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In vitro isolation and expansion of neural stem cells NSCs

Maeda H. Mohammad^{1*}  Aous Kahtan Almzaïen¹  Ahmad A. Al-Joubory²
Ahmed M. Al-Shammari¹  Ayser A. Ahmed¹ Hiba K. Shaker¹ Aseel K. Abedalsattar¹

¹Experimental Therapy Department, Iraqi Center for Cancer and Medical Genetics Research, Al-Mustansiriyah University, Baghdad, Iraq.

²Neuroscience Hospital, Baghdad, Iraq.

*Corresponding Author: maeda.mohammad@iccmgr.org

E-mail addresses: aous.kahtan@iccmgr.org, Ahmed.aljuboori@iccmgr.org, ahmed.alshammari@iccmgr.org, aysarayed78@gmail.com, hebakareem1982@gmail.com, aseelkhalid19799@gmail.com.

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Abstract

Neural stem cells (NSCs) are progenitor cells which have the ability to self-renewal and potential for differentiating into neurons, oligodendrocytes, and astrocytes. The *in vitro* isolation, culturing, identification, cryopreservation were investigated to produce neural stem cells in culture as successful sources for further studies before using it for clinical trials. In this study, mouse bone marrow was the source of neural stem cells. The results of morphological study and immunocytochemistry of isolated cells showed that NSCs can be produced successfully and maintaining their self-renewal and successfully forming neurosphere for multiple passages. The spheres preserved their morphology in culture and cryopreserved to be a ready source for use in experiments as a model for neurological disorders.

Keywords: Cryopreservation, Isolation, Nestin, Neural stem cells, Neurosphere, Stem cells.

Introduction:

Cell therapy refers to biological activates of the cellular material that cause a desired effect in the *in vitro* or *in vivo* or both. For over two decades, there has been a huge expansion variety of many cell types used in neurological disorders in animal models and humans with very interesting results. These results were precursory but important for proofing the grafting cellular tissue as an effective method for the treatment of many neurological disorder¹.

Over two hundred clinical studies applying numerous stem cell approaches to treat neurological disorder are registered thus far (Clinicaltrials.gov), the bulk of that are for multiple sclerosis, stroke and spinal cord injuries².

Different sources of stem cells were used such as umbilical cord, bone marrow, embryos, tissues and many other sources, all these sources contain many different types of cells that promote recovery and reduce injuries in many of animal models and humans such as neurodegenerative disorders,

traumatic brain injury, stroke, and multiple sclerosis³.

In cell therapy, the neural stem cells (NSCs) are very promising source in almost neural and CNS disease. It's very important for the future of cell-based therapy, these cells are reprogrammed by neural stem cell-specific transcriptional factors and should be established to be as identical to those *in vivo*⁴.

Three defining properties about NSCs biology are important: clonality, multipotency, and self-renewal. The neurosphere assay and cell culture tools were the key features of NSCs used since 1992. Also, much improvement has been made in the field of stem cell by demonstrating its actual potential, revisiting the neurosphere concept and limits⁵.

The NSCs result is to produce the central nervous system during the developmental stages, also these cells contribute to the cellular plasticity of the adult mammals brain. Therefore, it's a very useful

source in treatments. Therefore, the aim of this study is the isolation, identification, and cryopreservation of neural stem cells in laboratory from easy source to be ready to use in our Iraqi center for cancer for the next neurological experiments.

Materials and Methods:

This study was done in the Experimental Therapy Department Laboratories / Iraqi Center for Cancer and Medical Genetics Research (ICCMGR) / Al Mustansiriyah University in 2021.

Animals

All animals used in this study were housed at 23-25°C in a very controlled environment with free access to food and water under a 12h light/ dark cycle, they were purchased from the Animal House Unit of ICCMGR / Al Mustansiriyah University (all the work was approved by the ICCMGR animal care and committee). Healthy Swiss Albino mice at age 3-6 weeks old with weights ranging from 10-15 g were used as a source of NSCs production.

