Morphological and Growth Properties of Albino Rat Bone Marrow Stromal Cells *In Vitro*

I. M.Mnati and B.H. Mutlak
Deparatment of Biology, College of Education , Ibn Al- Haitham , University of Baghdad

Abstract

The potential use of bone marrow stromal cells as a cellular therapy for chronic diseases relies on the ability of the cell to replicate extensively *in vitro*.For this reason the present study investigated the replication lifespan and examined the growth properties of albino rats mesenchymal stem cells(MSC_S)*in vitro*.

To establish an *in vitro* system for isolating and culturing the MSCs of albino rats and to provide research data for its further application,the bone marrow (BM)was collected from young male rats and separated by gradient centrifugation.Then, the mononuclear cells(MNCs) were retrieved from the buffy layer and cultured in Modified Eagle's Medium (MEM) supplemented with 10%Fetal Calf Serum (FCS)and incubation at $37C^{o}$ in humid air with $5\%CO_2$.

The non-MSCs were screened out.A passage culture was undertaken while the monolayer of adherent cells formed .The spindle –shaped MSC_S with a single nucleus formed a monolayer after the 10-12 days of primary culturing,and the cells appeared in an oriented array with a swirling growth trend.

In the anaphase of passage culture ,the cell proliferation rate was decreased and the morphology changed into broad flat appearance.These results suggested that MSC_S of the albino rats can be passaged *in vitro* with the established optimized culture system,in addition, the growth curves can be divided into three phases:the initial quiescent phase,the logarithmic replication phase and after logarithmic replication phase .

Introduction

Bone marrow is the major source of stromal cells and adult hematopoietic stem cells (HSC_S) that renew circulating blood elements;these cells can also be found in other tissues .Adult BM also contains MSCs. MSCs are thought to be multipotent cells that can replicate as undifferentiated cells and have the potential to differentiate to lineages of mesenchymal tissues,including :bone ,cartilage muscle, nerves and marrow stroma [1,2].Considering their advantages ,such as ease of obtaining by a simple routine BM a spiration ,ability to self –renew and recently reported potential to differentiate into cardiomyogenic cells, MSCs have been considered as one of the most promising candidates for this purpose and the apparent multipotent nature of MSCs makes them excellent candidates for tissue engineering [3].

The MSCs were first described in 1970 by Friedenstein *et al.*, [4]who discovered that the cells adherent to tissue culture plates resembled fibroblasts *in vitro* and formed colonies. He and others demonstrated MSCs could differentiate into multiple pathways that include cardiac myogenesis[5,6].Among the most extensively studied of adult stem cells from BM, if plated at low densities, MSCs generate cell single derived colonies and the colonies can be differentiated into several cell phenotypes in culture.These characteristics have been identified from numerous species including humans, rats ,mice, and monkeys [7,8].

Interest with BMSCs has begun since 1960 and since those several techniques have been developed to proliferate these cells and then to differentiate them into the required cells to benefit from them in the basic and clinical researches.Recent attention has focused on BM as a

source of stem cells for transplantation into the heart.At present, the BM transplantation are normal operation which are used for treatment of many diseases [9].The use of MSCs in such therapeutic strategies relies on the ability of the cell to replicate extensively *in vitro* .For this reason the present study systematically examined the growth properties of rat MSCs *in vitro* and we report here the isolation ,expansion and characterization of the multipotent rat MSCs.

Materials and Methods

- Isolation of MSCs from albino rats bone marrow

This study comprised 30 young male rats(*Rattus rattus norvegicus albinos)* aged (180-200g) were used for the isolation of MSCs from the BM and were culturing *in vitro*, ,these animals were obtained from the Medical Research Unit College of Medicine in Al-Nahrain University. The primary culture of MSCs were performed according to Dodson [10].In brief,after rats were killed with an of diethyl ether,the femoral and tibial bones were collected for isolation of MSCs. The marrow pellet was washed in phosphate buffer saline(PBS),centrifuged at 2000 rpm for 10 min and then resuspended in Modified Eagle's Medium(MEM) .The cell suspension was loaded on 60% percoll and then centrifuged in a cooling centrifuge for 20 min at 2000 rpm at $4C^{o}$.After centrifugation, the mononuclear cells (MNCs)was removed from the interface and resuspended in MEM. MSCs were plated at concentration of $1X10^{6}$ cells/ml in 50 cm^{2} plastic tissue culture flask.

