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Assess The Pluripotency of Caprine Umbilical Cord Wharton's Jelly Mesenchymal Cells By RT-PCR Analysis of Early Transcription Factor Nanog

Homayoon Babaei [1], DVSc
Mojgan Moshrefi [2], DVM
Mehdi Golchin [3], PhD
Seyed Noureddin Nematollahi-Mahani [4*], PhD

[1] Developmental Biology and Regenerative Medicine Research Nucleus, [2] Graduated Student, and [3] Department of Pathobiology, Faculty of Veterinary Medicine, Shahid Bahonar University of Kerman, Kerman, Iran,
[4] Neuroscience Research Center, Kerman University of Medical Sciences, Kerman, Iran.

Abstract

Objective- In the present study we investigated the isolation protocol, population doubling time (PDT) and the expression of a pluripotential gene by RT-PCR analysis of early transcription factor Nanog in caprine umbilical cord (CUC) Wharton's jelly mesenchymal cells ($WJMC_s$).
Design- Experimental in vitro study.
Animals- Four mix breed goat.
Procedures- CUC_s were collected from abattoir pregnant uteri and their Wharton's jellies (WJ_s) were cut into 2×2 mm^2 segments for explanting. 8-10 segments were explanted into each 35 mm culture dish. WJ explants were removed 5 days after plating and the remaining adherent cells were cultured for another 5 days. Isolated cells were histochemically assayed for the presence of alkaline phosphatase (AP) activity. RT-PCR was used to assess the presence of Nanog mRNA. Besides, in this study the growth kinetic was evaluated for the isolated cells.
Results- CUC isolated cells displayed spindle-form and small round-shape with high nucleus. Confluent cells formed colonies that indicated AP activity. Initial seeding concentration of 2×10^4 CUC-$WJMC_s$ resulted in shorter PDT compared to fetal fibroblasts (46.57 vs. 54.29 hours, respectively). Expression of Nanog was undetectable in 9th passage of CUC-$WJMC_s$.

* **Corresponding author:**
Seyed Noureddin Nematollahi-Mahani, PhD
Neuroscience Research Center, Kerman University of Medical Sciences, Kerman, Iran.
E-mail address: nnematollahi@kmu.ac.ir

Conclusion and Clinical Relevance- CUC contains an easily obtainable source of mesenchymal cells which exhibit stem cells properties but probably these cells are not pluripotent.
Key Words- Wharton's Jelly; Mesenchymal Cells; Alkaline Phosphatase; Growth Kinetic; Nanog.

Introduction

The umbilical cord is an extraembryonic formation essential to provide feeding for the fetus during the intrauterine development. It is formed early during gestation and encloses the yolk sac, which is the embryonic source of two different kinds of mesenchymal stem cells. This structure also contains mesenchymal stem cells or unrestricted somatic stem cells isolated from fresh umbilical cord blood at the time of birth[1] and fibroblastoid mesenchymal stem cells isolated from umbilical cord matrix[2] which can be collected and stored after birth for therapeutic uses or biotechnology purposes.

WJ is the gelatinous connective tissue from umbilical cord and it is composed of myofibroblast-like stromal cells, collagen fibers and proteoglycans.[3] Previous studies have shown that mesenchymal cells derived from human[2] or porcine umbilical cord matrix are capable of expressing a variety of stem cells characteristics.[4] The stem cells in WJ of the umbilical cord have properties that make them of interest. For example, they are simple to harvest through non-invasive methods, provide large numbers of cells without risk to the donor, the stem cell population may be expanded in vitro, cryogenically stored, thawed, genetically manipulated and differentiated in vitro.[2,4-6] Recently it has been demonstrated that porcine umbilical cord mesenchymal cells resembled pluripotent cells, since they express early transcription factors Oct-4, Sox-2 and Nanog.[4] Nanog is a newly identified homebox transcription factor and is transcribed specifically in pluripotential cells.[7] The loss of Nanog in epiblasts of embryos soon after implantation and differentiation in Nanog-deficient embryonic stem cells[7] and also Nanog over expression-dependent clonal expansion of embryonic stem cells via the bypassing of regulation by Oct-4 levels indicate that Nanog is an important regulator for maintaining the capacity of pluripotency and self-renewal in embryonic stem cells.[8] To date no reports have been found about pluripotential competence of CUC-$WJMC_s$. In this report, we investigated Nanog mRNA expression in CUC-$WJMC_s$. Moreover, the isolation protocol and PDT were analyzed via in vitro cell culture of isolated CUC-$WJMC_s$.