Isolation of bone marrow mesenchymal stem cells

A modified protocol was used in this present study⁶. The male mice donor were killed (in clean place) by cervical dislocation and sterilized then transported to the laminar air flow cabinet in the Animal House Unit. In the laminar air flow cabinet (under very sterilize conditions), the donor was washed with 70% alcohol, then removed the femurs and tibias by sterile surgical tools and collected in a tissue culture petri dish containing Minimum Essential medium MEM (US Biological, Massachusetts USA) as transport media that contain MEM medium with fivefold antibiotics streptomycin (Cox Pharmaceutical CO., LTD, UK) as 500 µg /ml, and ampicillin (Kontam Pharmaceuticals CO., LTD, China) as 500 µg /ml. The femurs and tibias were transported to the laminar air flow cabinet in the tissue culture laboratory (under sterile conditions) and the bone end was cut off as close to the end and bone marrow was flushed out from the femur and tibias, then the cell suspensions was cultured into 25-cm² tissue culture flasks in MEM medium with 2% Fetal bovine serum FBS, streptomycin (Cox Pharmaceutical CO., LTD, UK) and ampicillin (Kontam Pharmaceuticals CO., LTD, China) as 100 µg /ml. Finally, the cultures were maintained at 37°C in humidified 95% air and 5% CO₂ incubator.

Maintenance of bone marrow mesenchymal stem cells

After 24 hrs., many isolated cells were adherent, the non-adherent cells were washed out (by changing media) using maintenance media (MEM with 20% FBS). Then the cultures were maintained by exchanging culture medium with a new culture media for each 2-3 day until developing cells colonies (3-7 days). After getting one confluent cells (monolayer cells), the cells were recovered (sub cultured) by using trypsin-EDTA (US Biological, Massachusetts USA) 0.25% and with MEM media (20% FBS) to get a passage one (P1), then the MSCs of passage one (P1) were ready to use in the next experiments⁷.

Stem cells detection.

The MSCs were used in passage one (P 1) for Immunophenotypic analysis (ICC). Tissue culture chamber slide (Eight- well) (IWKA, Japan) and MEM media with 20% FBS was used for cultivating cells. Cells were allowed to develop through 3-5 days in chamber slide, then media was removed and the cells were washed two times with Phosphate Buffer Saline PBS, then the cells were fixed with 4% paraformaldehyde (diluted in PBS) for 10 minutes and the cells were washed with PBS and leaved to dry. The slide was used in this ICC as triplicate.

In the present study, four specific markers were used in the immunocytochemistry analysis: CD90 (1:100; Mouse anti-human antibody, US biological, USA, C2441-06), CD105 (1:100; Mouse anti-human antibody, US biological, USA, C2446-50B), and CD44 (1:100; Rat anti-mouse antibody, US biological, USA, C2398-01T) as positive markers and CD34 (1:100; Goat anti-human antibody, Santa Cruz Biotechnology, SC-7045) as negative markers.

The fixed cells were incubated with 1% hydrogen peroxide (H₂O₂) in a humidified chamber for 10 minutes, then washed 2-3 times with PBS and incubated for 30- 40 minute at room temperature with 1.5% blocking serum (the kit from Immuno Cruz mouse ABC Staining System, SC-2017, Santa Cruz Biotechnology, Europe). Then the primary antibody of each CD markers used in this study was added and incubated in a humidified chamber overnight at 4 °C. After that PBS was washed many times and incubated for 30 minute with biotinylated secondary antibody (anti-mouse for all CD markers except in CD34 anti-goat) then washed with PBS 2-3 times. The horse radish peroxidase (HRP) was conjugated to avidin and added for each slide and incubated for 30 minute in a humidified chamber,

then washed 2-3 times with PBS. liquid DAB chromogen solution mix was added for visualizing the peroxide to each slide at room temperature for 2-5 minutes then washed with PBS for many time and counter stained for 30- 60 second in Hematoxlin stain. Finally, all slides were mounted with DPX and inspected by using light microscope and photographed by using digital camera.

Neural stem cells induction and maintenance

The neural stem cells were induced according to the results of Maeda *et al*⁸. Passage one of MSCs were induced to differentiation in to NSCs by added 1 mM β -Mercaptoethanol (BME) as neural inducer in MEM media with 5% FBS, 50 ng/mL for each basic mouse fibroblast growth factor b-FGF (USBiological, USA) and mouse Epidermal growth factors EGF (USBiological, USA) for 6h exposure time in CO2 incubator to be ready to maintenance in culture.

Neurosphere formation and maintenance

After induction of neural stem cells (as mentioned above), the induced cells were maintained in culture to form the neurosphere (as the main shape of neural stem cells in culture) in MEM media with streptomycin and ampicillin as 100 μ g/ml, and Amphotersine 100 μ g/ml. And supplemented with 5% FBS and 50 ng/mL for each EGF and basic b-FGF. The induced cells were maintained in a small tissue culture petri dish, 96-well flat-bottomed microtitration plate, and tissue culture flasks (25 cm²) for 3 days, 1 week, 2 weeks, and 5 weeks to allow for neurosphere formation.