-Culture and expansion

Following plating ,MSCs were maintained in a humidified incubator at $37C^{o}/5\%CO^{2}$ in MEM +10%FCS . The medium was changed after 24 hours to remove nonadherent cells and twice weekly thereafter. Culture MSCs were observed under inverted microscope to assess the level of expansion and to verify the morphology at each culture medium change.

To prevent the MSCs from differentiating ,each primary culture was replated for subculture. Cells were passaged with 2 ml of 0.25% trypsin- 0.1%Ethylene diamine tetra acetic acid (EDTA) upon reaching 70-80% confluency and expanded until passage 3,where upon they were analyzed.

-Quantification of MSCs growth

To determine the initial density of MSCs in primary cultures,the adherent cells were detached with 0.25% trypsin- 0.1% EDTA and stained with 0.2% trypan blue .Triplicate samples from each well of 4-well plastic plates were resuspended in 2ml of PBS and count with a hemocytometer under light microscope [11].

The average cell number calculated from four wells of each of three plates ($1X10^{4}$ cells/well ; n=12) was taken as the initial density of MSCs in primary cultures and the seeding density for every following subcultures .This cells counting procedure was repeated every 48 h until the cells almost ceased proliferation.The growth curves and the precise growth properties of MSCs in the primary cultures (P0)and the three subcultures (P1-P3) were determined .The numbers of cell doublings (NCD) were calculated with the formula : $NCD= \log_{10} (Y / X) / \log_{10} 2$

where X represents the initial seeding density of the cells and Y the end of the logarithmic replication phase in every passage .

Results

- Rat MSCs primary culture

The bone marrow isolates were removed by changing the medium at 24 h . In the first hours of culturing ,the adherent cells grew in a round shape with few morphological changes.Under an inverted microscope, morphological conservation was observed , the results showed that the parental MSCs were characterized by an enlarging appearance.After three days of primary culture,the MSCs were observed attach to the culture flask sparsely and these cells began to proliferate in culture medium(Fig.1 A).

At day 7,spindle shape was observed in well- spread colony –forming cells.Subsequently ,most adherent cells grew in a spindle shape characteristic of fibroblasts (Fig.1 B). By day 10,the MSCs was duplicating rapidly and the cell morphology was mainly spindle-shaped(Fig.1C). Observation on day 10-12 revealed that the cell monolayer was forming with constant orientation and a whirling tendency , which means the cells have the potency to be passaged in ratio 1: 2 .

- Rat MSCs passage culture

Passaged MSCs behaved similarly to those in primary cultures. The MSCs in subcultures could be divided into two types: small spindle- like cells and broad flattened cells.The flattened cells seldom proliferated and were gradually surrounded by the small –like cells that replicated faster and were more inclined to form colonies.It seemed that the spindle-like cells gradually transformed into broad flattened cells with later passages.P1 of the mesenchymal cells was characterized by the ability to proliferate in culture with an attached well-spread morphology, reaching confluence on day 6-8.In this passage,the cells continued to duplicate rapidly (Fig.2 A). Microscopic observations showed that P2 ,cells remain fast growing and reach70-80%confluence by day 6 (Fig.2 B). However the cell proliferation rate decreased in P3 compared with the earlier passages,the cell proliferation obviously slowed down on day 6.In this case,a few typical spindle cells can be observed under microscope and more flat cells were found in the field (Fig.2 C). In this study, the criterion for passaging was noticed a half of colonies reached 70-80% confluence, usually 10-12 days after seeding for primary culture and 6-8 days after subculture.