Materials and Methods

Materials

All chemicals except those otherwise indicated were purchased from Sigma-Aldrich Company (St. Louis, MO, USA).

Umbilical cord acquisition and Wharton's jelly matrix cells isolation

All experimental protocols were approved by the Research Ethic Committee of the Kerman Neuroscience Research Center. Uteri of pregnant goats (n=4) were collected from abattoir and transported within 2 hours to the Cell Culture Research Laboratory of Afzalipour School of Medicine, Kerman, Iran. Umbilical cords were obtained from the late-gestation fetuses and

placed in sterile phosphate buffer saline (PBS, composition in mM: 140 NaCl; 2 KCl; 1.5 KH_2PO_4; 15 Na_2HPO_4) supplemented with 2 µg/mL amphotericin B (Bristol-Myers Squibb), 200 IU/mL penicillin and 200 µg/mL streptomycin. Umbilical cord segments, 5 cm in length, were cut longitudinally then the umbilical cord arteries and vein were cleared off. The remnant tissue of umbilical cord including WJ was cut into 2×2 mm^2 segments by using small sharp-sharp scissor. 8-10 segments were transferred to each 35 mm disposable Falcon culture dish (Becton Dickinson & Company Franklin lakes) containing 1 mL of cell culture medium (α-MEM; Alpha modification of Minimum Essential Medium Eagle) supplemented with 20% fetal bovine serum (FBS, Gibco), 2 µg/mL amphotericin B, 200 IU/mL penicillin and 200 µg/mL streptomycin and maintained at 37 °C in a humidified atmosphere of 5% CO_2. Adherent WJ_s of CUC were observed 24 hours after plating and their cell culture mediums were reached to 3 mL. WJ_s explants were removed from dish cultures 5 days after plating then the remaining adherent cells were culture for at least another 5 days and the medium was refreshed every 72 hours. Then adhered CUC cells were dissociated with 0.1% trypsin + 1.0 mM EDTA in PBS for 2 min and then FBS was added to stop trypsinization. Cells that had been detached at this time were subcultured in a new 250 mL Falcon flask (Becton Dickinson & Company Franklin lakes) and denoted as passage 1. For long term storage, CUC cells were cryopreserved in a freezing medium consisting of 20% FBS and 10% dimethyl sulfoxide (DMSO) in α-MEM.

Fetal fibroblast cells isolation

As a comparison with CUC cells, fetal fibroblasts were isolated from a 4-5 month old goat fetus collected from slaughterhouse. The surface of uterine horns was thoroughly disinfected with 75% ethanol and fetus was obtained through an incision. About 1 cm^2 of subdermis connective tissue was placed in sterile PBS supplemented with 2 µg/mL amphotericin B, 200 IU/mL penicillin and 200 µg/mL streptomycin. Small pieces of subdermis tissue were explanted in culture dishes with 2 mL culture medium and allowed to proliferate. Explants were removed at day 5 and the proliferating fibroblasts were allowed to reach desired confluency.

Population doubling time

CUC cells collected by trypsinization from 5^{th} passage were diluted to 1×10^4 cells/mL and 35 mm Falcon culture dishes (n=30) were seeded with 2 mL of 1×10^4 cell suspension and incubated for a period of 10 days when culture terminated. In the course of cultivation, culture medium was refreshed every 72 hours. Every 24 hours, three culture dishes were removed from the incubator, and following trypsinization, cell concentration (cells per mL of culture medium) was counted visually by using hemocytometer (Neubauer). Semilog curve of the increase in cell concentration was plotted and PDT was calculated using the following equation:

$PDT = t \log 2/\log(N_t/N_0)$, where N_0 = initial cell number and N_t = cell number at culture period [9]. Fetal fibroblast cells were also cultured and counted parallel to CUC cells.

Alkaline phosphatase assay

CUC cells from 5^{th} passage were grown in a 35 mm culture dish for several days until colony formation and the medium was refreshed every 72 hours. AP activity was detected by using an AP Kit (Sigma-Aldrich Chemie GmbH, Germany, Catalog No. 86-1) according to the manufactures instruction. A red reaction product following 15 min of exposure to alkaline dye

mixture confirmed AP activity. As a positive control, a blood smear from patient with pyogenic leukocytosis was prepared and stained.

