Original Article

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An Evaluation of the Influence of Royal Jelly on Differentiation of Stem Cells into Neuronal Cells Invitro

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ABSTRACT

BACKGROUND AND OBJECTIVE: P19 carcinoma stem cells are able to differentiate into embryonic cells with three germ layers. Given the fact that differentiated cells can reduce complications associated with degenerative diseases of the nervous system, in this study, we aimed to investigate the differentiation of P19 stem cells into neuronal cells under the influence of royal jelly.

METHODS: In this basic-applied research, embryoid bodies, prepared in P19 cell suspension culture, were transferred to gelatinized containers and classified in six groups, receiving 25, 50, 100, 150, 200, and 300 mg/ml of royal jelly, respectively; five wells were allocated to each group of cells. Morphological evaluation of cell differentiation was performed via cresyl violet staining. Also, immunofluorescence technique was used to track the expression of neuronal marker proteins such as synaptophysin and β-tubulin III. Finally, the findings were analyzed.

FINDINGS: The present findings showed that cells exposed to royal jelly responded positively to specific staining of nerve cells. In groups receiving different concentrations of royal jelly, the mean percentage of cell differentiation was significantly higher than the negative control group (9±2.3). The highest percentage of cell differentiation was observed in groups treated with 200 and 300 mg/ml of royal jelly, respectively (98±4.8 and 99.3±2.2, respectively) ($p<0.05$). Based on the findings, the mean percentage of cell differentiation in the group receiving 200 mg/ml of royal jelly was not significantly different from the group receiving a concentration of 300 mg/ml.

CONCLUSION: The results of the present study showed that P19 cells are able to differentiate into neuronal cells, and therefore, they could be used in cell-based therapy for neurological diseases.

KEY WORDS: *Carcinomatous stem cells, Differentiation of neural stem cells, Neural markers.*

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Introduction

Degenerative diseases of the nervous system, such as Alzheimer's disease, are caused by the degeneration of nerve cells and aggregation of toxic proteins in the central nervous system (1). So far, various treatment methods, e.g., decreasing the level of accumulated proteins, have been proposed, which involve reducing the blood-brain barrier permeability for the treatment of these disorders (2). Cell-based therapy, which incorporates the use of stem cell differentiation into neurons, is one of the methods which has been highlighted in scientific communities in recent years. So far, differentiation of various stem cells (e.g., embryonic stem cells), triggered by different factors, has been evaluated in multiple studies (3).

In general, according to the literature, neural cells can be produced through the differentiation of human and mouse embryonic stem cells (4). P19 cells are multipotent, embryonic carcinoma cell lines, which can grow in a serum medium. These cells can be triggered to differentiate into all three lineages, i.e., mesoderm, endoderm, and ectoderm (5). When these cells are exposed to retinoic acid, they differentiate in the neuroectoderm, and consequently, neurons and glial cells fill the medium (6).

Several studies have been conducted on the adhesion of retinoic acid-exposed P19 cells to the striatum of immunocompromised rats (7). In this regard, a previous study, which evaluated the effects of deprenyl on the induction of P19 cell differentiation into neurons, revealed that this agent could induce neuronal differentiation in a dose-dependent manner (8). Besides chemical compounds which are used to control and reduce neurological complications, a number of natural compounds are used by indigenous people to manage and treat neurological diseases (6). Royal jelly is one of the substances with protective and anti-apoptotic effects on nerve cells. It is a viscous jelly substance, with a milk-white or pale yellowish color, a characteristic odor, and unpleasant taste (9, 10). In a previous study, it was revealed that cells, treated with adenosine monophosphate (AMP) N1-oxide of royal jelly, have the ability to express specific proteins of mature nerve cells. In addition, Hattori and colleagues showed that AMP N1-oxide in royal jelly could enhance the proliferation of progenitor cells and neural stem cells (11). Overall, the results of previous research in this area suggest that AMP N1-oxide could facilitate the production of astrocytes in neural progenitor cells by promoting the phosphorylation of STAT3 transcription factors. Social and economic damages, caused by degenerative diseases of the nervous system, are sharply rising in developing countries. Therefore, further research is required to control the prevalence of these disorders and reduce the associated complications. In this study, we aimed to induce neuronal phenotype in P19 cells by using royal jelly.