-Growth properties of MSCs in culture

The growth curves of MSCs in primary culture and in the three passages are shown in (Fig.3).For primary cultures, the cells remained quiescent during the first two days of culture and then quickly replicated until day 10 when the average cell number reached $13.4X10^4$ cells/well, the number of cells did not increase during the following five days of culture 10-14 days.The patterns of the growth curves of MSCs in P1 and P2 were similar to those of primary cells.However, the cells showed a shortened quiescent period 1-2 days before proliferation and the cells number kept increasing for a shorter period 4-6 days in P1 and P2.The increase of average cell numbers was considerably slowed in P3 and it is expected the cells almost ceased proliferation in later passages.

To determine the changes in precise growth properties of MSCs with passages, the growth course of MSCs in the primary culture and in each successive subcultures was divided into three phases according to their growth curves: the initial quiescent phase,the logarithmic replication phase,and after logarithmic replication phase.Rat MSCs(rMSCs) could be expanded up to 15.35cell doublings of successive subcultures.The results of this assay are summarized in Table 1,which show the number of cell doublings(NCD) from P0-P3,and the accumulative NCD with passages.

Discussion

Mesenchymal stem cells are thought to be multipotent cells,which are present in adult marrow.They can replicate as undifferentiated cells and have the potential to be induced to

differentiate exclusively into chondrocytic or osteocytic lineages[8].This study has important limitations which are represented by the use of young animals as the source of BM-derived MSCs. Our data is in line with the study made by Tomita *et al*., [12] who found that BM of young and growing pigs may have contained progenitor cells than would be found in older animals.Primary cell cultures from young animals grow more rapidly than do cells obtained from older animals.

However,the expandability of MSCs *in vitro* varied dramatically among different species and different methodologies for isolation and plating of the cells.It has been reported that murine MSCs were much more difficult to grow than other species[6] and this was probably due to the higher sensitivity of the murine MSCs to the initial seeding density[13].Here,the present study showed that at seeding density of $1X10^4$cells/well,rat MSCs could be expanded to 10.4 cell doublings after 17 days of culture(P0-P1)and up to a maximum 15.35 cell doublings in 30 days,this resembles the reported *in vitro* lifespan of human MSCs[14].

Multiple factors,such as medium ,serum selection ,seeding ratio and micro-environment (pH and temperature)can affect the cell yield and passage.In our experience, MSCs can be isolated by their adherence properties to the plastic surfaces of culture flasks,displaying fibroblast-like growth characteristics and increased proliferation[15].After two days of culture,MSCs appeared sparsely and began to proliferate in culture medium, and in the end of the second week they formed the monolayer of adherent cells .These results correspond to other studies results [11,16 ,17] who have arrived at regarding forming the monolayer of primary culture approximately in the second week of culturing. In our pilot experiments,it was found that if the cells were grown over two weeks before subcultures,they quickly lost the ability to proliferate within 1-2 passages. It is known that proliferation of cultured cells is usually promoted when cells begin to release their own cytokines and inhibited by cell-to-cell contacts upon confluency.Although a more frequent subculture can delay the senescence of MSCs,they gradually lost the ability to proliferate with the increasing of cell doublings,and this was accompanied by a gradual morphological conversion of the small spindle-like MSCs into the broad flattened cells,together with the evidence from several other laboratories [11,14].

Because the amount of MSCs is too low (0.001-0.01%) in adult BM(1),the best way to obtain sufficient MSCs is in expanding in culture.The growth kinetics of these cell populations revealed rapid adherence through to P2,but the cell proliferation rate decreased in P3 compared with the earlier passages, which was similar to[11,17].

According to these results, it appeared that the nature of rMSCs have several properties as follows:-

1. MSCs are more sensitive to plating density.
2. They expand more rapidly after low-density plating.
3. They require frequent culture medium changing.
4. They form confluent cultures after low-density plating.

From the results of this study,it was concluded that,the BM-derived MSCs of albino rats can be isolated,proliferated and maintained them in active state for several weeks and examined the growth properties of MSCs *in vitro*.

