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INVESTIGATING THE EFFECTS OF VITREOUS HUMOUR (CRUDE EXTRACT) ON GROWTH AND DIFFERENTIATION OF RAT MESENCHYMAL STEM CELLS (rMSCs) AND HUMAN NTERA2 CELLS

Two main characteristics of all types of stem cells are their potency for differentiation and self renewal capacity. There is a lot of interest to find the conditions and factors, which govern these behaviours of stem cells. It is very well documented that retinoic acid (RA) reduces growth rate by induction of cell differentiation in certain conditions and cell lines. On the other hand, hyaluronic acid (HA) is known for its growth induction on cultured cells. A natural source of HA, rabbit vitreous humour (VH), was previously shown to promote wound repair in model animals. In search for its possible mechanisms, VH extract was tested on the cultured mesenchymal stem cells and NTERA2 as human embryonal carcinoma cells in the presence of RA. Changes in some cellular and molecular markers (A2B5, Oct4, Sox2) showed that VH and possibly HA interfere with differentiating effects of RA. Therefore, this reagent may affect cell proliferation and tissue regeneration by inhibition of cell differentiation.

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Introduction. Extracellular matrix is very well known for its effects on cellular behaviours including proliferation, migration and differentiation. Vitreous humour (VH) is a gelatinous, colorless and shapeless mix of different substances containing a mass of extracellular matrix, especially hyaluronic acid and collagen fibrils [1].

Recent progresses in isolation and culture of stem cells is a step forward to study cell behaviours *in vitro*.These cells can be divided into two major groups of embryonic stem (ES) cells and adult stem cells [2]. ES cells are able to differentiate and produce almost all cell types in proper conditions [3], but due to many technical limitations in using these cells and also because of many similar properties, human embryonal carcinoma cells (EC) are used in some experiments instead [4]. EC cells are very similar to the ES cells [5] while their growth and maintenance are easier and less expensive. For example a human EC cell, NTERA2/D1 or NT2 for short, is easily differentiated upon retinoic acid (RA) treatment [6]. Mesenchymal stem cells (MSCs) derived from bone marrow are another group of stem cells which are also widely used because of their easy culture and therapeutic significance. MSCs are clonogenic, non-haematopoietic stem cells present in the bone marrow and are able to differentiate into multiple mesoderm-type cell lineages, e.g. osteoblasts, chondrocytes, endothelial-cells, and also non-mesoderm-type lineages, e.g. neuronal-like cells [7].

The capacity of each reagent to induce differentiation in these cells, are examined using certain cellular or molecular markers. *Oct4* and *Sox2* are among the molecular markers that rapidly respond to differentiation. The transcription factor Oct4 (also referred to as Pou5f1), has significant expression in ovulated oocytes, mouse pre-implantation embryos, ectoderm of the gastrula (but not in other germ layers) and primordial germ cells. This protein has also been reported from embryonic stem cells [8]. SOX2, an SRY-related HMG box transcription factor (TF), is also expressed in the ICM and ES cells, and has a co-activator role in OCT4 activity [9].

Based on the data from *in vivo* experiments, VH is considered as a suitable candidate to be tested for its possible effect on cell proliferation and differentiation. This could be due to its high content of glycosaminoglycans [10], which seem to play an important role in early embryogenesis, can increase the cell growth *in vitro* [11], and in bovine blastocysts [12].

Materials and methods. All experiments were performed in strict compliance with the Ferdowsi University of Mashhad Animal Ethics Guidelines in accordance with Iran Animal Welfare Act.

Culture of NTERA2 Cells. NTERA2-clone D1 (NT2/D1) is a hEC cell line derived from a testicular teratocarcinoma [13], which closely resembles hES cells and the inner cell mass of human blastocyst stage embryos. The NT2 cells were successfully defrosted from a liquid nitrogen stocked batch and grown in a standard T25 flask with Dulbecco's Modified Eagles Medium (DMEM, Gibco) supplemented by 10 % fetal bovine serum (FBS, Gibco), 100 U/ml penicillin (Gibco), and 100 µg/mL streptomycin (Gibco) and left for growth to 80 % confluency at 37 °C and 10 % CO_2 in the air. For passage, the NT2 cells were scraped by glass beads and reseeded 1:3, continuously until they were needed for experiments.