Methods

Materials and Methods: In this basic-applied study, the effect of royal jelly on the differentiation of P19 stem cells into neuronal cells was studied under laboratory conditions.

Cell culture: P19 teratocarcinoma cells were used in this study. The cells were obtained from the Cell Bank of Pasteur Institute of Tehran, Iran and proliferated in adhesive culture. Afterwards, the cells were cultured in alpha-minimum essential medium (α-MEM) (Gibco, Cat. No.: 11900-073), containing 10% fetal bovine serum (FBS) (Sigma, Cat. No.: 10270-106), 50 µg/ml of penicillin (Sigma, Cat No.: P3032), and 50 µg/ml of streptomycin (Sigma, Cat No.: S1277). The first step in the induction of differentiation is the formation of embryoid bodies. For this propose, P19 cells were cultured for 24 h in Petri dishes with no adhesive properties. The obtained embryoid bodies were transferred to gelatinized 12-well Petri dishes for cell culture. Afterwards, they were exposed to 25, 50, 100, 150, 200, and 300 mg/ml of royal jelly, respectively. For morphological evaluation of cell differentiation, specific staining of nerve cells was performed, using cresyl violet (Sigma, Cat. No.: 234-043-3).

The stained cells were counted in order to determine the optimal concentration of royal jelly, which could trigger the differentiation of more cells into neuronal cells. Moreover, a squared graticule was used to count neuronal cells which were stained purple with cresyl violet. The immunofluorescence technique was used to track the expression of specific markers of neuronal cells in differentiated cells. In this study, we used primary monoclonal β-tubulin III (Abcam, ab7751) and primary monoclonal synaptophysin (Abcam, San Francisco, CA, ab8049) antibodies. Also, fluorescein isothiocyanate-conjugated immunoglobulin G (IgG) (Sigma, Cat. No.: F9137) was used as the secondary antibody.

Preparation of different concentrations of royal jelly: Royal jelly was purchased from beekeepers in

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Shahrekord, Iran and mixed with phosphate-buffered saline (PBS) at a ratio of 0.025. The mixture was slowly shaken overnight at 4°C in an incubator; then, it was centrifuged at 12,000 g for 10 min at 4°C. In the next step, the supernatant was collected after centrifugation and its concentration was determined by Bradford assay. Finally, by using the culture medium, concentrations of 25, 50, 100, 150, 200, and 300 mg/ml were prepared, respectively.

Preparation of embryoid bodies: The first step in the induction of stem cell differentiation is the formation of embryoid bodies. For this purpose, P19 cells were cultured for 24 h in Petri dishes with no adhesive properties. Then, embryoid bodies were transferred to gelatinized six-well Petri dishes for cell culture. The cultured cells were divided into seven groups, each exposed to 0, 25, 50, 100, 150, 200, and 300 mg/ml of royal jelly, respectively. After 24 h, the culture medium was changed and the cells were cultured in a medium containing 10% serum for two weeks; it should be noted that the cell culture medium was replaced in all groups every two days. Neuronal phenotypes appeared in the cultured cells within ten days following the treatment.

Cresyl violet staining: Cresyl violet staining was used to evaluate the morphology of differentiated cells. For this purpose, the cells were washed with PBS and then fixated with 4% paraformaldehyde. Cellular hydration was performed in a solution containing ethanol and acetic acid. Afterwards, the cells were washed three times with PBS and incubated for 10 min in a mixture containing cresyl violet (0.25%; Sigma, Cat No.: 234-043-3), glacial acetic acid (0.8), sodium acetate (0.6 mM), and water (100 ml). Finally, the cells were washed with PBS and the cell plates were mounted on a slide, using Canada Balsam glue.

Determination of the optimal concentration of royal jelly: The stained cells were counted in order to determine the best concentration of royal jelly, which could differentiate more cells into neuronal cells. To count neuronal cells which were stained purple with cresyl violet, a squared graticule was used. For this purpose, five fields of view in the light microscope were randomly evaluated at 20X magnification for each sample.

