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Isolation and cultivation of MSC-like population from the rabbit amniotic membrane

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Despite the advantages of studying the therapeutic potential of autologous and allogeneic mesenchymal stem cells (MSCs) on the rabbit model, the cultivation of rabbit amnion MSCs has not been described in the literature. **Aim.** To study the possibility of isolation, multiplication, and cryopreservation of amnion-derived rabbit MSCs. **Methods.** The amniotic membrane was obtained surgically. Both enzymatic and explant methods of cell isolation have been tested. The obtained primary cultures were passed, and their proliferation capacity and morphology characteristics were studied. **Results.** The viable clones were obtained on the 2nd day of cultivation using an enzymatic method. At all passages, the cells showed adhesion to the culture plastic and fibroblast-like morphology. At the first and second passages, after 7 days of cultivation, the population doubling occurred 4.4 and 4.3 times, respectively. Evaluation of the viability after cryopreservation showed that after thawing, more than 90 % of cells were alive, and 4.3 doubling occurred in 7 days of cultivation. No cells with the atypical phenotype were detected during cultivation. **Conclusions.** The methods of extraction, multiplication, and cryopreservation of MSCs-like rabbit amnion cells were optimized. This can promote further studies of the MSCs characteristics and regenerative potential.

K e y w o r d s: rabbit amnion, MSC, primary culture

Introduction

Mesenchymal stem cells (MSCs) are considered one of the most promising types of stem cells in regenerative medicine. They can be obtained from a wide range of tissues and multiplied *in vitro* [1]. Their unique immunoregulatory properties and low immunogenicity, enable the study of xenogeneic MSC transplantation effectiveness on animal mo-

dels [2]. However, the experimental therapy of diseases, modeled in laboratory animals, includes the studies of the effects of allogeneic or autologous MSCs. This makes the search for methods of cultivating MSCs from various tissue sources of laboratory animals an actual task.

Despite the advantage of rabbit models for studying the therapeutic effects of allogeneic MSCs from neonatal tissues, only one experimental attempt to isolate and cultivate MSCs from the umbilical cord of rabbits is described [3]. The methods of isolation and characteristics of the stem cell populations from the human amnion are well described in the literature, similar data are also known for the amnion of mice [4]. However, for rabbits, this issue is practically unexplored. The facts of the isolation of cells from the amniotic membrane of rabbit are described, however, as a stage of the main task of the research. It should be noted that none of the works includes a detailed description of the properties of the obtained population [5, 6]. The task of present work is to check the possibility of isolation, multiplication, and cryopreservation of MSC population from the rabbit amniotic membrane.

Materials and Methods

Obtaining rabbit amnion

The rabbit amniotic membrane was surgically removed. An adult doe was operated under local anesthesia (Sol. Lidocaine, 2 %). Placenta, umbilical cord and remnants of the amniotic membrane were mechanically separated. Next, the tissues were placed in physiological solution.

Obtaining the primary culture of MSCs from amniotic membrane by the enzymatic method

Fragments of the amniotic membrane were washed in PBS and maintained in antibiotics solution (benzylpenicillin (20 units/ml (“Arterium”, Ukraine) and streptomycin (20 μg/ml (“Arterium”, Ukraine). Next, they were divided into fragments (5x5 mm size), placed into a solution of enzymes (1:1 v\v mixture of 0.2 % collagenase and 0.1 % trypsin) and incubated at 37 °C for 1–2 hours, until the maximum visual degradation of the tissues. After that, the solution was inactivated using fetal bovine serum (FBS), and centrifuged at 1500 rpm for 10 min. The obtained pellet was resuspended and placed in a culture flask, 25 or 75 cm^2 (SPL, South Korea), with aMEM (Biowest) or high-glucose DMEM (RAA, Austria) nutrient medium supplemented with benzylpenicillin (2 units/ml (“Arterium”, Ukraine) and streptomycin (2 μg/ml (“Arterium”, Ukraine), and 10 % FBS (Gibco), and incubated at 37 °C, under standard CO_2 incubator conditions.

Obtaining a primary MSC culture from the amniotic membrane by the explant method

The amniotic membrane fragment was mechanically separated from the placenta and umbilical cord, chopped into pieces (5x5 mm in size), washed with PBS, and maintained in a solution of antibiotics (benzylpenicillin (20 units/ml (“Arterium”, Ukraine) and streptomycin (20 μg/ml (“Arterium”, Ukraine). Next, it was minced to fragments of 1 mm, transferred to a culture flask, 25 or 75 cm^2 (SPL, South Korea), with nutrient medium

aMEM (Biowest) or DMEM with a high glucose content (RAA, Austria), with the addition of benzylpenicillin (2 units/ml ("Arterium", Ukraine), streptomycin (2 μg/ml ("Arterium", Ukraine) and 10 % fetal calf serum (Gibco), and incubated at 37 °C, in standard CO_2 incubator conditions.