Isolation and expansion of MSCs. Male Wistar rats, 4–6 weeks old, were sacrificed by cervical dislocation and their femurs and tibia were carefully cleaned from adherent soft tissues. The tip of each bone was removed with a rongeur, and the marrow was harvested by inserting a syringe needle (27-gauge) into one end of the bone and flushing with DMEM. Cells were plated at a density of 10^2 per T25 flask in 5 ml DMEM containing 15 % FBS, 2 mM L-glutamine (Gibco), 100 U/ml penicillin (Gibco), and 100 µg/mL streptomycin (Gibco). Cultures were kept at 37 °C in a humidified atmosphere containing 95 % air and 5 % CO_2. After 24 hrs, the non-adherent cells were discarded and adherent cells were washed gently with the fresh medium. The medium were replaced twice a week. When primary cultures became nearly confluent, the culture was treated with 0.5 ml of 0.025 % trypsin containing 0.02 % EDTA for 5 minutes at room temperature, and the detached cells were harvested and cultured in a T25 flask. Once the culture reached to 70–80 % confluency, the cells were harvested for further experiments.

Vitreous humour extraction. Seven skeletally mature male, New Zealand white rabbits (age, 6 months, weight 2.5–3 kg) were purchased from Razi Institute, Mashhad, Iran. They were kept in soft bedding cages with free access to food and water. General anaesthetize was performed by chloroform, and followed by sodium thiopental for euthanized. Their eye lips were cut open and the eye balls were taken out very smoothly. The balls were rinsed thoroughly in physiological serum and transferred to laminar flow hood. The vitreous humour was sucked out from the dorsal part of the ball using a fine syringe, with special care to avoid any contamination from the eye lens and retina. Each extract, about 0.5 ml in volume, was filter-sterilized using the standard micro filters (0.22 µm), and stored in a sterile microtube at –20 °C for further use.

RNA extraction and RT-PCR. About 10^2cells/ml were subjected to RNA extraction using Tri-reagent (Sigma) following manufacturer's protocol. To remove any DNA contamination, the RNA was treated with DNase (Ambion) and analysed on 1 % agarose gel. The first strand of cDNA was extended using 5 µg of total RNA and 100 pmol of oligo(dT)primer, 200 units of M-MLV reverse transcriptase (Promega) plus its reaction buffer, and 1.25 mM dNTPs in final volume of 40 µl, followed by 2 hrs incubation at 37 °C and 5 min inactivation at 80 °C. 1 µl of this reaction mixture was used as template for polymerase chain reaction (PCR) following standard protocols. PCR was performed using 1 µl of the cDNA in final volume of 25 µl containing 15 pmol of each primer, 0.1 mM dNTPs, and 0.3 units Taq polymerase (Promega).

The primer sequences and conditions of these reactions were as follows: human Oct4 forward (hOct4-F) (5'-GAGAATTTGTTCCTGCAGTGC-3'); human Oct4 reverse (hOct4-R) (5'-GTTCC-CAATTCCTTCCTTAGTG-3'); human Sox2 forward (hSox2-F) (5'-CCCCCGGCGGCAATAG-CA-3'); human Sox2 reverse (hSox2-R) (5'-TCG-GCGCCGGGGAGATACAT-3'); rat Oct4 forward (rOct4-F) (5'-AAGCTGCTGAAACAGAAGAGG-3'); rat Oct4 reverse (rOct4-R) (5'-ACACGGTTCT-CAATGCTAGTC-3'); internal control forward (β-actin-F) (5'-ATCTGGCACCACACCTTCTACA-ATGAGTGCG-3'); and internal control reverse (β-actin-R) (5'-CGTCATACTCCTGCTTGCTG-ATCCACATCTGC-3'). The number of cycles were 31 for hOct4, 30 for hSox2, 33 for rOct4 and 28 for β-actin.

