Research Paper

The Effect of Rosmarinic Acid on Neural Differentiation of Wartons Jelly-derived Mesenchymal Stem Cells in Two-dimensional and Three-dimensional Cultures using Chitosan-based Hydrogel

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ABSTRACT

Introduction: Numerous studies have shown the positive effects of rosmarinic acid on the nervous system. Rosmarinic acid as a herbal compound with anti-inflammatory effects can prevent the destructive effect of inflammation on the nervous system. Furthermore, various studies have emphasized the advantages of three-dimensional (3D) culture over the two-dimensional (2D) culture of cells.

Methods: In this study, thermosensitive chitosan (CH)-based hydrogel as a 3D scaffold with the combination of chitosan, beta-glycerol phosphate and hydroxyl ethyl cellulose (CH-GP-HEC) loaded with rosmarinic acid was used to induce neuronal differentiation in human Wharton jelly stem cells. Also, cells were divided into eight groups to evaluate the effect of 3D cell culture and to compare gene expression in different induction conditions.

Results: The results of gene expression analysis showed the highest expression of neuronal markers in Wharton’s jelly-derived mesenchymal stem cells (WJMSCs) cultured in chitosan, beta-glycerol phosphate and hydroxyl ethyl cellulose (CH-GP-HEC) loaded with rosmarinic acid. According to the results of gene expression, rosmarinic acid alone has a positive effect on the induction of expression of neural markers. This positive effect is enhanced by cell culture in 3D conditions.

Conclusion: This study shows that rosmarinic acid can be considered an inexpensive and available compound for use in neural tissue engineering. The results of this study indicate that rosmarinic acid can be considered a cheap and available compound for use in neural tissue engineering.
1. Introduction

Ulcerative colitis (UC) inflammation is one of the causes of damage to the central nervous system (CNS). Therefore blocking the molecular pathway of inflammation can enhance nervous system regeneration (Rust & Kaiser, 2017). Rosmarinic acid is one of the herbal phenolic compounds known for its anti-inflammatory, antioxidant, anti-bacterial, and neuroprotective properties (Adomako-Bonsu et al., 2017; Fonteles et al., 2016; Hase et al., 2019; Nieto et al., 2018). The neuroprotective effects of Retinoic Acid (RA) have been investigated in various diseases, such as Alzheimer’s and Parkinson’s diseases using animal models (Hamaguchi et al., 2009; Wang et al., 2012). Studies have shown that RA increases the expression of neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), which has an essential role in the plasticity of synapses (Hwang et al., 2016; Lu et al., 2015).

On the other hand, cell-based tissue engineering has been widely considered to repair the central nervous system. Various types of mesenchymal stem cells (MSCs) have been studied for this purpose (Simorgh et al., 2019). Wharton’s jelly-derived mesenchymal stem cells (WJMSCs) as a critical source of mesenchymal stem cells have higher proliferation and differentiation potential than bone marrow mesenchymal stem cells (Bharti et al., 2018; Wang et al., 2004). They preserve their stem cell properties even after frequent passages (Allahbakhshi et al., 2013). The immune response to these cells is very low and they are not tumorigenic, and due to the role of modulating the immune response from these cells, it can be used in allogeneic transplantation (Vawda & Fehlings, 2013). Many studies have demonstrated the differentiation of Wharton jelly mesenchymal stem cells to neurons in vitro and in vivo. One study showed that WJMSCs can differentiate into Schwann cells in the presence of glial growth factors. The same study also found the medium condition of differentiated WJMSC can increase the neurite outgrowth in the pheochromocytoma (PC)-12 cell line (Peng et al., 2011). Differentiated WJMSC has also been shown to improve neuronal function in the rat brain after ischemic stroke (Zhang et al., 2006).