References

1-Minguell,J.;Erices,A.andConget,P.(2001).Exp.Biol.Med.,226:507-520.
2-Pittenger, M.F. and Martin, B.J. (2004). Circ. Res., 95:9.
3-Makino,S.;Fukuda, K;Pan, and Ogawa,S.(1999).J. Clin. Invest., 103(5): 697-705.
4-Friedenstein,A.J.; Deriglasova, U.F. and Kulagina, N.N. (1974). Exp. Hematol., 2: 83-92

5-Piersma, A.H.; Ploemacher, R.E. and Brockbank, K.G.M. (1983). J. Haematol., 54(2): 285-290.
6- Clark, B. and Keating, A. (1995). Ann. N. Y. Acad. Sci.,770: 70-78.
7-Hai-jing, S.H.I. ;Zheng, Z.H.U.; Long-ding,L.I.U. and Yun-zhang,H.U. (2007).Zool.Res., 28(2):213-216.
8- Prockop, D.J. (1997). Sci., 276: 71-74.
9-Nilsson, S.K.; Simmons, P.J. and Bertoncello, I. (2006). Exp. Hematol., 34: 123-129.
10-Dodson, S.A. (2000). Cell suspension comparisons of bone production in bone marrow stem cells of young and adult aged rats as measured *in vitro* by bone colony assays .Thesis.www.termpaperpower.com./ stem-cell-thesis.
11-Liu,Y.;Song,J.;Liu,W.;Wan,Y.;Chen,X.andHu,C.(2003).Cardiovasc. Res.., 58: 460-468.
12- Tomita,S.;.; Weisel, R.D.; Jia, Z.Q.; Tumiati, L.C.; Allidina ,Y. ; and Li,R.K. (2002). J. Thorac. Cardiovasc. Surg., 123:1132-40.
13- Javazon, E.H.; Colter, D.C.; Schwarz, E.J. and Prockop, D.J. (2001). Stem Cells, 19 (3): 219-225.
14-Di Girolamo, C.M.; Stokes, D.; Colter, D.; Phinney, D.G.; Class, R. and Prockop, D.J. (1999). Br. J. Haematol., 107(2): 275-281.
15-Friedenstein,A.J.;Gorskaja,U.andKalugina,N.N.(1976).Exp.Hematol., 4: 267-274.
16- Xu, W.; Qian, H.; Zhu, W.; Sun, X.; Hu, J.; Zhou, H. and Chen, Y. (2004). Exp. Biol. Med., 229: 623-631.
17- Mnati, I. M. (2007).*In vivo* and *in vitro* studies of adult bone marrow stem cells and its role in induced myocardial infarction in albino rats. Ph .D. Thesis,College of Education/Ibn-Al- Haitham, Baghdad University: 105-111p.

Table (1): Growth properties of MSC_s in primary and passaged cultures

Passage number	Intitial quiescent phase(x)	Logarithmic replication phase (y)	Numbers of cell doubling(NCD)	Accumulative NCD
P0	$1X10^4$	$13.4X10^4$	6.7	6.7
P1	$1X10^4$	$7.4X10^4$	3.7	10.4
P2	$1X10^4$	$6.5 X10^4$	3.25	13.65
P3	$1X10^4$	$3.4 X10^4$	1.7	15.35