Detection of neurosphere

After neural stem cells induction, the produced cells (neurosphere) were detected by using Immunophenotypic analysis ICC using Nestin marker (as the neural stem cells main basic marker) with the same protocol that mention above in (stem cells detection) section.

Maintenance and Cryopreservation

The neural stem cells were maintained in culture using MEM media with streptomycin and ampicillin as 100 μ g/ml, and Amphotersine 2.5 μ g/ml and supplemented with 5% FBS and 50 ng/mL of EGF and b-FGF (as a maintenance NSCs media). Then the formed neurosphere were cryopreserved by freezing medium according to⁵ in Liquid nitrogen for later use.

Results:

Stem Cells Culture

The MSCs was isolated and cultured in MEM (with 20% FBS). A few cells were attached to the plastic culture flasks after 24h and formed adherent cells, while the other cells (non-adherent cells) were discarded usually after 24 or 48 h by the first medium changing as showed in Fig. 1 A&B. The results showed that the adherent cells began to proliferate through 2-3 days and formed a numerous fibroblasts like-cells, and grow gradually to form a small individual colonies displaying fibroblast-like morphology with short and long processes as well as, a small round cell with active nuclei can also be seen as showed in Fig. 1 C&D. The cells in culture started to proliferate to formed a larger colonies after 5- 6 days, these colonies were gradually expanded in size with adjacent ones interconnected with each other. After that a confluent monolayer was formed and became ready to be passaged to perform first subculture as showed in Fig. 1 E&F. After first subculture (Passage one), the cells began to grow and form colonies that expanded later after few days, a homogeneous confluent monolayer of cells occupied the whole plastic surface as showed in Fig. 1 G&H. Finally, the cells can be reseeded in same conditions for the second passage subculture (Passage two), and be readily to expanded *in vitro* without visible morphologic alteration by successive cycles of trypsinization, seeding and culture every five to seven days.