The cycling conditions were as follows: 94 °C for 45 sec, 58 °C (hOct4), 56 °C (hSox2), 57 °C (rOct4) and 62 °C (β-actin) for 1 min, 72 °C for 1.5 min, with a final extension at 72 °C for 10 min.

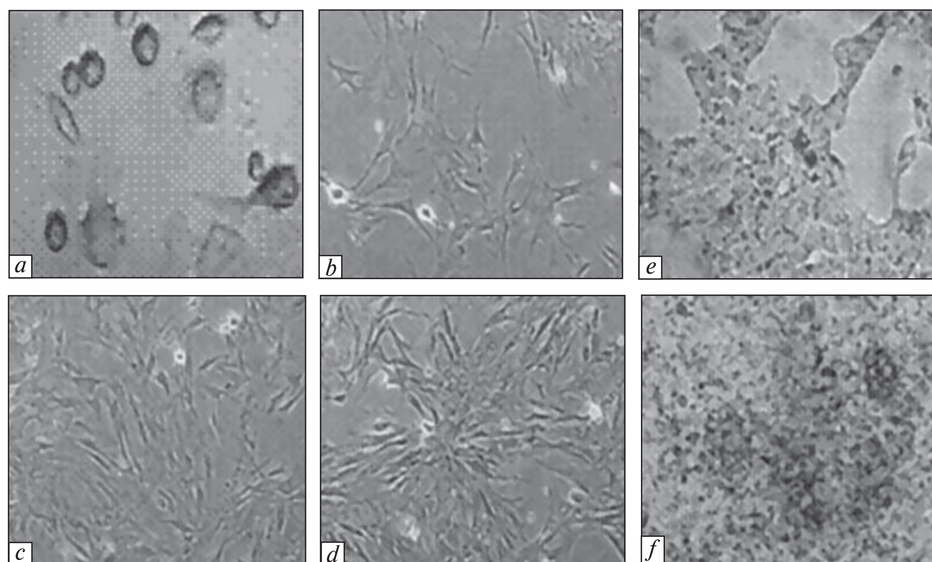

Fig. 1. Morphology of rat bone marrow mesenchymal stem cells (rMSCs) and NT2 cells. The picture shows a time course morphological changes of MSCs from round-shape on day one (*a*) to spindle-shape on days 8–15 (*b–c*) after isolation, and a perfect spindle-shape at first passage (*d*). The morphology of NT2 cells after 4 (*e*) and 7 (*f*) days in culture are also shown. The original magnification is 400×

PCR products were separated on 1 % agarose gel, stained with ethidium bromide, and visualized under the UV light. The graphs from the gels were subjected to intensity measurement using LabWorks program. In this method, the intensity of the RT-PCR products for the control sample is assumed 100 and the treatments are compared relative to the control one. The semi-quantitative RT-PCR experiments were repeated at least two or three times for expression assay of each gene.

VH and retinoic acid treatment of NTERA2 cells. NTERA2 cells were seeded at a density of 10^6 cells per T25 flask and treated with either VH (150 μl) or RA (10^{-5} M) for 7 days and the effects of both compounds on the gene expression were determined by semi-quantitative RT-PCR at different time points. Medium was changed with fresh RA, VH and DMEM supplemented with 10 % fetal bovine serum, every 2 days. Cells were harvested at different time points with Tri-reagent and total RNA was extracted from each sample.

Flow cytometry analysis. The cells were detached from culture dish with trypsin/EDTA. Cell surface antigen expression was assayed by flow cytometry as previously described [6]. Briefly, the cells were incubated in primary antibody (A2B5 anti-ganglioside GT3) [14] for 1 h at 4 °C. After three