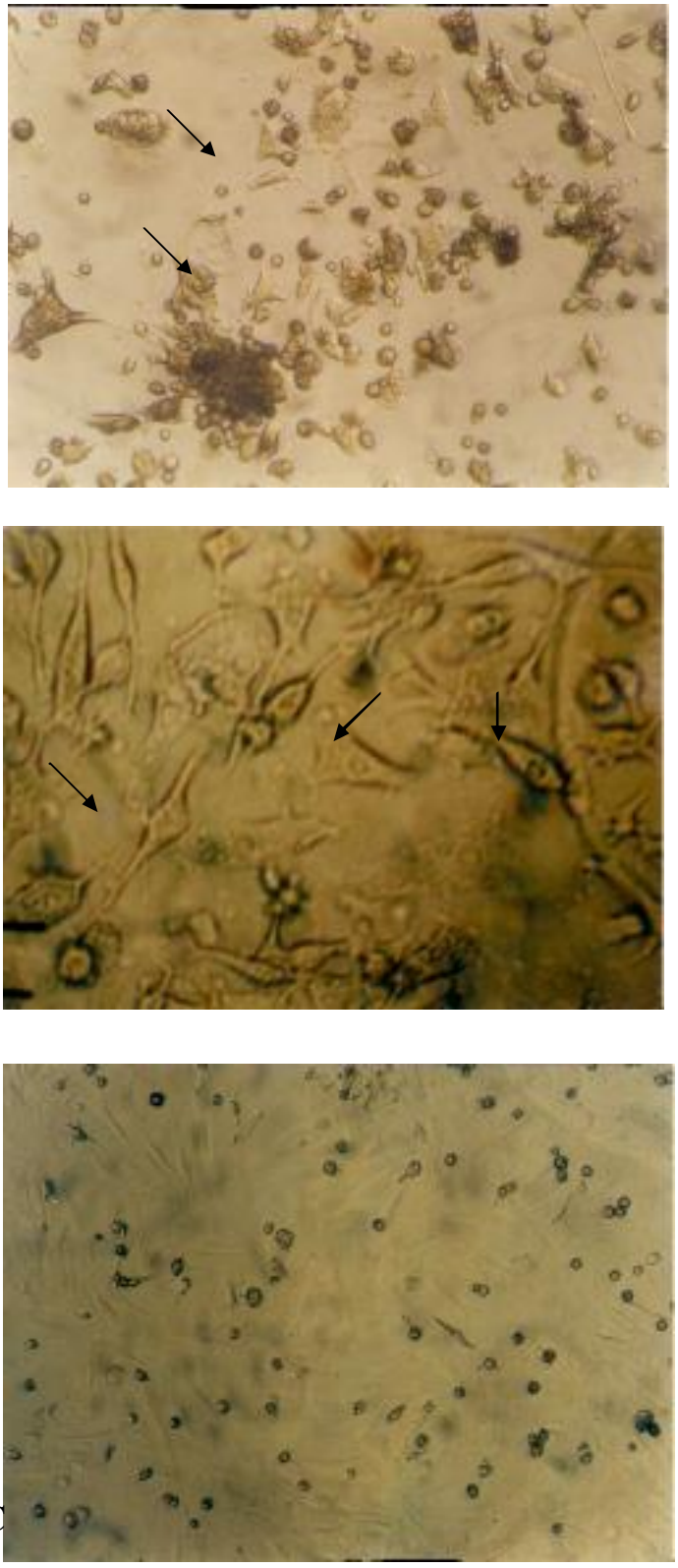

Fig.(1): Morphology of Rat MSCs primary culture.(A):The cells after three days in culture,the MSCs began to proliferate in culture medium(arrows)(X63).(B): The cells at seven days in culture,the MSCs were appeared a spindle-like shape(arrows)(X160). (C): The cells after ten days of culture,the cells formed a monolayer of adherent cells (X63)