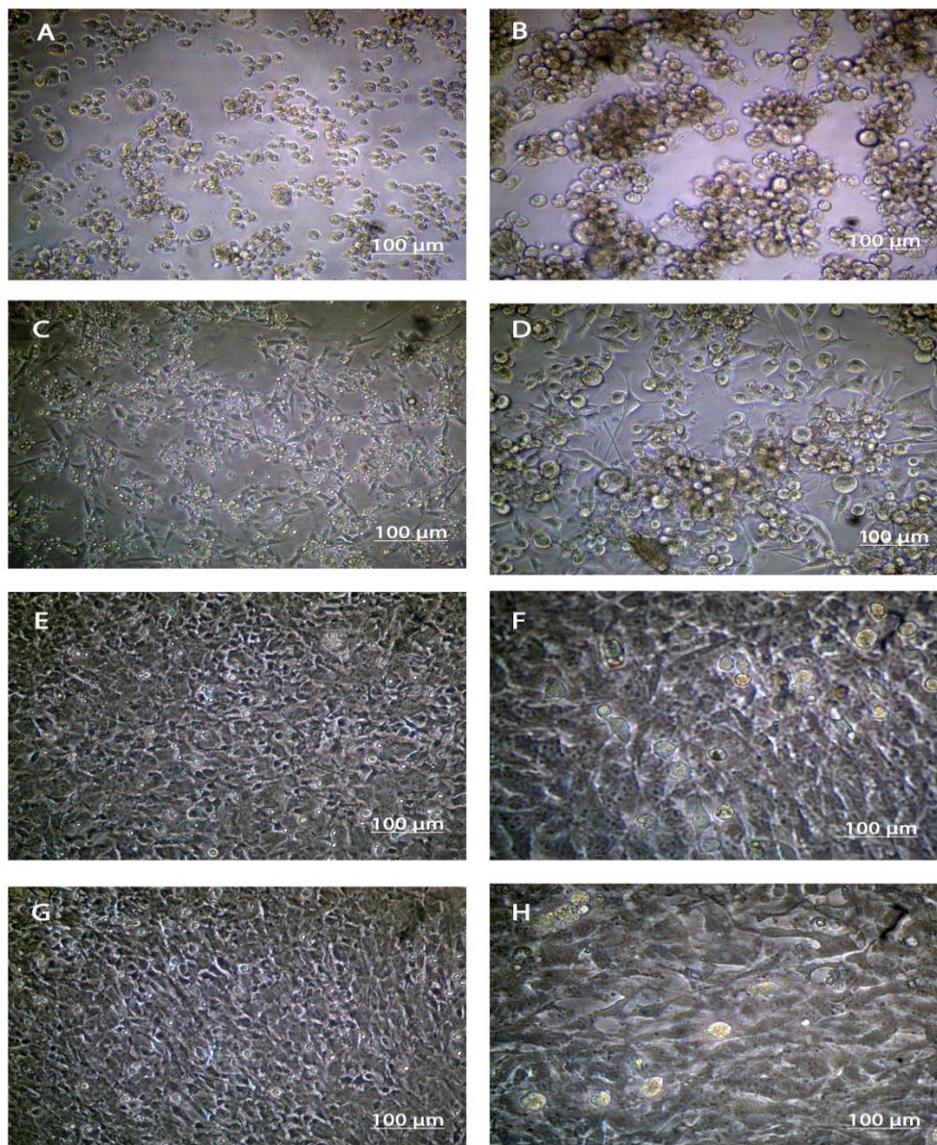


Figure 1. Morphological characterization for isolation of mouse MSCs cultured in MEM +20% FBS as revealed under inverted microscope as 10X and 20X respectively. (A&B) after 24h, (C&D) after 72h, (E&F) a confluent monolayer formed after 5-6 days, (G&H) maintenance of mouse MSCs in culture (after first passage P1).

Stem cells detection.

The MSCs were used in passage one (P 1) for Immunophenotypic analysis (ICC) for each positive markers (CD90 , CD105 , and CD44) and negative markers (CD34). The results showed that CD34 was negative results by cell stained with blue color

(from Hematoxlin stain) as shown in Fig. 2-A&B. While, each CD44, CD90, and DC105 showed a positive results by cell stained with brown color (from DAB stain) as showed in Fig. 3. Therefore, its conformed the origin of isolated cells (from mesenchymal stem cells origin).

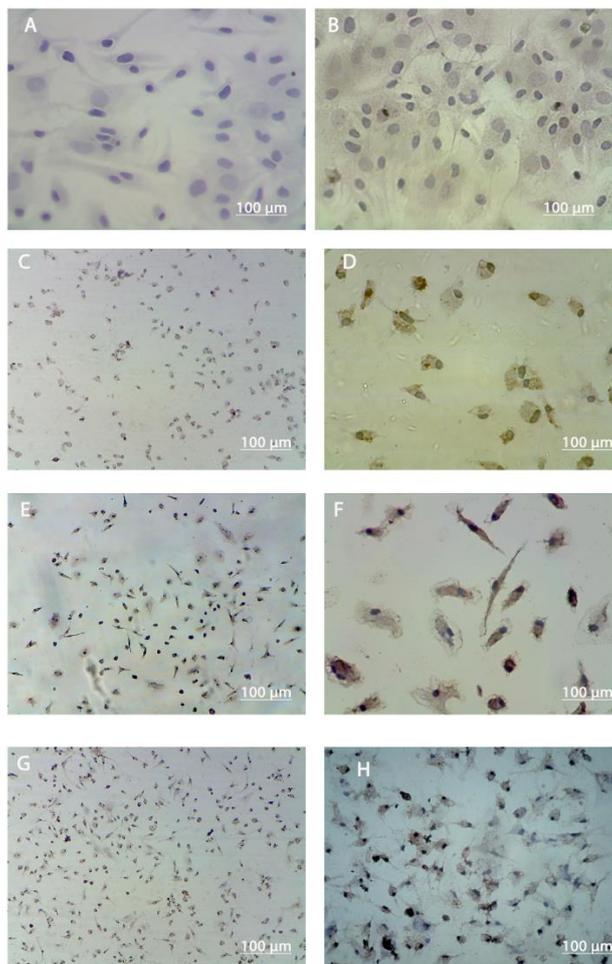


Figure 2. ICC analysis of stem cells at passage one as revealed under light microscope, the results showed that these cells were negative with blue color as negative control as 40X (A), and with blue color for CD34 as 40X (B). While the cells were positive with brown color for CD 44 (C&D), for CD90 (E&F), and CD105 (G&H) as 10X and 40X respectively.

Neural stem cells NSCs induction and maintenance:

After confirming the phenotypic of isolated cells by ICC, the MSCs were induced to differentiation in to neural stem cells NSCs by a specific formula (mentioned in material and methods). The results showed that the induced cells kept their morphological shape with no morphological changing in cells as showed in Fig. 3.