Hydrogels are similar to soft tissues in terms of water content and mechanical properties. They also have good oxygen permeability and high biocompatibility. Chitosan (CH) is a natural biocompatible and biodegradable polysaccharide obtained by the deacetylation of chitin. CH can be dissolved in aqueous acidic solutions due to its chemical structure (Supper et al., 2014). It has an anti-free radical and neuroprotective effect and an adjustable degradation rate that support neural cells to adhesion and growth (Gnawi et al., 2013). CH has an anti-bacterial feature (Fregnan et al., 2016) and supports the neural differentiation of stem cells (Zhang et al., 2016). Various studies have been performed on the use of chitosan and its derivatives in tissue engineering, including nervous system tissue engineering.
In a study, photocrosslinkable chitosan-based hydrogels were used for nerve tissue engineering and it was shown that chitosan-based hydrogels can induce neuronal differentiation in neural stem cells (Valmikinathan et al., 2012). Chitosan has also been widely used to produce thermosensitive hydrogels. Thermosensitive hydrogel scaffolds can be used to transport stem cells and drugs (Liu et al., 2016). They mimic the natural extracellular matrix and provide a suitable environment similar to three-dimensional (3D) tissue for cell growth (Shen et al., 2015; Supper et al., 2014). Crompton et al. showed that polylysine-functionalized thermoresponsive chitosan hydrogel is a suitable scaffold for nerve tissue engineering (Crompton et al., 2007). Studies have shown that β-glycerol phosphate (β-GP) at 37 °C can act as a catalyst in the sol-gel transfer of chitosan solution (Pankongdisak & Suwantong, 2018). Furthermore, hydroxyethyl cellulose (HEC) is used as a protective colloid in the polymerization process (Zulkifli et al., 2019). Various studies have used CH--β-GP-HEC (to make thermosensitive hydrogels for use in tissue engineering). Due to its low cytotoxicity, this hydrogel can be used as a stem cell carrier for liver tissue engineering (Haddad-Mashadrizeh et al., 2013). Karimpour Malekshah and colleagues also found that CH-β-GP-HEC can be used to induce chondrogenic differentiation in adipose tissue-derived mesenchymal stem cells (Karimpour Malekshah et al., 2016).

To investigate the difference between two-dimensional (2D) and 3D cell cultures, various culture systems can be selected. Khodabandeh et al. used 2D collagen films and 3D collagen scaffolds for the culture of HWJMSCs. (Khodabandeh et al., 2016). In another research, Hosseini et al. used 3D alginate scaffolds and a 2D monolayer system to differentiate WJMSCs into neurons (Hosseini et al., 2015). In a study, perinatal MSC from Wharton’s Jelly of the umbilical cord (UC-MSC) was used for their differentiation capacities due to partial expression of pluripotency markers in bone tissue engineering. To this end, UC-MSCs were cultured on 3D collagen I/III gels and osteogenic differentiation occurred, showing all features needed for effective bone fracture healing (Schneider et al., 2010). Bagher et al. investigated the effect of the 3D nano-scaffolds on (WJMSCs) differentiation into neuronal motor lineages in the presence of RA and Sonic Hedgehog (SHH). The results showed adhesion, proliferation, and differentiation of WJMSCs (Bagher et al., 2016). In another study, a 3D biomimetic nano-hydroxyapatite/chitosan/gelatin (nHA/CH/Gel) scaffold was used to differentiate WJMSCs. Results indicated that WJMSCs attached to the scaffold surface and uniformly spread throughout the contacting surface (Jamalpoor et al., 2019).

This study was conducted to assay the effect of rosmarinic acid in the neural differentiation of WJMSC in three dimensions culture using a thermosensitive chitosan-based hydrogel (CH-hydrogel).