RNA extraction and RT-PCR

Total cellular RNA was extracted from lung carcinoma, fetal fibroblast and the 9th passage of CUC-WJMC$_s$ using RNeasy mini kit (Qiagen Inc., Valencia, CA) according to the manufacture protocol. To eliminate genomic DNA contamination, the samples were treated with 2.0 IU of DNase I (Fermentas) for 1-2 µg of template RNA at 37 °C for 30 min followed by inactivation with the addition of 2 µL of 25 mM EDTA at 65 °C for 10 min. Expression of Nanog gene was assessed using OneStep RT-PCR kit (Qiagen Inc., Valencia, CA) and gene specific primers. The Nanog primers (FWD: 5'-CAG GAC AGC CCT GAT TCT TCC-3' and REV: 5'-TTC TGG AAC CAG GTC TTC ACC TG-3'; annealing temperature = 57 °C, expected product length = 248 bp) described in this report were designed based on common regions between the predicted caprine (Genbank: AY786437.1) and human (Genbank: NM024865.2) Nanog gene. GAPDH primers were selected as an internal positive control (FWD: 5'-TCA CCA TCT TCC AGG AGC GAG-3' and REV: 5'- CTT CTG GGT GGC AGT GAT GG-3'; annealing temperature = 57 °C, expected product length = 338 bp). The reactions were assembled using 1 µg of total RNA, 400µM of each dNTP, 400 nM FWD and REV primers, 2 µL Enzyme mix and 10 µL buffer in a 50 µL reaction volume.

The PCR thermocycling programs were the following: 30 min reverse transcription step at 50 °C, 15 min denature step at 95 °C followed by 35 cycles of 94 °C for 30 sec, annealing step for 30 sec and elongation at 72 °C for 45 sec with a final extension at 72 °C for 10 min using a thermal cycler (MJ Mini, BIO RAD, USA). Reactions conducted without reverse transcriptase (-RT) and RNA template (-RNA) were run in parallel using Super Taq DNA polymerase (Super Taq Co., UK) and 'RNase and DNase" free water in place of the OneStep RT-PCR enzyme mix and template RNA respectively. PCR products were identified by 1.5% Tris-Boric acid-EDTA agarose gel containing 1µg/mL ethidium bromide.

Results

Cellular isolation and morphology

Figure 1 shows two panels of representative inverted (Olympus, IX71) micrographs of CUC cells at the different days. Primary CUC cells were observed at the second (Fig 1A) or third (Fig 1B) days of primary culture of WJ explants. These cells reached a good confluency after about 10 days so they were subcultured and considered as passage 1.

Cells isolated from caprine umbilical cord matrix explants displayed a heterogeneous morphology including fusiform or spindle-form cells and small round cells with a large and prominent nucleus. Confluent cells were arranged in parallel arrays. As CUC cells reached considerable confluency, colonies of cells began to form (Fig 2).

Population doubling time

Figure 3 shows semilog plot of the increase in CUC-WJMC$_s$ concentration. Initial seeding concentration of 2×10^4 cells resulted in mean population doubling times of 46.57 and 54.29 hours for CUC-WJMC$_s$ and fibroblastic cells respectively. However, nearly on the tenth day of

cultivation, both CUC-WJMC$_s$ and fibroblast cells were entered to the plateau or stationary phase.

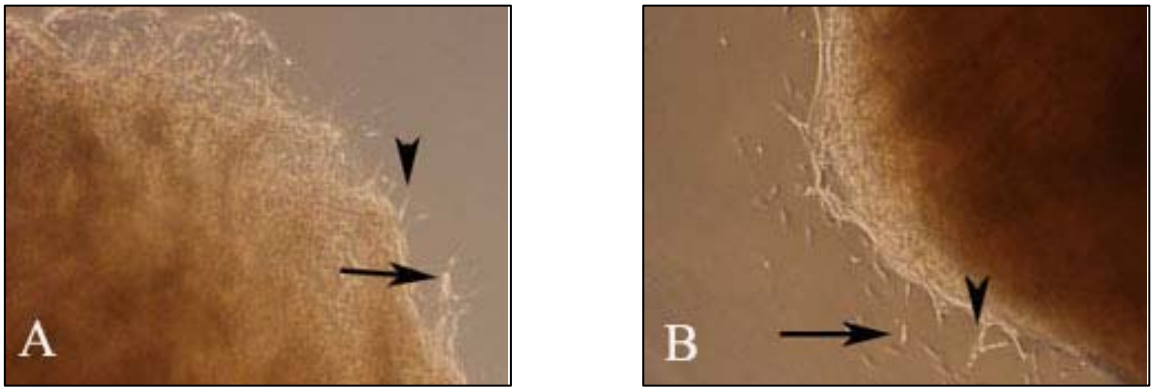

Figure 1. Inverted micrographs of CUC cells isolated at the second (A) or third (B) days of culture (arrow and arrow head). Magnification ×100.