Then these induced cells were maintained in culture. The results showed that these induced cells were successfully maintained in culture for many passages with keeping their morphological shape as shown in Fig. 4.

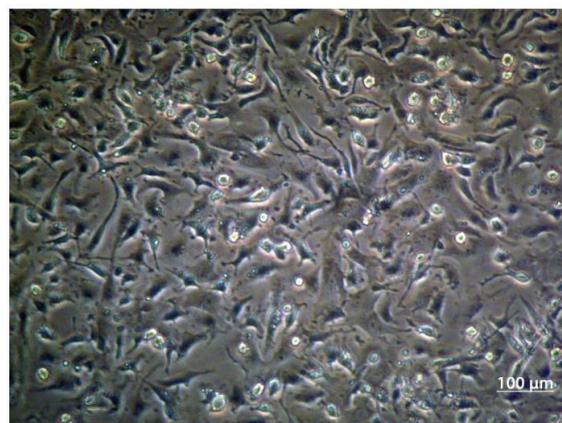


Figure 3. Neural stem cells formation after added the NSCs formula (1 mM BME, 50 ng/mL b-FGF and EGF) as showed under inverted microscope as 10 X.

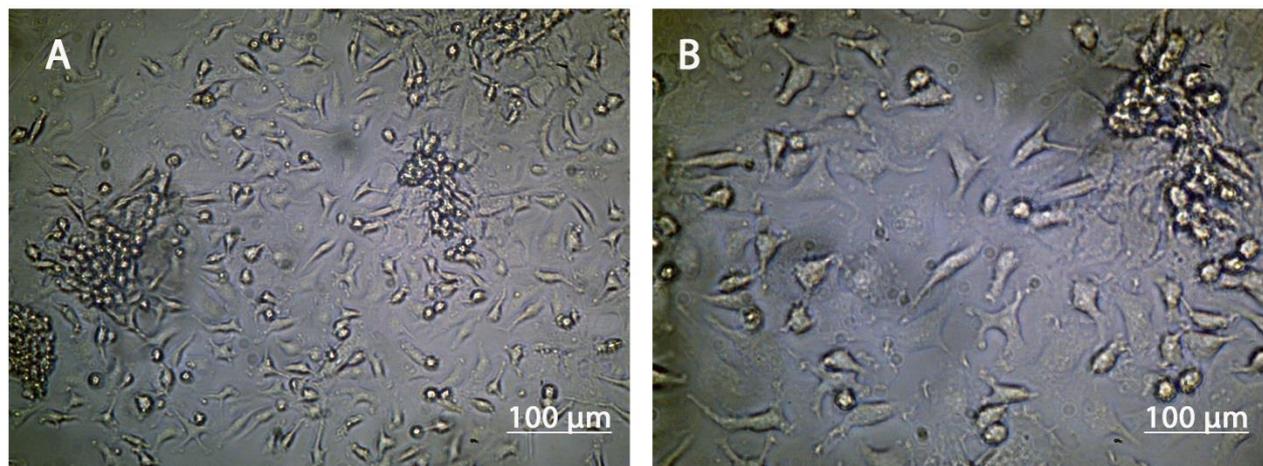


Figure 4. Neural stem cells maintenance in culture by passaging as revealed under inverted microscope (A):10X. (B):20X.

Neurosphere formation and maintenance

During maintaining the induced cells, the results showed that these induced cells started to form aggregations in the culture (after 3 day, 1 week, 2 week) as shown in Fig. 5 A-F, then continue to maintain in culture and cells aggregation until formed a neurosphere after 5 weeks as showed in Fig. 5 G &H.

Therefore, from the results above, the results showed this method was successful to produce neural stem cells and maintain of induced cells in culture. These neurosphere showed with a round cell clusters, small, and clear boundaries, which lose the ability to adhere to the bottom of as shown under inverted microscope as showed in Fig. 5.

