المعلق الخلوي خارج الجسم الحي في الوسط ألزرعي DMEM/Ham's F12 (عالي الكلوكوز) بوجود 20 نانوغرام/مل من عامل النمو البشروي (rHuEGF) و 20 نانوغرام/مل من عامل النمو الليفي-القاعدي (FGF-2) . أظهرت النتائج الفعل التأزري لعاملي النمو (rHuEGF وFGF-2) في حث تكاثر وتوالد الخلايا الجذعية العصبية المشتقة من الدماغ. لذا فقد لوحظ بعد ثلاثة أيام من الزراعة، وجود أعداد من خلايا جذعية عصبية طافية في الوسط ألزرعي ، و الذي بدأت أعدادها بالازدياد في الوسط ألزرعي مع تقدم وقت الزراعة. وبعد 7 أيام من الزراعة بدأت بعض هذه الخلايا بالتجمع والتكتل لتكوينNeurosphere لخلايا غير متمايزة (المرحلة المبكرة) ، و بعد ثلاثة أسابيع من الزراعة لوحظ تكّون الـ Neurosphere الناضج كبير الحجم. ولما تم عزل الـ Neurospheres من الوسط ألزرعي وإعادة زراعتها على طبق بتري مغطى بطبقة من الجلاتين، لوحظ التصاقها ومن ثم هجرة العديد من الخلايا العصبية من Neurosphere لتحتل المنطقة المحيطة.تؤكد هذه النتيجة اشتقاق الـ Neurosphere من الخلايا الجذعية العصبية ومن ثم قابليتها على التمايز للخلايا العصبية. من هذه الدراسة تبين أن الطريقة المستعملة هي أكثر سهولة وأقل تكلفة من الطرق السابقة ، إلا أنها تحتاج إلى وقت أطول للحصول على Neurosphere من الخلايا الجذعية العصبية. كما أن استخدام الطريقة الميكانيكية كان له دورا ناجحا في الحصول على معلق الخلايا المنفردة بدلا من الطريقة الإنزيمية.

بوخته

مەرەما ڤێ ڤەكولینێ ئەنجامدانا گهونێ ل سەر رێكا جوداكرن و چاندنا خانێن دەمارگیری یێن كەلێژێ ونیوروسفیر ژ مێشكی مشكان بوو ، ژبو دەستڤەئینانا رێكەكا سادە و كێمتر بها شانەی لەش مێشكی هاتە پرتكاندن بكارئینانا رێكا میكانیكی پیش رێكا ئەنزیمی ڤە ژبو پەیداكرنا راوەستانێ خانێن یەكانە. ئەڤ راوەستانە هاتە چاندن ل ناڤنجی جاندنی (DMEM/Ham's F12) (ل سەر كلوكوزێ) ب هەبوونا 20 نانوگرام/ملیلیتر ژ فاكتەرێ گەشەبیدن یا مروڤان (rHuEGF) و 20 نانوگرام/ملیلێتر ژ فاكتەرێ گەشەبیدن یا فایبری تفتی (FGF-2). ئەنجامان دیاركر ئێكو پشتگیریكرنا هەردوو فاكتەرێن گەشەبیدنێ rHuEGF و FGF-2 ژ بو هاندانا زیدەبوونا خانێن كەلێژێ یێن دەمارگیری (NSCs) ئەوێن هاتینە جودا كرن ژ مێشكی. خانێن كەلیژێیێن دەمارگیری هاتنە دیتن ب سەركەتی ل ناڤ ناڤنجی چاندنێ بشتی سێ روژان ژ جاندنی، هەروووسا ژمارا خانێن كەلیژێ دەمارگیری زیەدبن ب بورینا دەمی، وبشتی 7 روژان ژ جاندنێ هندەك خانێن كەلێژێ یێن دەمارگیری دەست بێكیر وەك كومێن خانان دیاربوون و نبیوروسفیر ب ڤەبارێن بچیك پەیداكرن ئەوێ كو ژ بێك دهێن ژ خانێن نەجودەكرێ هەروسا نیروسفیریێ ڤەبارە مەزن وگەهشتی دروست بوو بشتی بوورینا سێ حەفتیان ژ جاندنێ، و بشتی جودا كرنا نیوروسفیری ژ ناڤنجی جاندنێ،حارەكادی هاتە چاندن دناڤ بێتری دشەكێ نخافتی ب جیلاتنی ، ونهینا نیوروسفیری ب ئەردێ بێتری وشێڤە هاتە ویتن،هەروەسا مشەختبوونا خانێن دەمارگیری ژ نیوروسفیر بو دەرڤە. دیاربوو ژ ڤێ ڤەكولینێ كو رێكا هاتیە ب كارئینان ساناهیتر وكیمتر بها بوو ژ رێكێن كەڤن، بەلێ پا پێتڤی ب دەمەكێ درێژتر هەیە ژ بو پەیداكرنا نیوروسفیری ژ خانێن كەلیژێ یێن دەمارگیری. هەروەسا ب كارئینانا رێكما میكانیكی رولكێ سەركەفتی هەبوو ژ بو ب دەستڤە ئینانا راوەستانێ خانێن یەكانە بەراورد دگەل رێكا ئەنزیمی.