Proliferation analysis

On the first and following passages, the cells were seeded in a number of 50×10^3 per vial (25 cm^2), and cultivated for 7 days. After that, they were passed using 1:1 EDTA (0.02 %) and trypsin (0.1 %) mixture, and counted in Goryaev's chamber. For the each next passage, the procedure was repeated. Number of population doublings was estimated according to the formula: PD = log (Nf / Ni) / log 2, where Nf = final number of cells; Ni = initial number of cells.

Analysis of culture morphology

At each passage, starting from the primary culture, the cultures were observed by light and interference microscopy, using a Leica DMIL inverted microscope. Microphotographs were taken using a Cannon PowerShot 640A camera, ×40 and ×100, camera magnification ×1.4.

Cryopreservation of MSCs and assessment of its effectiveness

After the first passage, MSCs were passed using EDTA (0.02 %) and trypsin (0.1 %), centrifuged at 1000 rpm and placed in cryopreservation medium (15 % DMSO, 85 % FBS) in an amount 200,000 per 500 ml. According to the standard method, they were placed in a low-temperature refrigerator. After 6 months of storage at –80 °C, microtubes were thawed at 37 °C for 1 minute, then centrifuged, resuspended in full media, stained with trypan blue, and counted.

To study the proliferative activity of cryopreserved cells, they were seeded in the amount of 50×10^3 per flask (25 cm^2), and cultivated for 7 days, after which their number was counted.

Statistics

Experimental data are presented as mean ± standard deviation, unless otherwise indicated. The number of repetitions of independent experiments is 3.

Results and Discussion

In this study, taking into account the histological features of the amnion known from the literature, both the explant and enzymatic methods were used to isolate MSCs.

The amnion of rabbits is a dense translucent film that is anatomically connected to the umbilical cord and tightly adheres to the placenta (Fig. 1).

Since the aim of the work was to obtain the population of MSC from amnion, first it was important to separate the tissue from the umbilical cord mechanicaly.

As mentioned in "Materials and Methods", two methods of the primary culture obtaining were tested. The explant method appeared to be not effective, as no adherent cells were detected after 10 days of cultivation. However, the observation showed that the cells remained viable in the tissue itself at least for 10 days (Fig. 2).

Therefore, the effectiveness of the enzymatic method was tested. As a result, the visible multi-cellular clones could be detected

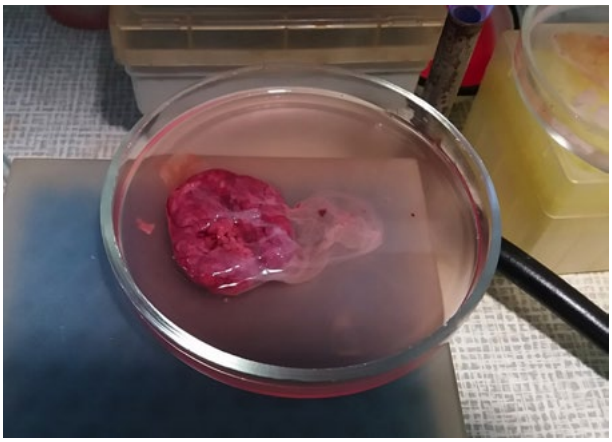

Fig. 1. Rabbit placenta, amnion, and umbilical cord

already on the 2nd day of cultivation. After 10 days of cultivation, the cells formed a monolayer with varying density and organization (Fig 3).

Interestingly, after treatment with enzymes, the viable cells could be observed in fragments of tissue. Placed in another culture flask, they began to form adhesive clones approximately on the 5th-7th day of incubation (Fig. 4).

In general, on the 10th day of incubation, it was possible to collect 600–800 thousand cells from a 25 cm^2 flask.

The microscopy showed that the obtained cells had a fibroblast-like morphology, "classical" for MSCs, and did not contain the cells with atypical features (binucleate, with the detectable signs of senescence, *etc.*). Though, the visual observation showed, that the cells slightly varied in size.

After the primary culture stage, the cells were passed according to a standard method. At each passage, their number was counted and the number of population doublings was calculated. It was found that at the first passage, after 7 days of cultivation, the number of cells obtained was approximately 1050000 ± 105 365.4, at the second — 1007333.33 ± 28023.8. Thus, after 7 days of cultivation, the populations doubled approximately 4.4 and 4.3 times, respectively.