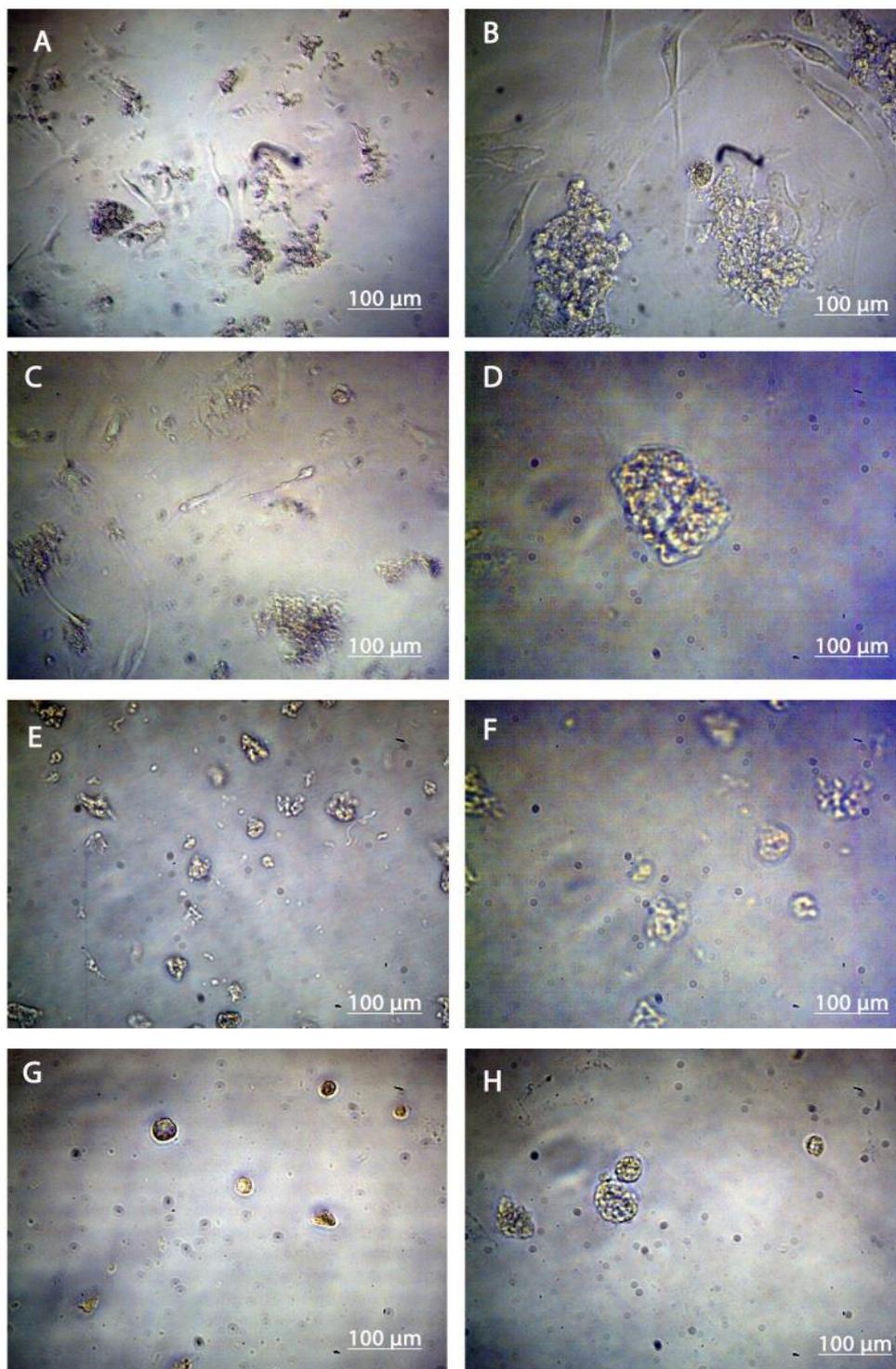


Figure 5. Neural stem cells induction that showed the aggregation of NSCs to formed a neurosphere cells as revealed under inverted microscope as 10X & 20X respectively. (A&B): after 3 days. (C&D): after 1 week (E&F): after 2 weeks as (G&H): after 5 weeks.

Detection of neurosphere

The detection of induced cells by using immunophenotypic analysis ICC using Nestin marker (as the neural stem cells main basic marker) showed that these produced cells (neurosphere)

were neural stem cells with a positive results by cell stained with brown color (from DAB stain) as showed in Fig.6. It has been confirmed that the induced cells were NSCs.