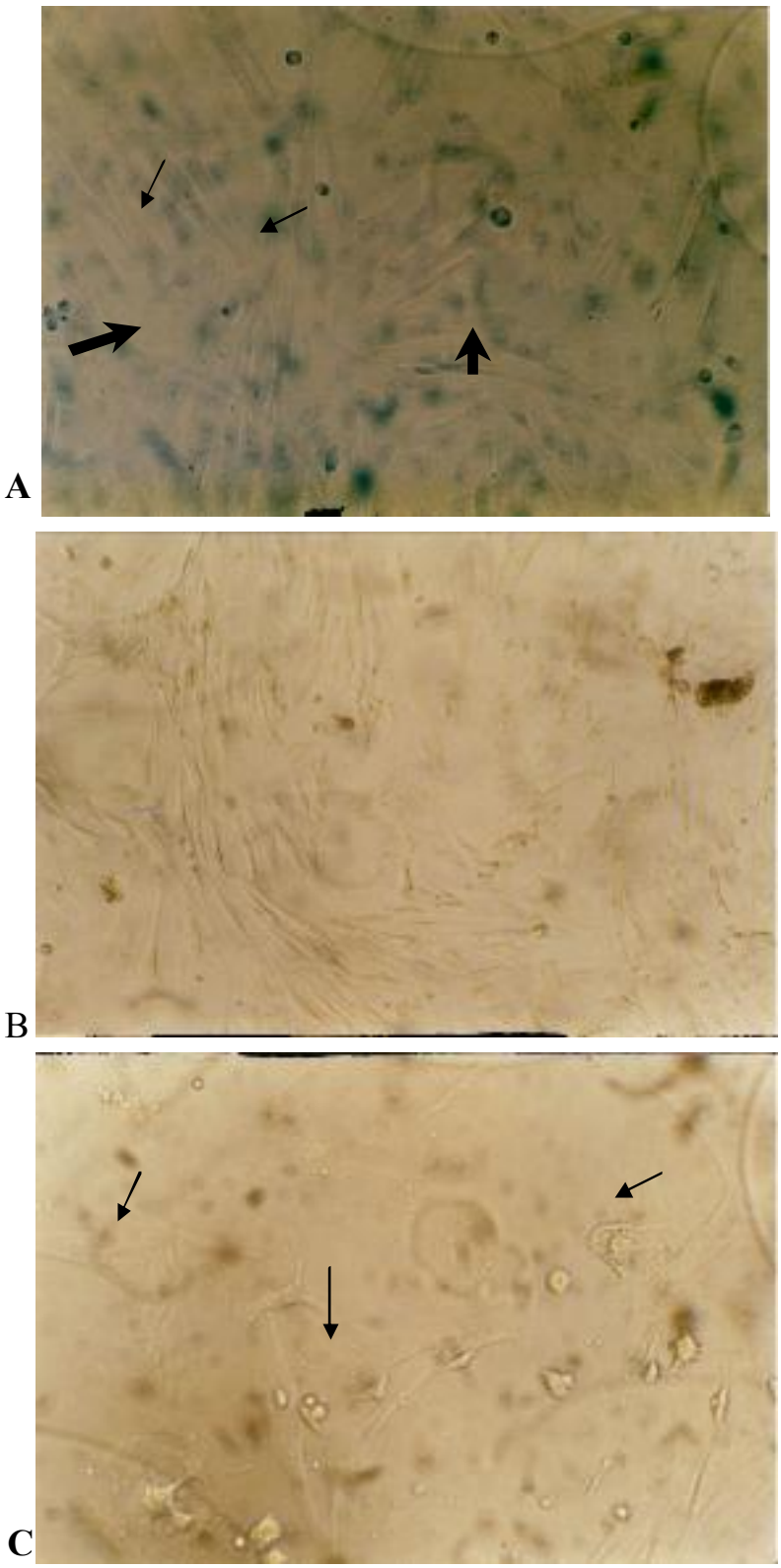

Fig.(2): Morphology of Rat MSCs passage culture.(A):The cells from P1 of culturing, the flattened cells (thick arrows) surrounded by number of spindle cells(thin arrows). (X100.8).(B):The cells from P2 of culturing,the cells showed that continued to duplicate rapidly (X160).(C): The cells from P3 of culturing,the cells proliferation rate was decreased and the morphology changed into flat appearance (arrows)(X160)