2. Materials and Methods

Study design

To evaluate the effect of RA-loaded CH-hydrogel on the induction of neural differentiation in WJMSCs, WJMSCs were divided into several groups, including A) WJMSCs (as a control group)

B) WJMSCs+neural differentiation medium
C) WJMSCs+CH-hydrogel loaded with RA
D) WJMSCs+CH-hydrogel loaded with RA+neural differentiation medium
E) WJMSCs+RA+neural differentiation medium
F) WJMSCs+RA
G) WJMSCs+CH-hydrogel
H) WJMSCs+CH-hydrogel+neural differentiation medium

WJMSCs isolation and characterization

Umbilical cord samples were collected from full-term newborns from healthy mothers under ethical consent and transferred into a lab in sterile phosphate buffer saline (PBS, Sigma-Aldrich, USA) containing 3% W/V penicillin (100 IU/mL, Sigma Aldrich, US)/streptomycin (100 μg/mL, Sigma Aldrich, US). Samples were washed twice using sterile PBS and then cut into small parts and vessels were removed. Afterward, Wharton jelly was chopped (into approximately 1 mm 3 pieces) and directly transferred into cell culture flasks containing Dulbecco’s modified eagle medium/nutrient mixture F-12 (DMEM/F-12) containing 10% fetal bovine serum (FBS; Gibco) and incubated at 37°C in a carbon dioxide (CO2) incubator. The cell was passaged using 0.25% W/V trypsin/ Ethylenediaminetetraacetic Acid (EDTA), and cells were used on passage 3. The isolated cells from passage 3 were characterized using flow cytometry (BD FACS Calibur Bioscience, USA) for CD90, CD44, CD105, and CD74 as mesenchymal stem cells positive markers and CD34, CD45 as negative markers. Accordingly, the cells were incubated with antibodies against CD90 (1:200), CD44 (1:300), CD34 (1:100), and CD45...
(1:100) according to the manufacturer’s instructions. The results were analyzed using FlowJo software (version 7.6.1)

**Fabrication of chitosan-hydrogel**

A total of 225 mg CH (Sigma Aldrich-448869-50g) was dissolved in 9 mL of 0.1 M hydrochloric acid by mechanical shaking. The prepared solution was autoclaved and after cooling, it was kept at 4°C until use. Next, cold βGP solution (3% W/V in deionized water sterilized via filtration using a 0.22 µm syringe filter) was added dropwise to the cold CH solution with continuous stirring. After adding βGP, the final volume of the solution reached 15 mL. For the preparation of the HEC solution, 125 mg of HEC (Sigma Aldrich) was dissolved in 10 mL of PBS. HEC solution and CH-β-GP solution were mixed with a ratio of 1:6 To prevent gelling formation, CH-β-GP was stored on ice and the HEC solution was added to CH-β-GP just before injection. Separately to prepare RA stoke, 10 mg of RA Sigma (R4033-10MG) was dissolved in 500 µL DMSO. RA and cells were also added to CH-β-GP-HEC before gelation.

**Scanning electron microscopy (SEM)**

The hydrogel morphology and porosity were investigated using scanning electron microscopy (SEM) (HI-TACHI-S4160). To complete the gelation process, the RA was added to CH-β-GP-HEC solution, and the final solution was transferred to 37°C for 1 h. Before freeze-drying, samples were kept at -80°C. samples were gold coated using a sputter coater (Technics, Hummer II, Japan) before SEM.

**Cytotoxicity assay**

A 2,5 diphenyl tetrazolium bromide (MTT) assay was performed to evaluate cytotoxicity three days after seeding cells on the hydrogel. 5×10^4 WJMSCs per cm² were seeded in a 96-well plate containing prepared rosmarinic acid-loaded hydrogels and culture media then incubated for 72 h. After removing the cell culture medium, 200 µL MTT solution (0.5 mg/mL in PBS) was added into each well, and cells were incubated at 37°C for 4 h. Afterwards, the MTT solution was removed and formed formazan crystals were dissolved with 100 µL of DMSO. After several times aspiration, the absorbance was measured at 570 nm using a Microplate Reader (Model 680 S/N 21116).