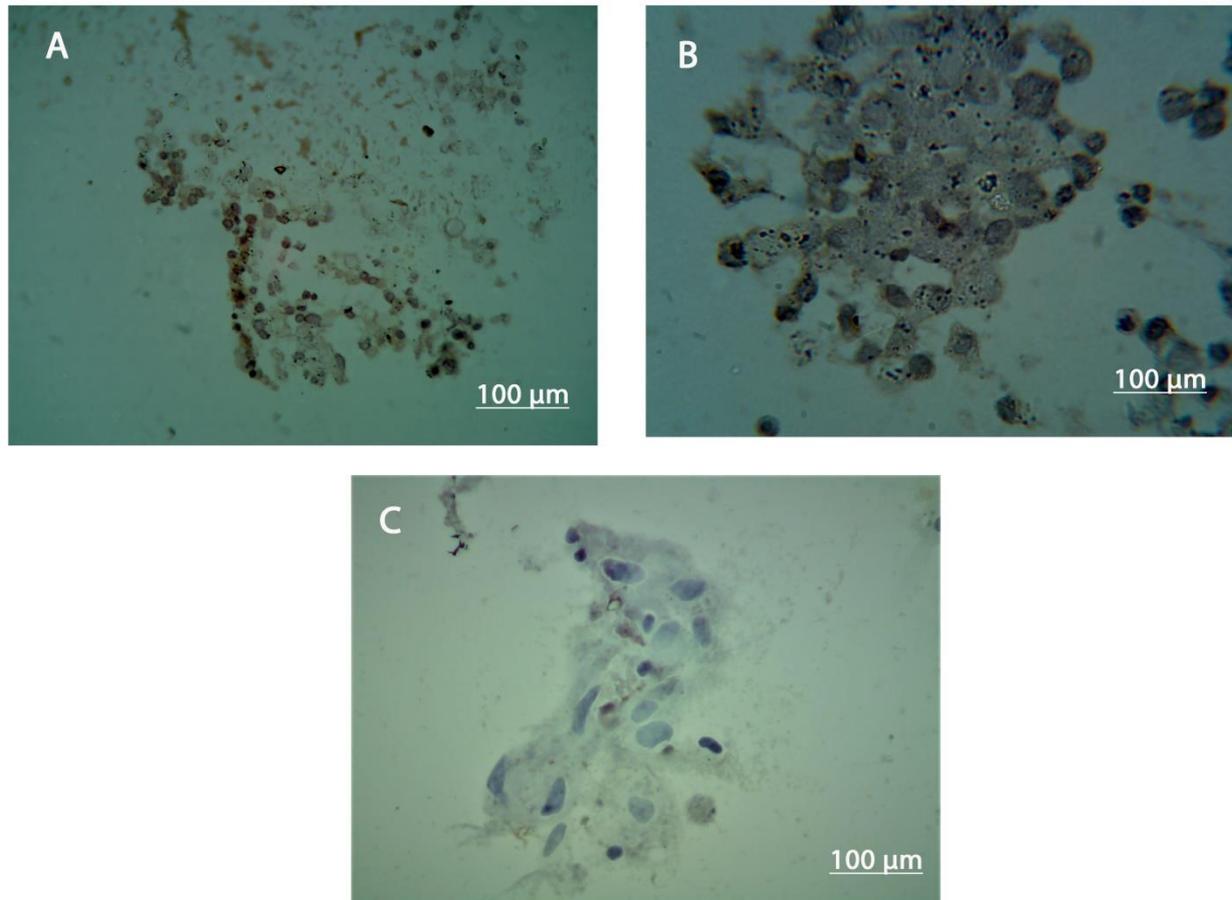


Figure 6. Immunophenotypic analysis of neural stem cells (during neurosphere formation) showed under light microscope that these cells were brown color for Nestin marker (as positive by cell stained) as 10X (A) and 40X (B) compared with blue color for control as 40 X (C).

Maintenance and Cryopreservation of NSCs

After conforming the produced cells by ICC, the results showed that the neural stem cells were successfully maintained in culture with MEM media with streptomycin and ampicillin as 100 µg/ml, and Amphotersine 100 µg/ml supplemented with 5% FBS and 50 ng/mL for and basic b-FGF (as maintenance of neural stem cells media). Then NSCs for all methods mentioned above were successfully cryopreserved in Liquid nitrogen (- 196 °C) to be used later as a ready source for neurological disease.

Discussion

Neural stem cells are a group of ectodermal progenitor cells considered to be uncommitted cells and the most primordial of the nervous system. They give rise to many types of more specialized cells of the peripheral nervous system (PNS) and the central nerves system CNS. The neural stem cells have many characteristics compared to the progenitor cell (i.e., cells have already become

lineage committed to give rise to only one category of neural component, e.g., neurons versus glial cells) that must have the ability to (1) self-renewal (2) able to give rise to cell types in addition to themselves through asymmetric cell division, and (3) the generation or differentiation to all neural lineages (oligodendrocytes, astrocytes, and neurons) over the nervous system^{9,10}.