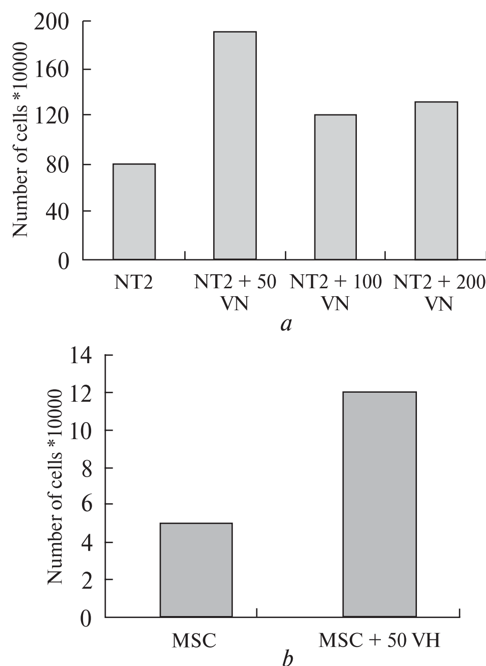

Fig. 2. Growth induction on NTERA2 and rMSCs upon 7 days of VH treatment: *a* – Growth of NT2 cells was significantly ($P < 0.05$) increased in all VH concentrations reaching to its highest in 50 μl per well; *b* – Growth of MSCs was almost doubled ($P < 0.004$) in wells treated with 50 μl of VH extract

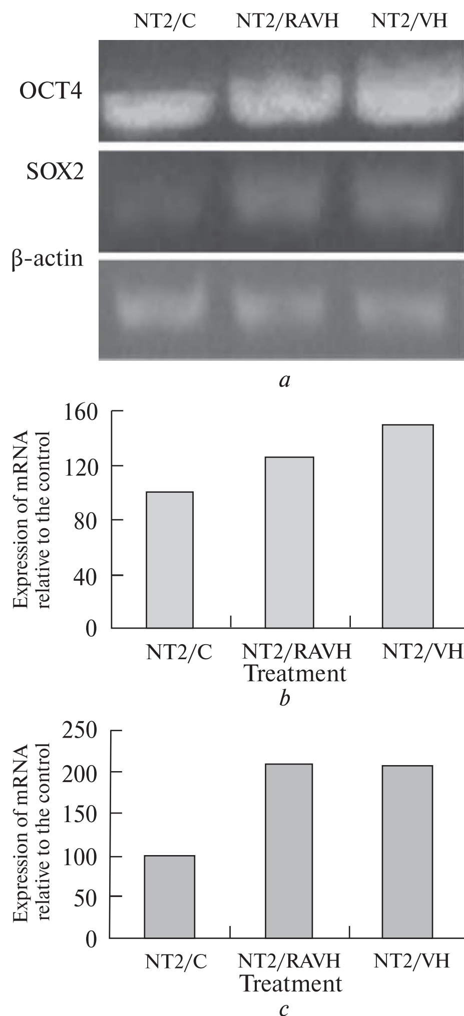

Fig. 3. Semi-quantitative RT-PCR analysis of OCT4 and SOX2 mRNA expression in human NT2 cells 7 days after treatment: *a* – pictures of the observed PCR products on the agarose gel; *b*, *c* – the relative intensities of the PCR products for OCT4 and SOX2 mRNAs respectively. These treatments included NT2 control cells (NT/C) and those treated with VH (NT2/VH) or a combination of RA and VH (NT2/RAVH). OCT4 and SOX2 mRNAs increased in both treatments. RT-PCR of β-actin mRNA indicates equal loading of the samples

washes in wash buffer (5 % FBS and 0.02 % sodium aside in magnesium and calcium free phosphate-buffered saline (PBS)), they were incubated in a FITC-conjugated secondary IgM antibody for 1 hr. They were then resuspended in the wash buffer, and analysed using a flow cytofluorimeter. The antibody produced from the myeloma P3X63Ag8 [15] was used as a negative control.

Statistics. The SPSS V. 13 statistical program was used to analyse the data. Significance of the data was examined in the level of ($p \leqslant 0.05$) using one way ANOVA and tukey test.

