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An Efficient Protocol for Embryonic Carcinoma Cells P19 Differentiation to Cardiomyocytes Using Oxytocin as Inducer

Majid Malekzadeh Shafaroudi [1, 2], Nourollah Rezaei [1, 2], Saeid Abedian kenari [2], Maryam Nazm Bojnordi [1], Mostafa Latifpour [3], Hatef Ghasemi Hamidabadi [1, 2*]

[1] Department of Anatomy and Cell Biology, Faculty of Medicine, Mazandaran University of Medical Sciences, Sari, Iran.
[2] Immunogenetics Research Center (IRC), Faculty of Medicine, Mazandaran University of Medical Sciences, Sari, Iran.
[3] Department of Anatomy, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran.

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Corresponding Author:
Hatef Ghasemi Hamidabadi
Department of Anatomy and Cell Biology, Faculty of Medicine, Mazandaran University of Medical Sciences, Sari, Iran.
Phone: +9811-33543081
E-mail: hatefdr@gmail.com

Abstract

Background: The capability of embryonic carcinoma cells P19 in differentiation to Cardiomyocyte was examined through inducing effects of Oxytocin (OT) and 5-Azacytidin (5Az) individually and compared with each other in laboratory condition.

Materials and Methods: P19 Embryoid Bodies (EBs) was formed through hanging drops method. Then, EBs were treated with (5Az) or (OT) and the EB medium (Ctrl) until 12 days. Morphology and beating number per minute were recorded every two days. Viability was carried out every three days. The expression of several cardiomyocyte-associated genes was assessed by RT-PCR.

Results: The beating area percentage of EBs in OT treatment group was more than that of the 5Az group in all days of experiment. However, only in final stage, a significant increase was observed in beating area of OT group. There was no significant difference in viability and morphological changes. OT induction expressed three more specific proteins in cell culture than 5Az.

Conclusion: Statistical analysis revealed that response to OT inducer was more excessive than 5Az in all treatment groups. The Oxytocin was found to be effective inducer of cardiomyocytes differentiation from embryonic carcinoma cells P19 than 5-azacytidine.

Keywords: P19 Cells; Embryoid Bodies; Cardiomyocytes; 5-Azacytidin; Oxytocin; Differentiation

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Introduction

Cardiovascular diseases are big concerns among human communities. In ischemic heart muscle problems, subsequently, myocardial infarction occurs that causes the impairment of normal function and damage in cardiomyocyte (1). Considering that damaged cardiomyocytes are not able to rebuild and repair, mortality rate in this group of patients is incredibly high. Therefore, finding the available cell reservoir provides evolutional change in treatment of these patients through injection of differentiated cardiomyocytes to the location of myocardial infarction (2).

Several studies have proven that a suitable cellular source for the treatment of these patients must have some characteristics such as easy access, high proliferation power, resistance to ischemic conditions, low effect on immune system response and capability of differentiation to cardiomyocytes (1-2)

Despite the easy access to allogen cells, no proper cell source is found due to the immunological reactions (3-4). However, it seems that Embryonic Carcinoma cells (ECs) could be an appropriate source for successful treatment of this group of patients. The ECs are one of the types of stem cells which has high proliferation rate in culture media. One of the ECs is P19 cells which causes carcinoma in mice. However, they have a normal karyotype. In this connection, there are several reports that P19 cells is capable to differentiate into a variety of cells including cardiomyocytes in vitro, so that, P19 cells can solve

some of the current problems in cell and tissue grafts (5). There are several factors in the process of cardiomyogenic induction during embryonic period, including Bone Morphogenetic Proteins (BMP) which belongs to the Transforming Growth Factor β (TGF-β), involved in transcription of genes that has a key role in cardiomyogenic process (6).

Oxytocin (OT), a nonapeptide largely expressed in the hypothalamus, has long been recognized as a female reproductive hormone (7).

OT also has an influence on developing heart and cardiovascular functions, and excess administration to the fetus may impair cardiac growth in humans and rats [8-10]. OT_OTR system in the heart is expressed at higher levels in developing hearts than the rate in adult hearts, leading to consider OT as a potential naturally occurring cardiomorphogen (11-12)

5-azacytidine, as an antimitotic and anticancer medicine, is used to cure acute non-lymphocytic leukemia and myelodysplastic syndrome. Makino's et al used the stromal bone marrow cells under 5-Azacytidin stimulation differentiated to beating cardiomyocytes that transferring them to MI locations, caused accelerate repair and recovery process in the heart (13-15).