**Induction of neural differentiation**

To induce neural differentiation, WJMSCs were cultured at 4*10^4 per well in a 48-well plate (SPL, China), including RA-loaded CH-hydrogel. After 24 h culture medium was replaced with differentiation medium containing (Zhang et al., 2006): DMEM/F-12 (DMEM/F12) (Gibco), 10% KnockOut serum replacement (KSR) (Gibco), 20 ng/mL epidermal growth factor (EGF) (Invitrogen), 20 ng/mL basic fibroblast growth factor (bFGF) (Invitrogen) and 10 mg/mL heparin (Invitrogen). After 4 days, the cell’s morphology was evaluated by Phase Contrast Microscope. Also, real-time-polymerase chain reaction (PCR) was performed to evaluate neural differentiation in treated WJMSCs.

**Ribonucleic acid (RNA) extraction and real-time polymerase chain reaction (PCR)**

Ribonucleic acid (RNA) extraction was performed using the Qiagen kit according to manufacturer protocols. The concentration of RNA in each sample was measured by the nanodrop (Thermofisher, USA). Afterward, total RNA was converted to complementary DNA (cDNA) using an RT2 HT First Strand Kit (Qiagen, Germany) according to the manufacturer’s protocol. Finally, for SYBR green-based real-time PCR, 10 µL SYBR green, 1 µL Rox, 0.7 µL F primer, 0.7 µL R primer, 1 µL complementary DNA (cDNA), and 6.4 µL RNase free water were mixed in 20 µL total volume and PCR performed in triplicate using ABI Step One device (Applied Biosystems, Sequences Detection Systems, Foster City, CA) for 40 cycles. (Table 1). The target gene expression level (nestin and β-tubulin III) was normalized to the reference gene (β-actin) using the ΔΔCT formula.

**Statistical analysis**

Data were expressed as Mean±SD of three separate experiments. One-way analysis of variance (ANOVA) was performed using SPSS software, version 23. Statistical significance was set at P<0.05.

3. Results

**WJMSCs isolation and characterization:**

Seven days after isolation, most of the attached cells were bipolar and spindle-shaped (Figure 2A). After 3 passages, cells expressed CD44, CD105, CD90, and CD73 (98.6%, 91.4%, 98.3%, and 95.9%, respectively) and most of the cells expressed CD45 and CD34 (4.75%, and 2.74%, respectively) were negative as hematopoietic markers (Figure 1).
Hydrogel characterization

SEM images showed a highly-connected porous structure in CH-hydrogel (Figure 3). The results of determining gelling time showed that immediately after transferring the hydrogel to the oven (constant temperature of 37°C), the hydrogel began to gel and became gelled completely after about 25 minutes.

Two, five diphenyl tetrazolium bromide (MTT) assay

MTT assay analysis showed no significant difference between the test and control groups (P<0.05). Results showed that using hydrogel had a direct effect on increasing the percentage of cell viability (Figure 4). During this period, the control group has a decrease in cell viability, while in other groups, it increased. The effect of the presence of RA is obvious in Figure 4.

Gene expression analysis

RT-PCR analysis demonstrated that Nestin and β-Tubulin III gene expression increased significantly in RA, CH-hydrogel, and RA-loaded CH-hydrogel groups without any additional treatment compared with the control group. Furthermore, nestin and β-tubulin III expression levels were higher in groups that were treated with

Figure 1. Flow cytometry analysis of wharton’s jelly-derived mesenchymal stem cells (WJMSCs) showing positive marker A.CD44: 98.60%; B.CD105: 91.40%; C.CD90: 98.30%; D.CD73: 95.90% and negative marker; E.CD45: 4.75%; F.CD34: 2.74%.
a neural differentiation medium compared to groups that did not treat with a differentiation medium. Results also showed that the highest expression level of nestin and β-tubulin III was detected in WJMSCs that were seeded on RA-loaded CH-hydrogel in the presence of neural differentiation medium (Figure 5). The results also show that treatment of cells with rosmarinic acid in two-dimensional conditions also increases the expression of neural markers. However, cell culture in three-dimensional conditions is more crucial than treatment with rosmarinic acid alone (*P<0.05, **P<0.01, ***P<0.005, ****P<0.001).