The assessment of viability after cryopreservation showed that after the thawing procedure in all cases more than 90 % of the cells were alive. It was shown that after the thawed cells were seeded on a culture flask, as indicated above, their number reached 10000000 ± 351567.92 in 7 days of cultivation. Therefore, in 7 days of cultivation, the population underwent approximately 4.3 doublings.

Noteworthy, the morphology of the cells did not visually change in all passages. The cells kept the typical fibroblast-like shape, and reached 100 % confluency of the monolayer. The cells with a senescent phenotype were not detected (Fig. 5).

As it was mentioned in the Introduction section, rare cases of the cells isolation from the amniotic membranes of rabbits are described, though no characteristics of their properties are given. For example, the work of Hinko & Soloff [5] describes the isolation of cells from rabbit amnion tissue using mechanical processing, centrifugation, and DNase I treatment. However, after several days of cultivation, the obtained cells were used to study the characteristics of oxytocin receptors associated with synthesis of prostaglandins, therefore no data are known about their multiplication, morphology and other features of the obtained population. In the work of Papadopulos *et al.* [6] the enzymatic isolation of cells from the amniotic membrane of a rabbit is described, using a 0.25 % solution of trypsin and versene, but no detailed data on the characteristics of cell pro-

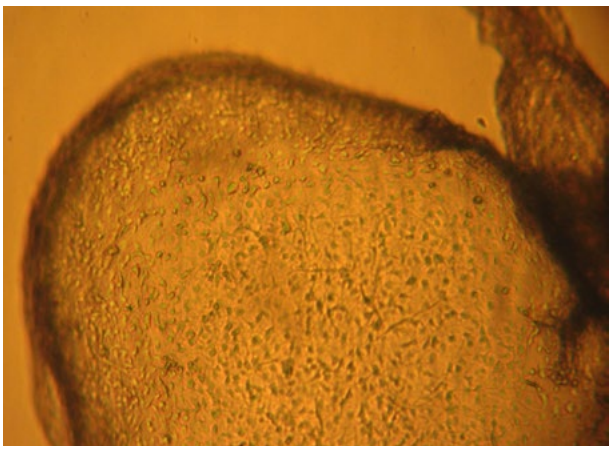

Fig. 2. Amnion fragments, day 10th of cultivation (×40, camera zoom ×1.4)

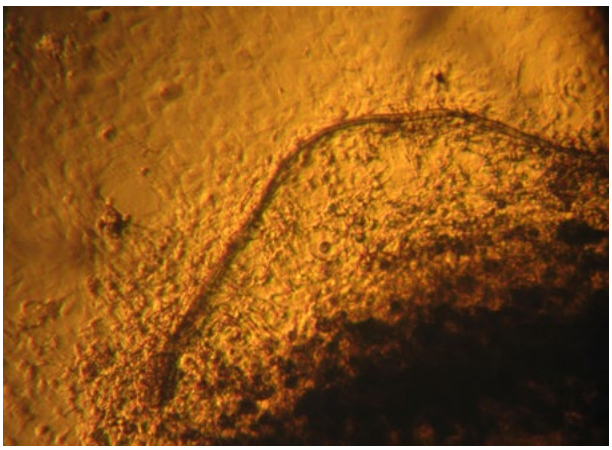

Fig. 3. Formation of a monolayer of MSC-like cells from the amnion, enzymatic method, 10th day of cultivation (×40, camera zoom ×1.4)

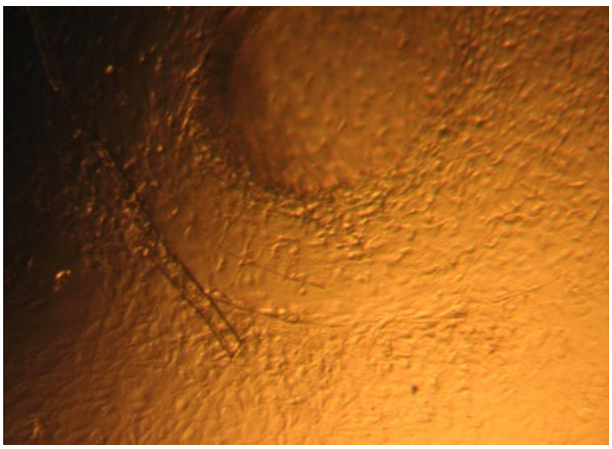

Fig. 4. Formation of a monolayer of MSC-like cells from the amnion, enzymatic method (×40, camera zoom ×1.4)