According to previous studies, both of the heart stem cells and non-heart stem cells after transplantation can differentiate into cardiomyocytes. It has been suggested that stem cells can stimulate angiogenesis through ischemic cardiomyocytes secretions of C-reactive protein (CRP), Interleukin 6 (IL6), and tumor necrosis factor (TNF) to keep ischemic cardiomyocytes alive and prevent the process of apoptotic progress. In addition, these phenomena stimulate proliferation of cardiomyocytes (16-17).

Also, several reports prove that cardiomyocytes differentiation depends upon concentration and duration of induction and several cardiogenic inducers have been described on mice heart developments. Now a days, several cardiomyogenic factors have been identified through the study of heart development in mice. In order to improve the efficiency of cardiomyocytes differentiation, some of the inducers such as 5-Azacytidin and OT are mainly used for in vitro differentiation (18).

In order to enhance the efficacy of cardiomyocytes differentiation through creating beating cardiac cells (BCs) in vitro, using differential inducers like Oxytocin (OT) and 5-Azacytidin (5Az) are helpful while considering the length of induction and concentration of inducer. The ability of the individual inducers has been proved. However, the comparison between OT and 5A is a novel process.

In this respect, we compared the effectiveness of oxytocin and 5-Azacyteidin induction on differentiation of P19 cells into a cardiomyocyte phenotype.

Materials and Methods

Primary Cell Culture

P19 cells (provided by Tehran Pasteur Institute of Iran) were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Inc., Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 2 mM l-glutamine, 1% w/w penicillin/ streptomycin (Gibco) in a 5% CO2 atmosphere at 37 °C. In this study, cultures of P19 cells were used with little variation at passage numbers.

Secondary Cell Cultures and Cardiomyocyte Differentiation Procedure

Cardiomyocyte differentiation of P19 cells were performed. Embryoid bodies (EBs) were created by hanging drop method (6). To induce the formation of EBs, the cells were dissociated into single cells, suspended again in the EB medium (primary cell culture medium) to a concentration of 800 cells per 20 µl. Twenty micro liter drops of the suspension were put on the inner surface of a petri dish lid. The cells were incubated at 37 °C in 5% CO2 for 2 days. Then, EBs were collected by aspirating with a 1 ml pipette and transferred to bacteriological dishes for 5 days to be either treated with 10 µM 5- azacytidine (5Az) (Sigma-Aldrich, Germany) or 10 nM Oxytocin (OT) (Wako Chemicals, Germany) and the EB medium (Ctrl). After 5 days in suspension, the (2+5; 7d) EBs were transferred to individual wells of 24-well plates until 12 days. The medium was renewed every 2 days.

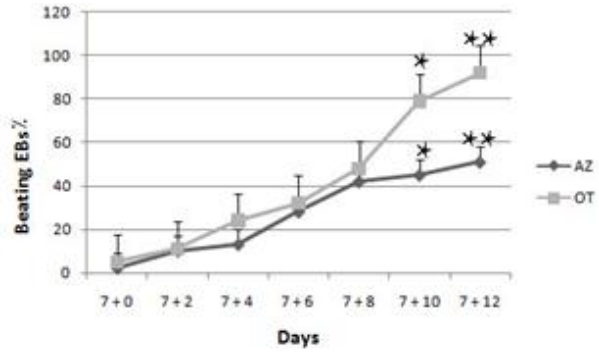

Figure 1. Time course of beating Embryoid Bodies (EBs) appearance after 5AZ and OT treatment in cultures of P19 cells (N = 7).

Quantitative analysis of EBs containing spontaneously contracting areas

7 d EBs exposed to the different treatments were transferred to individual wells of 24-well micro well

plates and monitored microscopically (Phase contrast microscopy,) every 2 days for the presence of contractions Until 12 days. The percentage of contracting EBs was examined.

Morphological features of cardiomyocyte differentiated of P19 cells

The morphological changes of 7 d EBs were observed by inverted microscopy daily after treatment with OT and 5Az.