4. Discussion

Our results showed that cells isolated from human Wharton jelly (by the method used in this study) express mesenchymal markers well and do not express CD34 hematopoietic marker and CD45 as a leukocyte marker. WJMSCs have a higher proliferation potential than BMSCs. The process of senescence in WJMSCs also occurs later than BMSCs (Batsali et al., 2013). Besides the advantages mentioned, human Wharton’s jelly-derived mesenchymal stem cells (hWJMSCs) have enhanced expression of neurotrophic factors, and a spontaneous tendency toward a neural lineage differentiation compared to MSCs isolated from adult tissues (Millán-Rivero et al., 2018). The advantages of WJMSCs have led to many studies focusing on the differentiation of these cells into neuronal cells. In a study, hWJMSCs were neutrally differentiated and expressed specified neural markers at both the protein and mRNA levels, confirming that it is possible to generate neural stem cell-like cells from

| Table 1. Sequence of forward and reverse primers |
|-----------------------------------------------|
| **Gene**                                    | **Forward and Reverse Sequence** |
| β-actin                                     | F: (5′-3′): acatca aggagaagctgtgcctac |
|                                             | R: (3′-5′): cottcagtaggtgaagaggt |
| Nestin                                      | F: (5′-3′): ggcttctctcagcatcttg |
|                                             | R: (3′-5′): aaggctgctagttgtggt |
| β-tubulin III                               | F: (5′-3′): cagagcaaaacacagtactt |
|                                             | R: (3′-5′): tgtaaactccagtctccagtccct |

**Figure 2.** The inverted microscope image of WJMSCs before and after treatment with neural differentiation medium
A) WJMSC before any treatment in culture medium, B) Four days after treatment with neural differentiation medium
hWJMSCs in a 2D culture (Kruminis-Kaszkiel et al., 2020). Cell-based tissue engineering is a promising way to treat neurodegenerative diseases, such as Parkinson’s disease (Alizadeh et al., 2019). The neuroprotective effects of rosmarinic acid have been investigated in various studies (Fazel Nabavi et al., 2015; Taram et al., 2018). In a study, Hwang and colleagues showed that rosmarinic acid has neuroprotective effects via reducing cholinergic activity and has a positive effect on neural plasticity (Hwang et al., 2016). Another research has shown that the treatment of cells with RA prevents H₂O₂-induced cell death in N2A cells (Ghaffari et al., 2014). Peng et al. demonstrated that treating WJMSC with a cocktail of growth factors leads to differentiation into Schwann-cell phenotype and promotes neurite outgrowth (Peng et al., 2011). In another study, the effect of RA on spinal cord injury, significantly reduced the neurological deficit, increased neuronal preservation, and decreased apoptosis were observed. Ma et al. proved that RA’s neuroprotective effect may be due to its antioxidant and anti-inflammatory properties (Ma et al., 2020).

The gelation time for hydrogels, including CH-hydrogel, can range from a few minutes to several hours. The optimal gelation time for clinical applications is approximately 20 minutes. Our results showed that the gelatin time of the CH-β-GP-HEC compound was about 25 minutes. In many studies, the combination of chitosan and glycerol phosphate has been used to produce thermosensitive hydrogels. MTT assay showed that CH-hydro-

**Figure 3.** The scanning electron microscopy (SEM) image of chitosan, beta-glycerol phosphate, and hydroxyl ethyl cellulose (CH-β-GP-HEC) hydrogel after drying

The right side shows the average pore size, which is less than 1 μm. Scale bar, 2 μM.