Results. *Growth rate of NTERA2 and rMSCs is increased upon VH treatment.* Rat MSCs (Fig. 1, *a–d*) and NT2 cells (Fig.1, *e*, *f*), at approximately 80 % confluency, were trypsinized and seeded into six well plates. Next day, NT2 cells were treated with different volumes, 50, 100 and 200 μl of the VH extract to evaluate the optimum concentration for the highest cell growth rate. The medium was replaced with fresh one, containing VH every 48 hrs, and finally the cells were counted after one week. This experiment was repeated three times independently. After seven days, statistical analysis revealed that there was a significant ($p \leqslant 0.05$) growth difference between NT2 control cells in comparison with the cells treated with VH (50, 100 and 200 μl). The maximum proliferation was observed at 50 μl of the VH extract (Fig. 2, *a*). As seen in Fig. 2, *b*, treatment of rMSCs with 50 μl VH extract increased cell proliferation almost 2 folds ($P < 0/004$) in comparison with the untreated ones.

Expression of Oct4 and Sox2 genes are affected by retinoic acid and vitreous homour treatments. As previously described, NT2 cells are among the cells expressing a high level of OCT4 and SOX2 transcription factors. As expected, the semi-quantitative RT-PCR analysis following RA treatment of these cells showed a decline in the expression levels of OCT4 and *SOX2* genes. Interestingly, this pattern was reversed upon treatment of the cells with VH extract. Both OCT4 and *SOX2* showed a significant increase in their expression when NT2 cells were treated with VH alone. In a separate experiment, results showed that VH could somehow inhibit the suppressing effects of RA on the expression of these genes when applied in combination with RA (Fig. 3, *a–c*). In other words, mRNA levels of *SOX2* and OCT4 were higher in combinational treatment of RA and VH (RA-VH) compared to that of the RA alone.

OCT4 gene is expressed in rat mesenchymal stem cells. mRNAs were extracted from rMSCs with or without RA treatment and subjected to semi-quantitative RT-PCR. *Oct4* mRNA was detectable in rMSCs. However, it disappeared upon treatment with RA (Fig. 4).

Differentiation of rMSCs upon treatment with RA and VH. As expected, rMSCs and NT2 cells, treated with a combination of RA and VH, did not display a significant decrease in their proliferation, despite presence of retinoic acid. RA alone signals a decline in growth of rMSCs and NT2 cells within 2 days, prior to the appearance of neuronal differentiation markers. rMSCs treated with RA (10^{-5} M) were first examined microscopically for any morphological changes. After 4 days of treatment the rMSCs showed a transition to the cells with asymmetric morphology, while the cells treated with RA and VH had a symmetric morphology. This change progressed to day 10, when the RA-treated rMSCs showed neuron-like structures. By day 26, the treated cells demonstrated structures very similar to neurons (data not shown) which remains to be confirmed more precisely. Treatment of the cells with combination of RA and vitreous humour extract however, reduced the progressive effect of RA to differentiate the MSCs to neuron-like cell types.

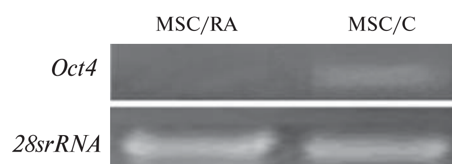

Fig. 4. Detection of *Oct4* mRNA in rMSCs (MSC/C) by semi-quantitative RT-PCR analysis. The level of Oct4 mRNA decreases upon RA treatment (MSC/RA). The lower panel represents the band for 28s rRNAs

RA treatment induces neural differentiation in NT2 and rMSCs. To confirm the morphological data