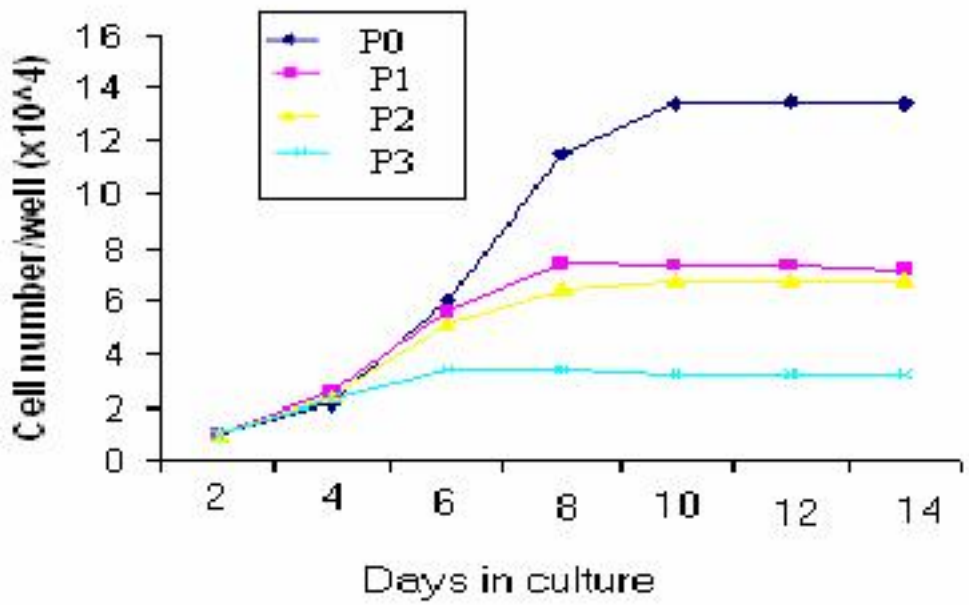

Fig .(3): Growth curves for MSC_s in primary culture (P0) and three successive subcultures (P1,P2,P3).

الخصائص المظهرية والنمو لخلايا سدى نقي العظم للجرذان البيض خارج الجسم الحي

انتظار محمد مناتي وبيداء حسين مطلك

قسم علوم الحياة ،كلية التربية-ابن الهيثم ،جامعة بغداد

الخلاصة

ان الامكانيات المحتملة لاستخدام خلايا سدى نقي العظم علاج خلوي للامراض المزمنة يعتمد على قدرة تكرار الخلايا بصورة كبيرة خارج الجسم الحي ،ولهذا السبب فان الدراسة الحالية بحثت تكرارالخلايا لمدى الحياة وفحصت خصائص النمو للخلايا الجذعية اللحمية للجرذان البيض خارج الجسم الحي .

ولغرض انشاء نظام لعزل وزراعة الخلايا الجذعية اللحمية للجرذان البيض خارج الجسم الحي ولتقييم البيانات البحثية لتكاثرها اللاحق، فقد جمع نقي العظم من ذكور الجرذان اليافعة وفصل بأستخدام النبذ المتدرج الكثافة لطرد معظم خلايا الدم ،وبعد عملية النبذ المتدرج الكثافة سحبت الخلايا الاحادية النواة من الطبقة الضبابية وزرعت في وسط زرعي MEM مضاف اليه 10% مصل جنين العجل وحضنت المزارع بدرجة حرارية 37م^0 وفي جو رطب مع 5%غاز ثاني اوكسيد الكربون .

عزلت الخلايا الجذعية غير اللحمية بالاستبدال المتكرر للوسط الزرعي ،وتم القيام بالزرع الثانوي في الوقت الذي تكونت فيه طبقة احادية من الخلايا الملتصقة .كونت الخلايا الجذعية اللحمية المغزلية الشكل الاحادية النواة طبقة احادية من الخلايا بعد مرور 10-12 يوما" من الزرع الابتدائي، وقد ظهرت الخلايا على شكل اشعة موجه بأتجاه نمو دوراني.وفي الاطوار النهائية للزرع الثانوي فان معدل تكاثر الخلايا بدأ بالانخفاض وتغير الشكل المظهري الى مظهر مسطح عريض ،وهذه النتائج تقترح بان الخلايا الجذعية اللحمية يمكن تكاثرها خارج الجسم الحي بتكوين نظام زرعي بشكل امثل ،يضاف الى ذلك فان منحنى النمو يمكن تقسيمه على ثلاثة اطوار :طور السكون الابتدائي، وطورالتكرار اللوغارتمي وطورالتكرارما بعد اللوغارتمي .