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**Figure 4.** The results of MTT Assay

It shows that treatment of WJMSCs with rosmarinic acid leads to an increment of cell viability.
The gel had no cytotoxic effects on cultured WJMSCs. The results of cytotoxicity tests showed that the concentration of glycerol phosphate (GP) used in this study have been appropriate for causing osmolarity and appropriate pH in chitosan. Chitosan amine groups were also well neutralized with glycerol phosphate (GP), which aids in the gelation process of the compound by HEC at body temperature. Similar concentrations have been used in other papers to produce CH-βGP-HEC hydrogels, and similar results have been obtained (Naderi-Meshkin et al., 2014). The morphology of CH-βGP-HEC hydrogels was observed by SEM (Figure 3). According to Fig. 3, the porous of the lyophilized hydrogels are homogeneous and have interconnected structures. This structure is suitable for maintaining cells and other compounds. For cell culture or tissue engineering applications, the number and size of hydrogels’ pores are essential to transfer sufficient oxygen, eliminate toxic constituents, and provide sufficient space for cell growth (Sung et al., 2013). The pore and size of pores determine the type of cell to be cultured and the hydrogel’s specific application (Bodenberger et al., 2016). Gene expression analysis showed that nestin and β-tubulin III expression as markers of neuronal differentiation increased in all groups compared to the control group, which included WJMSCs cultured in 2D conditions. Nestin is a neural stem cell marker and the tubulin marker is an early stage of neuronal differentiation. The highest expression of neuronal markers was observed in group D, which included WJMSCs cultured on RA-loaded CH-βGP-HEC hydrogel and treated with a neural differentiation medium. The results also show that treatment of cells with rosmarinic acid in 2D conditions also increases the expression of neural markers. However, cell culture in 3D conditions is more critical than treatment with rosmarinic acid alone. (*P<0.05,**P<0.01,***P<0.005, ****P<0.001)
gene expression to neural differentiation (Ferdousi et al., 2019). It has also been shown that RA and its derivatives have positive effects on the proliferation of neural stem cell (NSC), neural stem cell (NSC) differentiation into neural precursor cells, as well as the maturation of neural precursor cells (Habtemariam, 2018). The results also revealed that the expression of neural markers in the 3D groups was significantly higher than in the 2D groups. As can be seen from the results, three-dimensional cell culture in hydrogels alone and without any additional factor can induce neural markers’ expression. In addition, the effect of 3D culture on the expression of neural markers is more than that of rosmarinic acid. The impact of 3D culture on the induction of neural differentiation has also been shown in other studies (Chandrasekaran et al., 2017; Marchini et al., 2019; Song et al., 2018). Khodabandeh et al. used 2D collagen films and 3D collagen scaffolds for the culture of hWJMSCs. The cells showed a significant increase when cultured in 3D one compared to conventional monolayer culture and 2D films. They observed a significant increase in tight junction markers, such as claudin (Khodabandeh et al., 2016). Additionally, WJMSCs were differentiated into neurons in 3D alginate scaffolds and 2D monolayer systems. Hosseini et al realized that neural differentiation of embryonic stem cells was also more obviously increased in 3D culture compared to the 2D system. Furthermore, cells on alginate scaffolds exhibited a round appearance, which confirms the scaffold’s suitability for culturing (Hosseini et al., 2015). Given the increased expression of the nestin gene compared to tubulin, 3D culture and rosmarinic acid seem to differentiate WJMSCs into neural stem cells. As a result, additional treatments may also be needed to differentiate stem cells into mature neurons.

5. Conclusion

In addition to its anti-inflammatory role, rosmarinic acid can be used as a facilitator of neuronal differentiation. The results of this study showed that although rosmarinic acid alone can induce the expression of neural markers if used in combination with an inducer of neural differentiation, it will have a greater effect on the expression of neural markers. On the other hand, stem cell culture in three-dimensional conditions with rosmarinic acid helps to increase the expression of neural markers. The results of this study and such studies could lead to the production of inexpensive 3D scaffolds capable of successfully inducing neural differentiation in stem cells, which can be used in nerve tissue engineering.

Ethical Considerations

Compliance with ethical guidelines

All tests performed in this study was done under approval of Ethical Committee of Najafabad Branch, Islamic Azad University.

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Authors’ contributions

All authors equally contributed to preparing this article.

Conflict of interest

The authors declared no conflict of interest.

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