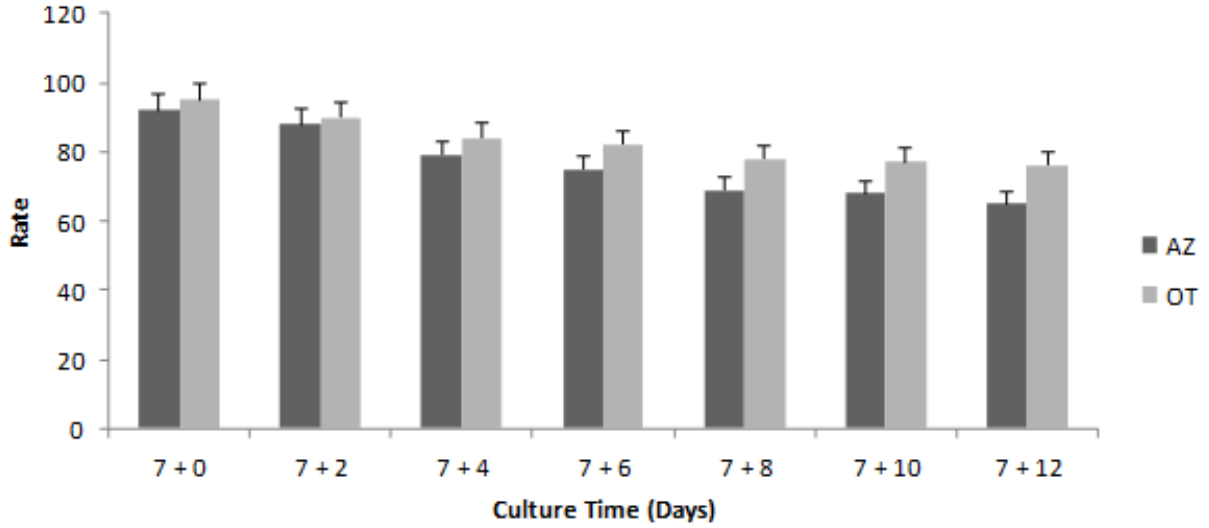

Figure 2. The comparison between the mean percent of viability rates in treatment with 5Az and OT.

Cell Viability

Viability of differentiated cells in different groups was carried out by trypan blue. Briefly, 4 × 104 cells per 200 ml medium were seeded into each well of a 24-well tissue culture plate for 24 hrs at 37 °C in 5% CO2. Viable cells in every 3 wells were counted by trypan blue exclusion in days 7+0, 7+3, 7+6, 7+9 and 7+12.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted by Trizol Reagent (Invitrogen Life Technologies) and transcribed into cDNA using oligo dT primer and reverse transcriptase (Fermentas). Primer sequences were as follows: NKX2.5 (Product size: 461bp) 5′-CCAAGGACCC TAGAGCCGAA-3′ (F) and 5′-ATAGGCGGGGTA GGCGTTAT-3′ (R), Atrial natriuretic factor (ANF, Product size:203bp) 5′-TGATAGATGAAGGCAGG AAGCCGC-3′ (F) and 5′-AGGATTGGAGCCCAG AGTGGACTAGG-3′(R), Cardiac α-myosin heavy chain (α-MHC, Product size:301 bp) 5′-CTGCTGG AGAGGTTATTCCTCG-3′ (F) and 5′-GGAAGAG T GAGCGGCGCATCAAGG-3′ (R), cTnT (Product size: 150bp) 5′-GGCAGCGGAAGAGGATGCTGAA-3′ (F) and 5′-GAGGCACCAAGTTGGGCATGAACG AC-3′ (R), α-actinin (Product size: 223bp) 5′-TGTT GGAGTGGACCGCCGCACAA -3′ (F) and 5′ - CAT CCTGCCCTCAGAGGGCATGAA-3′(R), (6); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Product size: 302bp) 5′-A GCCACATCGCTCAGA CACC-3′ (F) and 5′- GTACTCAGCGGCCAGCA TCG-3′ (R) exon specific oligonucleotide primers was used as internal standard. PCR products were separated on a 1.5% agarose (Fermentas) and stained with ethidium bromide, visualized and photographed on a UV transluminator.

Statistical methods

Quantitative data was expressed as mean + minus SEM from at least three experiments. Treatments were compared by unpaired Student's t test and P values less than 0.05 were considered significant.

Results

To determine the cardiomyogenic effect of 5Az and OT, the number of EBs that had beating areas was quantified every 2 days.