Immunofluorescence technique: Immunofluorescence technique was employed to track the expression of neural cell markers within differentiated cells. In this study, primary β-tubulin and synaptophysin antibodies were used; also, conjugated IgG antibody was used as the secondary antibody. To perform immunofluorescence, cells exposed to royal jelly were incubated for 30 min with 4% paraformaldehyde solution. Following fixation, the cells were washed three times with PBS. The cells were then incubated using a blocking solution containing 0.03% Triton X100 and 0.010 of normal goat serum (Sigma, Cat. No.: G9023:NGS) in PBS for 30 min at 37°C. These cells were incubated for 2 h with the primary antibodies at 37°C. Moreover, after washing the cells with PBS, they were incubated for 2 h with secondary antibodies. Finally, after washing the cells with PBS, glycerol (0.070) was used to mount the lamellae on the slide. By using the microscope, immunofluorescence confirmed β-tubulin and synaptophysin expression in differentiated cells.

Statistical analysis: Statistical analysis was performed, using SPSS. Kolmogorov-Smirnov test was used to check the normal distribution of the data. To assess the difference between the groups, analysis of variance (ANOVA) and Duncan's post-hoc tests were performed and $p<0.05$ was considered statistically significant.

Results

Morphological examination: Embryoid bodies were cultured after 24 h of exposure to royal jelly for 10 days. The proliferating cells were emitted from the embryoid bodies (fig 1).

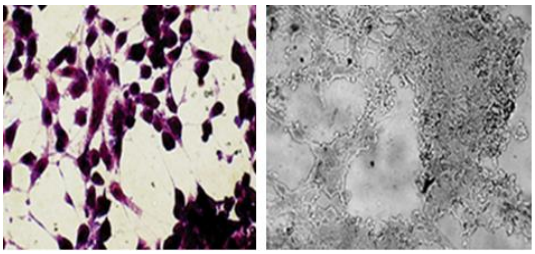


Figure 1. Photomicrograph of P19 cells differentiated into neuronal cells by cresyl violet staining. The stained nerve cells

Determination of the optimal dose of royal jelly for the induction of neuronal phenotypes: Neuron-like cells were stained purple, using cresyl violet. The mean percentage of neuronal differentiation in groups exposed to different concentrations of royal jelly was significantly higher than the negative control group (9±2.3). Based on the findings, maximum percentage of neuronal differentiation was reported in groups exposed to 200 and 300 mg/ml of royal jelly, respectively (98±4.8 and 99.3±2.2, respectively). The percentage of neuronal differentiation significantly

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increased in groups exposed to 200 and 300 mg/ml of royal jelly, compared to cells treated with 25 (21±2.2), 50 (32±2.3), 100 (71.3±5.3), and 150 mg/ml (83±5.9) of royal jelly, respectively ($p<0.05$). However, as the finding revealed, the mean percentage of neuronal differentiation in the treatment group receiving 200 mg/ml of royal jelly was not significantly different from the group exposed to 300mg/ml of royal jelly ($p<0.05$) (table 1).

Confirmation of neuronal cells by immunofluorescence: Based on the immunofluorescent assessment of differentiated P19 cells, these cells showed a positive immune response to β-tubulin III and synaptophysin after exposure to royal jelly. Neurons positive for synaptophysin and β-tubulin were traced as single cells or small clumps of cells (fig 2).

Table 1. Evaluation of the effects of different concentrations of royal jelly on the induction of neuronal phenotypes in P19 cells

Concentration of royal jelly (mg/ml)	Percentage of nerve induction (PNI) (Mean±SD)
25	21±2.2 [b,c,d,e,f*]
50	32±2.3 [a,c,d,e*]
100	71.3±5.3 [a,b,d,e,f*]
150	83±5.9 [a,b,c,e,f*]
200	98±4.8 [a,b,c*]
300	99.3±2.2 [a,b,c,d*]
Control	9±2.3 [a,b,c,d,e]