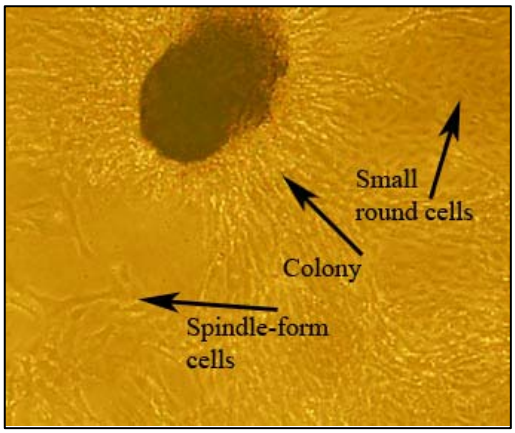

Figure 2. Two different cell types isolated from Wharton's jelly explants (spindle-form cells and small round cells) based on morphology. CUC isolated cells form colony. Magnification ×100.

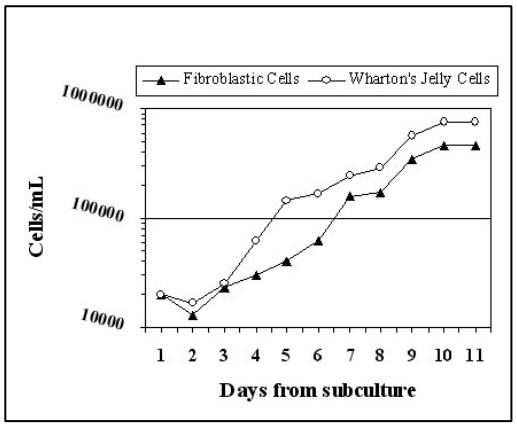

Figure 3. Semilog plot of the increase in CUC-WJMC$_s$ concentration in comparison with fibroblast cells concentration.

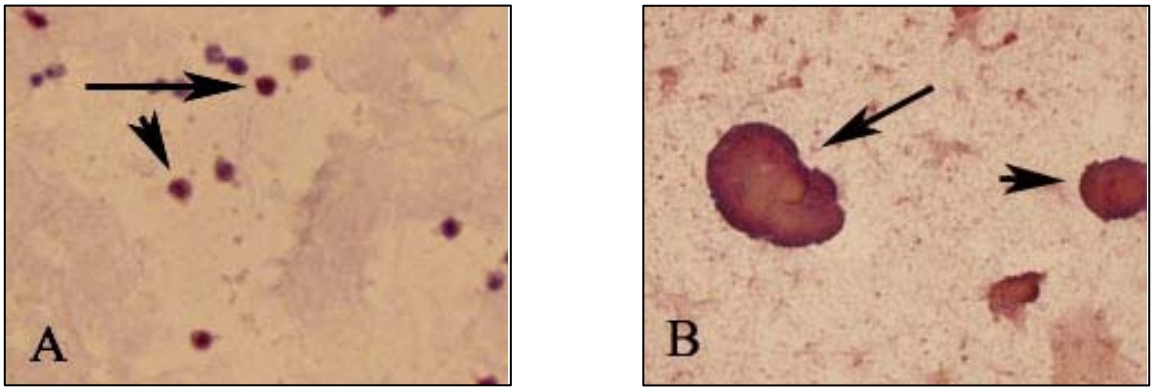

Figure 4. (A) Alkaline phosphatase assay in pyogenic leukocytosis specimen (arrow and arrow head) as a positive control (Magnification ×400) and (B) in CUC cells colonies (arrow and arrow head) (Magnification ×40).

Alkaline phosphatase

In our study a pyogenic leukocytosis specimen from a human patient was used as a positive control. According to the alkaline phosphatase kit used in our study a red colored reaction should be observed into the cytoplasmic granules of active leukocytes (Fig. 4A). In this regard, CUC-$WJMC_s$ colonies formed in culture exhibited positive AP activity. The reaction produced in the colonies formed by CUC-$WJMC_s$ was very intense at the border of colonies (Fig. 4B).