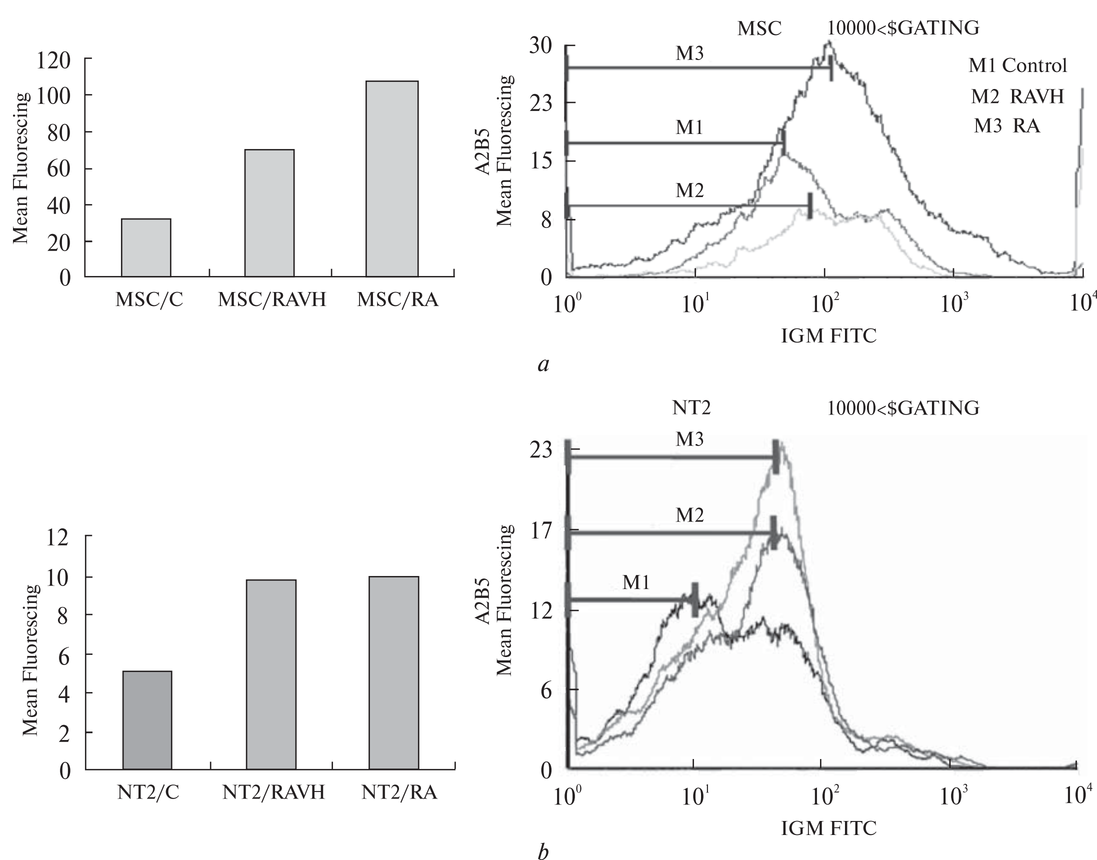

Fig. 5. Cell surface antigen (A2B5) expression (analyzed by flow cytometry) following retinoic acid and vitreous homour treatment in NT2 for 4 days and rMSC for 7 days: *a* – rMSCs express high level of A2B5 after RA treatment (MSC/RA), but not as high after RA plus VH treatment (MSC/RAVH) compared to the control (MSC/C); *b* – RA treatment of the NT2 cells (NT2/RA) and its combination with VH (NT2/RAVH) induces A2B5 expression to the same level in NT2 cells with no RA treatment (NT2/C)

of transdifferentiation, the RA-induced neuronal differentiation of MSCs and NT2 cells was also evaluated by flow cytometry based on the presence of specific cell surface antigens [4].

A prominent induction of the neuroectodermal marker, A2B5, appeared by day 7 in the RA treated MSC cells (Fig. 5). Terminal morphological differentiation, such as neuronal outgrowth, did not typically appear until approximately 3 weeks after RA treatment.

Discussion. As a natural source of intact HA, we were interested to test the differentiative and proliferative effects of VH on stem cells as an accepted model for *in vitro* studies. Previous experiments on mouse ears demonstrated that wounds treated with VH had an increase in fat cell production, angiogenesis and fibroblast proliferation [16]. This caused a significant increase in tissue regeneration and wound repair.

Our data indicate that treatment of stem cells with VH increased cell growth considerably, but the mechanism of such effect is open to speculation. In other line of experiments, our results showed that VH also interfered with cell differentiation.