*p<0.05

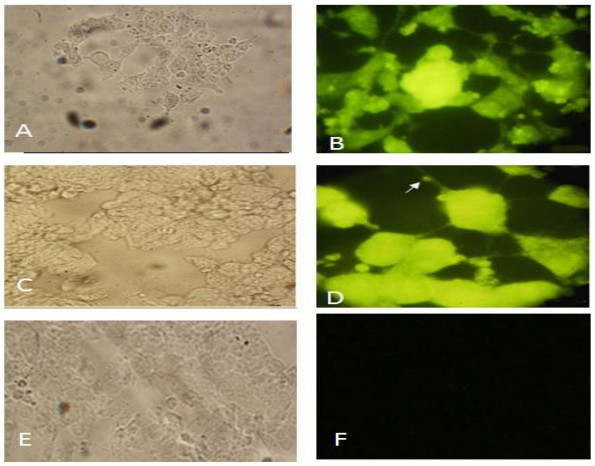


Figure 2. Fluorescence microscopic images of neuronal cells yielded by the differentiation of P19 cells under the influence of royal jelly.

A) Light microscopic image; B) fluorescence microscopic image of cells treated with royal jelly and exposed to primary synaptophysin antibody; C) light microscopic image; D) fluorescence microscopic image of cells treated with royal jelly and exposed to primary β-tubulin antibody; E) light microscopic image; F) fluorescence microscopic image of P19 cells exposed to primary synaptophysin antibody by not royal jelly.

Discussion

In the present study, ten days after the treatment of embryoid bodies with royal jelly, the morphology of a number of cells was shown to be similar to that of neuronal cells; also, these cells responded positively to specific nerve cell staining. The results showed that by increasing the concentration of royal jelly, the percentage of differentiated cells increased in a dose-dependent manner.

These cells expressed specific neuronal markers such as synaptophysin and β-tubulin. In various studies, P19 cells have been extensively used for differentiation into neuronal cells. Chou and colleagues showed that differentiation of these cells into neuronal cells is influenced by retinoic acid. The differentiated cells showed the expression of nestin gene on the eighth day, which is a characteristic marker of neuronal cells (12).

Moreover, in a study conducted by Martin and colleagues, it was revealed that P19 cells, exposed to non-toxic concentrations of dimethyl sulfoxide, exhibited a similar morphology to neuronal cells (13). In addition, in a previous study, neural differentiation of P19 cells, triggered by cell growth factors, was reported (14). Furthermore, in a study by Bakhshalizadeh et al., deprenyl could induce dose-dependent neural differentiation in P19 cells transfected with green fluorescent protein (GFP). Moreover, they revealed that GFP-positive neurons derived from P19 cells could migrate in the chick embryo nervous system and form synapses with the host tissue cells (15).

In a study by Abdanipour and colleagues, by extracting adult stem cells, neuronal differentiation was induced by deprenyl at 10^{-6} and 10^{-11} mM concentrations (16). In addition, Karadeniz et al. showed that cisplatin, a chemotherapy agent, could result in a significant increase in hepatic cell death. On the other hand, royal jelly exerted protective and anti-apoptotic effects on hepatocytes by increasing the production of superoxide dismutase in liver cells (17). According to the literature, use of cisplatin might lead to the emergence of risk factors and reduce the antioxidant activities of superoxide dismutase and glutathione peroxidase in the blood. However, concomitant use of royal jelly and cisplatin resulted in a decline in oxidative stress factors, while increasing the level of antioxidants (18). Overall, oxidative stress is not only one of the major contributing factors for cancer, but is also a major inducer for other disorders

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(19-28). Hashimoto showed that oral administration of royal jelly could increase the expression of neurotrophic factors, their receptors, and neural cell markers in the hippocampus of adult mice (29). Additionally, a study by Hattori and colleagues showed that Amp N1-oxide in royal jelly could elicit neuronal differentiation in PC12 cells and trigger an increase in phosphorylation and astrocyte-specific transcription factors (30). In conclusion, the present findings suggested P19 cell line as an appropriate and cost-effective source for studying the effects of various factors involved in the growth and differentiation of neurons *in vitro*; moreover, P19 cells could be regarded as an interesting model for mass production of neural cells. The results of this study also showed that royal jelly could trigger the differentiation of P19 stem cells into neuronal cells.

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