The length of cell differentiation was divided into three stages: the first one was shortly after initiation contraction at the early developmental stage (days: 7+0 to 7+4), the second or intermediate stage (days: 7+4 to 7+8) and the final stage (days 7+8 to 7+12). The count results of EBs revealed firstly; the EBs derived from P19 cells in both OT and 5Az treatments provided differentiated beating cardiomyocytes. Secondly, beating area percentage of EBs increased during first to final stages from days 7+0 to 7+12. Thirdly, beating area percentage of EBs in OT treatments group was more than 5Az group in

all days of experiment. However, only in the final stage a significant increase was observed in beating area of EBs of OT group (Figure 1).
The viability percentage of EBs in two groups was gradually decreased from 7+0 to 7+12, but there was no significant difference between the two groups (Figure2). The morphology of OT induced cells gradually transformed to cardiomyocytes.
The slight elongation of cells was detected around day 7+4. Shortly after initiation of contractions, differentiated cells begun thinning out and tube-like cells and the nucleus slightly positioned away from the center. The morphological analysis of differentiated cardiomyocytes at day 7+8 in intermediate stage demonstrated that mononuclear cells have a large euchromatin nucleus. In intermediate stage, the cells expanded their processes and created myotube like organization through formation of connections to the neighborhood differentiated cells.

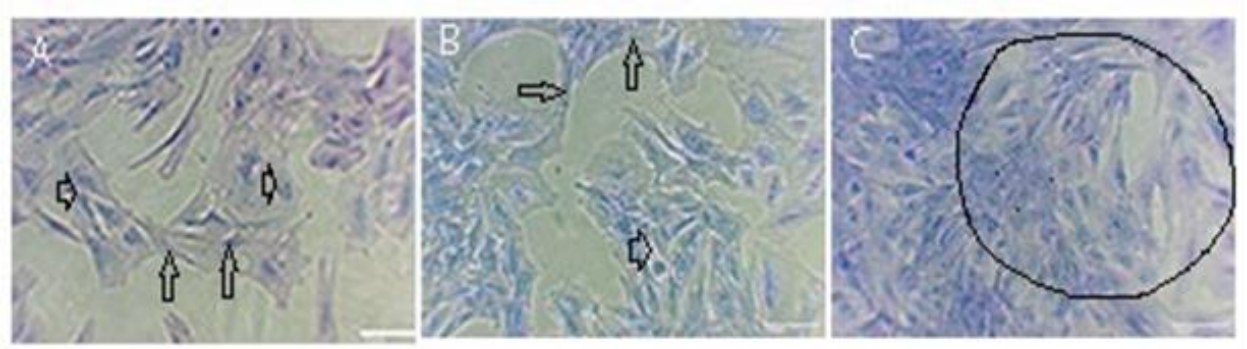

Figure 3. The morphological appearance of differentiated P19 cells. (A) Polygon-like morphological features on day 7+4 (arrow heads). (B) Adjoining cells forming myotube-like structures on day 7+8 (arrow). (C)Stick-like appearance and aligned parallel to each other on day 7+12 (circle area).

The morphological changes were seen at the terminal stage of induction; at day 7+12. Differentiated cells became thinner and longer formed clusters of 8-10 cells aligned parallel to each other. Differentiated cells seemed striated with eccentric nucleus and end branched (Figure 3).
A similar phenotypic change was already reported in treatment with 5Az.

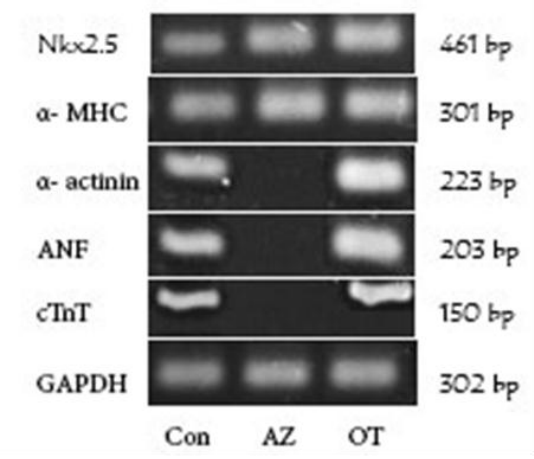

Figure 4. RT-PCR analysis on cardiac-specific markers (Nkx2.5, α- MHC, α- actinin, ANF, cTnT) expression in 7 days EBs. Mouse heart ventricle mRNA was used as a positive control.