RT-PCR detection

Primers for GAPDH were included in the control positive (human lung carcinoma cells), the control negative (fetal fibroblastic cells) and the main experiment (CUC-$WJMC_s$) as a loading control which produced sharp bands. Reactions omitting the reverse transcriptase (-RT) and done without reverse transcriptase and template RNA (-RT & -RNA) produced no product in each experiment. Single amplification product of expected size was observed using human lung carcinoma RNA and primers for Nanog. Expression of Nanog transcription factor was not detected in CUC-$WJMC_s$ similar to the fetal fibroblastic cells (Fig 5).

Discussion

In recent years, parallel to the great efforts for exploring the novel and alternative sources of stem cells in the human and animal body, the umbilical cord appeared to be a promising reservoir of fetal cells that could be readily used as multipotent stem cells. Many reports have shown the characterization and stem cell potency of mesenchymal cells isolated from the human umbilical cord,[10] but a few reports were observed in animals.[11] Here we have report some characteristics of the isolated CUC-$WJMC_s$ and their AP activity. However we could not find any expression of Nanog gene in cultured CUC-$WJMC_s$. To the best of our knowledge, this is the first investigation of pluripotential competence of $WJMC_s$ by the detection of Nanog transcription factor in goat. The gene expression analysis and reverse transcription polymerase chain reaction have indicated that mesenchymal stem cells from the human umbilical cord express genes found in cells derived from all three germ layers to some extent.[12] Only one report indicates that, in one species examined, the pig, $WJMC_s$ resemble pluripotent cells, since they express transcription factors including Nanog, Oct-4 and Sox-2 at low levels relative to embryonic stem cells and are AP positive.[4] Our finding about pluripotency of CUC-$WJMC_s$ following Nanog transcription factor analysis contradicts the data previously obtained by Carlin et al., 2006. Previous observation revealed that the Nanog protein is easily fragmented, so it indicates this possibility that Nanog turns over rapidly.[7] In the present investigation, cryopreserved CUC-$WJMC_s$ were used after thawing for RNA extraction which

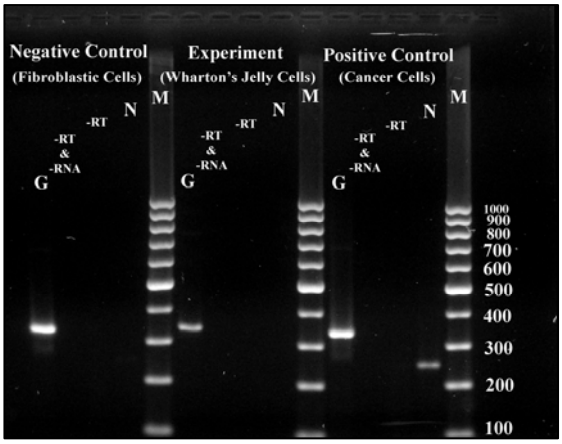

Figure 5. Transcription factor Nanog (N) expression in caprine umbilical cord Wharton's jelly mesenchymal cells which assessed by RT-PCR. Reactions omitting the reverse transcription (-RT) and without RNA (-RNA) produced no bands. As an internal control, endogenous 'housekeeping' gene GAPDH (G) were used in all reactions. M, marker.

may affect Nanog gene expression. Likewise, in a recently study, it has reported that thawed and freshly plated embryonic stem cells were negative for Nanog, but following a number of passages, the phenotype of the vigorously proliferating embryonic stem cells was changed to Nanog positive.[8] Another possible explanation is that maybe the transcription of Nanog was suppressed with continued cell passages. In another recently study, it has indicated that transcription factor Nanog is expressed in the initial days of inner cell mass explants culture but diminished in the course of cultivation and at the same time, markers of differentiation are appeared on all of cultured cells.[13] However, in the present investigation, passage 9 of CUC-$WJMC_s$ was prepared for RNA extraction which may explain above mentioned result about Nanog expression pattern and its down regulation during the different passages.