The octamer-binding transcription factor-4 (*Oct4*) and *Sox2* are expressed in human pluripotent stem cells, but not in their differentiated derivatives. Therefore, their expression is rapidly downregulated in response to retinoic acid-induced differentiation [17] and markers associated with neu ral cells are co-ordinately up regulated during the differentiation [18]. We used expression level of these transcription factors as indicator of VH effects on the cells. These markers were considerably increased in mRNA level upon the VH treatment. As they are key genes in determination of cell fate during embryogenesis, it is possible that VH can make a suitable cell nitch for certain cells with activation of primitive and upstream signalling pathways [19]. The undifferentiated cells become committed to differentiation with RA, unleashing a genetic program that involves the differential expression of more than 3000 genes [17]. Therefore, components of the VH may take an antagonistic approach to prevent cell differentiation. As we show here, VH reverses differentiative effects of RA, with a consequence of increased cell proliferation.

As reported before, during cell differentiation the level of peroxides also increases. Alternatively, reduction of peroxides could be a possible mechanism of VH action in differentiating cells. This again could be due to the high content of HA, which is believed to have antioxidative effects in various systems [20]. VH is known for its high content of glycosaminoglycans specially hyaluronan. As the effect of hyaluronan on proliferation of cord blood progenitor cells is well known [21, 22], it seems reasonable to investigate the effect of different chemical fractions of VH extract on cell proliferation and differentiation.

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ИЗУЧЕНИЕ ВЛИЯНИЯ ГРУБОГО ЭКСТРАКТА СТЕКЛОВИДНОГО ТЕЛА НА РОСТ И ДИФФЕРЕНЦИАЦИЮ МЕЗЕНХИМАЛЬНЫХ СТВОЛОВЫХ КЛЕТОК КРЫСЫ (rMSCs) И КЛЕТОК NTERA2 ЧЕЛОВЕКА

Двумя основными характеристиками всех типов стволовых клеток является их способность к дифференциации и самообновлению. Значительный интерес вызывает раскрытие условий и факторов, которые управляют таким поведением стволовых клеток. Хорошо известно, что ретиноевая кислота (RA) снижает темпы роста, индуцируя дифференциацию клеточных линий в определенных условиях. Вместе с тем известно, что гиалуроновая кислота (НА) индуцирует рост культивируемых клеток. Ранее было показано, что естественный источник НА, стекловидное тело (VH) кролика, вызывает заживление ран у модельных животных. В поисках возможного механизма этого процесса экстракт стекловидного тела был исследован на культивируемых мезенхимальных стволовых клетках и клетках NTERA2 эмбриональной карциномы человека в присутствии RA. Изменения некоторых клеточных и молекулярных маркеров (A2B5, Oct4, Sox2) показали, что VH и, возможно, НА влияют на дифференцирующие эффекты RA. Таким образом, это вещество может влиять на пролиферацию клеток и регенерацию тканей, ингибируя дифференциацию клеток.

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ВИВЧЕННЯ ВПЛИВУ ГРУБОГО ЕКСТРАКТУ СКЛОПОДІБНОГО ТІЛА НА РІСТ ТА ДИФЕРЕНЦІАЦІЮ МЕЗЕНХІМАЛЬНИХ СТОВБУРОВИХ КЛІТИН ЩУРА (rMSCs) І КЛІТИН NTERA2 ЛЮДИНИ

Двома основними характеристиками всіх типів стовбурових клітин є їхня здатність до диференціації та самооновлення. Значний інтерес викликає роз-

криття умов та факторів, котрі управляють такою поведінкою стовбурових клітин. Добре відомо, що ретиноєва кислота (RA) знижує темпи росту, індукуючи диференціацію кліткових ліній в певних умовах. Разом з тим відомо, що гіалуронова кислота (HA) індукує ріст культиво- ваних клітин. Раніше було показано, що природне джерело HA, склоподібне тіло (VH) кроля, викликає загоєння ран у модельних тварин. В пошуках можливого механізму цього процесу екстракт склоподібного тіла був досліджений на культивованих мезенхімальних стовбурових клітинах та клітинах NTERA2 ембріональної карциноми людини в присутності RA. Зміни деяких клітинних та молекулярних маркерів (A2B5, Oct4, Sox2) показали, що VH і, можливо, HA впливають на диференціюючі ефекти RA. Таким чином, ця речовина може впливати на проліферацію і регенерацію тканин, інгібуючи диференціацію клітин.

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