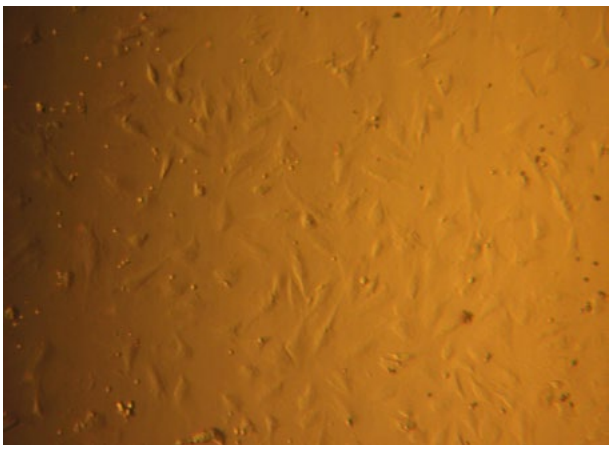

Fig. 5. Monolayer morphology, passage 2 (×100, camera zoom ×1.4)

liferation and morphology are given. It was only noted that they have a significant proliferative potential and the ability to proliferate in collagen matrices [6].

As it is known from literature, primary cultures can be obtained from tissue source by enzymatic and explant methods. In the enzymatic method, cells are isolated by tissue digestion with enzymes (mostly, different collagenases and trypsin). The technique can be applied to the tissues of various types. The main advantages of the method are shorter time of an isolation period of the obtaining procedure, and a large number of the obtained cells [7]. In the explant method, cells migrate from mechanically minced tissue pieces.

Though the procedure of cell extraction is more time consuming, comparing to enzymatic methods, some works show that it is less damaging to cells surface, and, particulary for MSCs, is beneficial for preservation of their paracrine potential [8].

In present study, we checked the possibility of obtaining of MSC-like population, and showed, that only the enzymatic method appeared to be effective. Noteworthy, the successful cases of human amnion MSC obtaining by the explant method are described in literature [9]. The fact that the explant method was ineffective in the present study may be connected to the histological peculiarities of rabbit amnion tissue, and needs additional studies to be explained.

As for the cells population itself, considering the lack of data, unfortunately, we cannot compare the obtained results with the results of other researchers. However, it can be confidently stated that the cells obtained in this study have a typical fibroblast-like morphology and retain it during several passages. Also, the cultures showed strong distinct adhesion to culture plastic. Thus, these data may indicate that, according to generally accepted criteria [10], the obtained population can be attributed to MSC. At the next stages of research we plan to conduct the study of their differentiation potential, immunological features, and regenerative potential, in order to solve this issue. Noteworthy, the obtained cells tolerate well the freezing and thawing procedures, that eases the conduction of the studies.

Conclusions

In the present work we optimized the methods of isolation, multiplication and cryopreservation of the MSCs-like rabbit amnion cells, and showed, that they have sufficient proliferative potential, and high post-thaw survival rate. All this makes it possible to conduct the further research of their characteristics and possibility of using them in the therapy of diseases on the animal models.

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Отримання та культивування МСК-подібної популяції з амніотичної оболонки кроля

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Незважаючи на переваги вивчення терапевтичного потенціалу аутологічних і алогенних мезенхімальних стовбурових клітин (МСК) на кролячих моделях, культивування МСК кролячого амніону не описано в науковій літературі. **Мета.** Вивчення можливості виділення, розмноження та кріоконсервації МСК, отриманих з амніону кроля. **Методи.** Амніотичну оболонку кроля було отримано хірургічним шляхом. Для отриманя первинної культури було перевірено ферментативний метод, та метод експлантів. Отримані первинні культури пересівали, вивчали їх проліферацийну здатність і морфологічні характеристики. **Результати.** Життєздатні клони було отримано на другу добу культивування ферментативним методом. На всіх пасажах клітини мали адгезію до культурального пластику та фібробластоподібну морфологію. На першому та другому пасажах через 7 діб культивування популяція подвоювалась 4,4 та 4,3 рази відповідно. Оцінка життєздатності клітин після кріоконсервації показала, що після розморожування більше 90 % клітин були живими, а за 7 днів культивування відбулося 4,3 подвоєння. Під час культивування не було виявлено клітин з атиповим фенотипом. **Висновки.** Оптимізовано методи отримання, мультиплікації та кріоконсервації МСК-подібних клітин амніону кроля. Це може стати основою для подальших досліджень їх характеристик і регенеративного потенціалу.

Ключові слова: амніон кроля, МСК, первинна культура

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