In the present investigation, the doubling time of CUC-$WJMC_s$ was shorter than fetal fibroblast cells. Shorter doubling time is a common feature for mesenchymal stromal cells derived from fetal blood.[14] Also, in the previous reports, it was mentioned that the doubling time of WJ cells and umbilical cord blood mesenchymal stromal cells is shorter than that of the adult bone marrow-derived mesenchymal stromal cells.[15,16] This common feature was thought to reflect the relatively primitive nature of mesenchymal stromal cells compared to the adult stromal cells.[11]

The ability to generate clones, e.g., cellular colony derived from a single cell, is a formal demonstration of the self-renewal ability, a characteristic of stem cell populations.[17] We demonstrated that CUC-$WJMC_s$ have clonogenic properties, therefore a formal demonstration of self-renewal was provided for our isolated umbilical cord cells. The Oct-4 gene has been proposed as a master regulator of the pluripotency of cells.[18] So, still further studies are necessary to determine the expression of some transcription genes such as Oct-4 in CUC-$WJMC_s$ to determine the pluripotency ability. Another nonspecific test for approving stem cells is expression of AP enzyme. AP is an enzyme that long-known to be expressed in embryonic stem cells as well as primordial germ cells.[19] In agreement with the previous report in pig,[4] in the present study AP activity was observed very intense in CUC-$WJMC_s$ colonies that was similar to the pattern observed in embryonic stem cells or other colony forming stem cells.

In summary, we demonstrated that WJ of caprine umbilical cord contains a large number of fibroblast-like cells which exhibit stem cells properties but the transcription factor Nanog are not expressed in passage 9 of CUC-$WJMC_s$. Present study contradicts with few previous reports. Since many factors such as environmental condition of culture, number of passages, cryopreservation, and breed may affect expression pattern of transcription factors. Thus, we suggest further exploration of these conditions on pluripotential genes expression in CUC-$WJMC_s$ as well as their abilities to differentiate into adult cells.

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چکیده:

ارزیابی پرتوانی سلول های مزانشیمی جدا شده از ژله وارتون بز با روش آنالیز RT-PCR ژن نسخه برداری اولیه نانوگ

همایون بابایی[۱]، مژگان مشرفی[۲]، مهدی گلچین[۳]، سید نورالدین نعمت اللهی ماهانی[۴]

[۱] هسته تحقیقات بیولوژی تکوینی و طب ترمیمی، [۲] دانش آموخته دکترای دامپزشکی و [۳] گروه پاتوبیولوژی، دانشکده دامپزشکی دانشگاه شهید باهنر کرمان، کرمان، ایران،
[۴] مرکز تحقیقات علوم اعصاب، دانشگاه علوم پزشکی کرمان، کرمان، ایران.

هدف - بررسی روش جدا سازی، زمان دوبرابر شدن سلول های جدا شده و ارزیابی بیان یکی از ژن های نسخه برداری اولیه به نام نانوگ به روش RT-PCR در سلولهای مزانشیمی جدا شده از ژله وارتون بز می باشد.

طرح مطالعه- مطالعه تجربی در شرایط آزمایشگاهی.

حیوانات - ۴ بز نژاد مخلوط.

روش کار - از رحم های کشتارگاهی بز ها تعداد ۴ عدد بند ناف جدا گردید و ژله وارتون آنها به قطعات ۲×۲ میلی متر مربع جهت کشت اکسپلنت بریده شد. در هر ظرف کشت ۳۵ میلی متری تعداد ۱۰-۸ عدد از قطعات بریده شده قرار داده می شد. این قطعات پس از ۵ روز از ظروف خارج شدند و سلول های جدا شده تا ۵ روز دیگر کشت داده می شدند. سلول های جدا شده از لحاظ هیستوشیمیایی برای وجود فعالیت آلکالین فسفاتازی ارزیابی می گردیدند. همچنین از روش RT-PCR برای وجود mRNA نانوگ استفاده شد. بعلاوه در این مطالعه منحنی رشد سلول های جدا شده نیز بررسی گردید.

نتایج- سلول های جدا شده از بند ناف بز دارای دو شکل دوکی و سلول های گرد کوچک با هسته درشت بودند. کلونی های آنها دارای فعالیت آلکالین فسفاتازی بود. در صورتی که با غلظت ۲۰۰۰۰ سلول کشت داده می شدند زمان دوبرابر شدن کوتاه تری نسبت به فیبروبلاست های جنینی داشتند (۴۶/۵۷ در برابر ۵۴/۲۹ به ترتیب) و در نهمین پاساژ آنها ژن نانوگ بیان نشد.

نتیجه گیری و کاربرد بالینی- بند ناف بز حاوی منبع قابل دسترسی از سلول های مزانشیمی است که خصوصیت سلول های بنیادی را از خود نشان می دهند اما احتمالا این سلول ها خاصیت پرتوانی را ندارند.

کلید واژگان- ژله وارتون، سلول های مزانشیمی، آلکالین فسفاتاز، منحنی رشد، نانوگ.