Neural stem cells in the adult brain remain undifferentiated and capable of division. Various signaling and growth factors are needed to stimulate their proliferation and differentiation toward particular types of neurons, astrocytes, and oligodendrocytes. When needed, neural stem cells were very important in brain cell maintenance¹¹.

The self-renewal characteristic of neural stem cells (NSCs) and their ability to differentiate into different neural types are used in many scientific studies and clinical applications. Culturing, maintenance, and cryopreservation for 7 days, or 1, 6 or 12 months of neural stem cells *in vitro* is very

important under controlled environmental conditions for characterization of their properties that may be modified and monitored accurately^{12,13}.

Beta Mercaptoethanol BME is a successful source to differentiate stem cells into neural stem cells from adult stem cells (from mouse bone marrow) with an easy way and with the ability to autologous transplantation from this source^{8, 14}.

In this study, we have been successful in the isolation, identification, and cryopreservation of neural stem cells in laboratory from an easy source from bone marrow mesenchymal stem cells compared with many isolation methods derived from the adult mouse brain^{15,16} or even embryonic cerebral cortices source¹⁵ to be ready to use it in the next clinical neurological experiments.

Conclusions:

This protocol described in this study was success to isolation, identification, and cryopreservation of neural stem cells in laboratory from easy source from bone marrow mesenchymal stem cells compared with the original brain tissue source to be ready to use in our Iraqi center for cancer and medical genetics research for the next neurological experiments.

Authors' declaration:

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for re-publication attached with the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee in Al-Mustansiriyah University. The authors signed the Animal Welfare Statement (no. 1/ paragraph 3 on January 25, 2021).

Author Contributions:

All contributions of this study was done by the members of experimental therapy department involved in the research. Maeda H.M in conception, design, acquisition of data, analysis, interpretation, drafting the MS, revision and proofreading, Aous K.A. in acquisition of data, analysis interpretation, drafting the MS, revision and proofreading , Ahmad A.A. in conception, design, acquisition of data, drafting the MS, revision, Ahmed M.A. in conception, design, acquisition of data, analysis, interpretation, drafting the MS, revision and proofreading, Ayser A.A. in acquisition of data,

Hiba K.S. in acquisition of data, Aseel KA. in acquisition of data.

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عزل وتنمية الخلايا الجذعية العصبية في المختبر

مانده حسين محمد¹ أوس قحطان المزين¹ احمد عدنان الجبوري² احمد مجيد الشمري¹
أيسر عايد احمد¹ هبة كريم شاكر¹ اسيل خالد عبد الستار¹

¹قسم العلاج التجريبي، المركز العراقي لبحوث السرطان والوراثة الطبية، الجامعة المستنصرية، العراق.
²مستشفى العلوم العصبية، بغداد، العراق.

الخلاصة:

الخلايا الجذعية العصبية (NSCs) هي خلايا مولدة لديها القدرة على التجديد الذاتي والقدرة على التمايز إلى خلايا عصبية نجمية astrocytes ، وخلايا عصبية قليلة التفرع oligodendrocyte ، و خلايا عصبية neuron . في هذه الدراسة تم عزل الخلايا الجذعية في المختبر ، وتنميتها في المختبر ، وتحديد هويتها ، ومن ثم إنتاج الخلايا الجذعية العصبية وحفظها بالنيروجين السائل كمصدر خلايا ناجح لاستخدامها لاحقاً في التجارب العلاجية. تم استخدام نخاع عظم الفأر كمصدر للخلايا الجذعية العصبية في هذه الدراسة. أظهرت نتائج دراسة الشكل المظهري والكيمياء المناعية للخلايا المعزولة أنه يمكننا إنتاج الخلايا الجذعية العصبية NSCs بنجاح والحفاظ على تجديدها الذاتي وتشكيلها بنجاح في الغلاف العصبي ولتعزيزات متعددة. تم الحفاظ على الشكل المظهري للخلايا الناتجة وتخزينها في النيروجين السائل لتكون مصدراً جاهزاً للاستخدام في التجارب كنموذج لعلاج الاضطرابات العصبية.

الكلمات المفتاحية: حفظ الخلايا بالتجميد ، العزل، النسجين، الخلايا الجذعية العصبية، الغلاف العصب، الخلايا الجذعية.