The expression of several cardiomyocyte-associated genes was assessed in 7 days EBs by RT-PCR. As shown in Figure 4. 7 days EBs differentiation with the Oxytocin expressed the Nkx2.5 as well as, α -MHC, α -actinin, ANF and cardiac troponin T (cTnT). However, Only the Nkx2.5 and α –MHC expression were expressed in the 5Az treatment (Figure 4).
Cardiac-specific genes expression revealed comparison of the cardiomyogenic effect of 5Az and OT using RT-PCR.

Discussion

The carcinoma P19 cell line has a specific role in determination of mechanism of evolutionary biology which makes it as an excellent cell differentiation model that mimics the events of early cardio-embryogenesis. It is essential to know about the optimum condition of P19 cell culture to induce differentiation to the individual cell. According to some reports, OT levels were significantly higher in the heart of fetuses compared to those in the adult animals. In addition, OT levels decreased during development (12). It reveals that the oxytocin / oxytocin receptor (OT/OTR) system is necessary for heart formation and function. On the other hand, OT could be considered as a serum-borne factor that modulates intracellular Ca2+ concentration that may affect the cardiac-specific ionic channels during heart development and also in vitro differentiation (16). One of the main reason increase in beating area of EBs and major morphological changes at the final stage of induction (at day 7+12) in OT treatment

compared with 5Az reflected to Ca2+concentration that lead to differentiation myogenic phenotype and formation of myotubes from P19 cells.

Cardiac differentiation of mesenchymal stem cells (MSCs) induced by the demethylating agent 5-azacytidine is controversially discussed (17). Martin-Rendon et al. reported that 5-azacytidin treated human mesenchymal stem cells derived from umbilical cord and bone marrow do not generate cardiomyocytes in vitro at high frequencies (19).

Trixi Hollweck et al. have demonstrated that cardiac differentiation of UCMSCs induced by oxytocin leads to a higher frequency of cardiac specific protein expression than treatment by 5-azycytidine (20).

Based on cardiac specific markers such as cardiac troponin T (as troponin complex subunit), ANF and α–actinin were expressed at the early developmental stage of OT treatment, it seems that OT can be make to more differentiation P19 cells into highly specialized phenotypes of the cardiomyocytes at the early developmental stage.

Epigenetic regulation should also be considered. Major DNA modifications occur during in vitro cardiogenesis from P19 that can influence differentiation procedure and mature cardiomyocyte (21).

Seung-Cheol Choi et al. have shown that P19 cells can differentiate into cardiomyocytes by a 5-Az treatment, a demethylation agent, without exposure to DMSO in the P19 confluent monolayer culture in the absence of prior EB formation, depending at least in part on alteration of BMP signaling cascade. This phenomenon reveals that the demethylation process is capable of altering gene expression or regulating cellular differentiation in cardiac differentiation (18).

In present study we used OT and 5Az to realize specification of differentiated cardiomyocytes from P19 in vitro.

Present results have shown that, P19 stem cells simultaneously in presence of two inducers, OT and 5Az differentiated to cardiomyocytes. The statistical analysis revealed the response to OT inducer is more excessive than 5Az and EBs beating area percentage significantly increased in OT treatment compared to 5Az in final stage. However, the response to OT in cell culture was always higher than 5Az.

The results of gene expression revealed that the Nkx2.5, α-MHC, α-actinin, ANF and cardiac troponin T (cTnT) were expressed at the early developmental stage of OT treatment. However, α –actinin, ANF and cardiac troponin T (cTnT) were not present at the early developmental stage of 5Az treatment. The cardiomyocyte-associated genes Nkx2.5 and α –MHC were expressed with OT and 5Az differentiation procedures. It should be noted that express cardiac myosin heavy chain lead to the movements of induced cells and EBs beating.

According to current results Oxytocin is more effective inducer of cardiomyocytes differentiation from embryonic carcinoma cells P19 than 5-azacytidine.

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Author contributions

All authors have made substantial contributions to the conception and design of the study, doing the experiments, acquisition or statistical analysis and interpretation of data. Drafting the article or revising it critically for important intellectual content have done by M-ShM, Gh-HH and N-BM. All authors also contributed in final approval of the version of manuscript which to be submitted.

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Conflict of Interest

The authors declare no conflict of interest.

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