THE RESVERATROL INCREASE OF HEPATOCYTE GROWTH FACTOR (HGF)

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THE RESVERATROL INCREASE OF HEPATOCYTE GROWTH FACTOR (HGF) AND EPIDERMAL GROWTH FACTOR (EGF) LEVELS IN WHARTON'S JELLY MESENCHYMAL STEM CELLS (WJ-MSCS) SECRETOME : TOWARD CELL FREE THERAPY IN DRY EYE DISEASE (DED)

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ABSTRACT : This study aims to explore and determine the effect of resveratrol at various doses on WJ-MSCs secretome. Wharton's jelly was extracted from umbilical cord, with the enzymatic-explant mixed method used to isolate the MSCs which were stained with CD 105, CD 73, CD 90, and CD 45 FITC conjugated antibodies. Furthermore, WJ-MSCs were treated with culture media supplemented with resveratrol at doses of 0.1 µM, 0.2 µM, 0.4 µM, 0.5 µM, 0.8 µM, and 1 µM, with 2% and 10% FBS used in the control group. The cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphyniltetrazolium bromide (MTT) assay., while the level of EGF, HGF, and vascular endothelial growth factor (VEGF)-A were measured using Enzyme Linked Immunosorbent Assay (ELISA). Data analysis, were carried out using Analysis of variance (ANOVA) and post-hoc Tukey using SPSS 19.0 software. The viability of WJ-MSCs was maintained in all resveratrol doses compared to the control. This lead to a decrease in cell viability in resveratrol at 1 µM dose and at 0.1 µM it increased the HGF (130 ± 11.2 pg/mL) and EGF (76.67 ± 15.67 pg/mL) levels, and decreased the VEGF (65 ± 5.02 pg/mL) level compared to other groups. These cells secretome composition tend to have beneficial effect on ocular surface regeneration in DED. 0.1 µM resveratrol was the optimal dose used to increase EGF and HGF secretion from WJ-MSCs, while decreasing the VEGF level. In addition, the various compositions of growth factors within WJ-MSCs leads to a cell-free based regenerative therapy in DED.

Key words : Resveratrol, wharton's jelly mesenchymal stem cells, secretome, dry eye disease

INTRODUCTION

Dry eye disease is one of the most commonly encountered problems in ophthalmology, with its prevalence accounted between 5% to 35% with serious health and economic burdens (Villatoro *et al*, 2015; Bittencourt *et al*, 2016). It is a multifactorial disease with a dysfunction lacrimal function unit which leads to composition and stability of tear film thereby, creating visual disturbance prior to the severe potential damage of the ocular surface. The cytokines and inflammatory processes affecting both lacrimal gland and ocular surface are considered to be the main pathological process in DED (Villatoro *et al*, 2015; Murri *et al*, 2018). The inflammatory process in accordance with dysregulation of growth factors tends to maintain ocular surfaces such as EGF, HGF, basic fibroblast growth factor (bFGF), keratinocyte growth factor (KGF) and VEGF, thereby leading to ocular surface damage (Weng *et al*, 2012; Dudok *et al*, 2015). Vascular endothelial growth factor-A (VEGF-A), is a member of the cysteine super family known to increase in DED which results in corneal neovascularization (Seong *et al*, 2015; Peltz *et al*, 2012).

Currently, there are no curative treatments for dry eye disease to alleviate symptoms and its progression. According to research, there is a high probability of the advanced use of the regenerative therapy in the

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management of this pathology (Villatoro *et al*, 2017; Phinney *et al*, 2017). Wharton's jelly mesenchymal stem cells are multi-potent with the ability to differentiate many mesoderm cells from their lineages. Currently, MSCs consists of secretory profiles of various bioactive molecules with trophic, paracrine, and immunomodulatory functions (Villatoro *et al,* 2015; Konala *et al*, 2016; Lavoie *et al*, 2013). Mesenchymal stem cells and its secretome are powerful regulators of the immune response and have been shown to be effective in treating wide variety of immune disorders. Bittencourt *et al* (2016) demonstrated the safety and efficiency aspects of allogenic MSCs transplantation in dogs using DED (Bittencourt *et al*, 2016; Sivian *et al*, 2016; Turner *et al*, 2014). However, the production of MSCs secretome for DED treatment remains a challenge owing to its high amount of VEGF levels.

Resveratrol is a phytoalexin agent, with a significant effect in reducing VEGF secretion in various cell lines (Seong *et al*, 2015). This study, therefore aims to explore and determine the optimal dose of resveratrol preconditioned WJ-MSCs to produce secretome with increased level of EGF and HGF, while reducing the VEGF levels as the candidate for regenerative therapy in DED.

MATERIALS AND METHODS

Materials

The main reagents were Type IV collagenase (Roche USA), alpha modified eagle mediμM (α-MEM, Gibco-Life Technologies, USA), nonessential amino acid (NEAA, Sigma, USA), Trypsin-EDTA (Gibco-Life Technologies, USA), fetal bovine serum (FBS, Biowest, USA), resveratrol (Sigma, USA), bFGF (Gibco- Life Technologies, USA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphyniltetrazolium bromide (MTT; Gibco- Life Technologies, USA). FITC conjugated antibodies for CD 105, CD 90, CD 73 and CD 45 were purchased from BIOSS (USA), while Human VEGF, EGF, and HGF ELISA kit were from Bioassay Technology Laboratory, China.

Methods

This is an *in vitro* study which investigated the effect of various doses of resveratrol on WJ-MSCs secretion, for EGF, HGF and VEGF-A levels. It was conducted at Stem Cell Research and Development Center, Universitas Airlangga with all experiments confirmed by the institutional ethics review board.

Cell isolation

Wharton's jelly MSCs were isolated from single donor of umbilical cord, obtained from a healthy baby through caesarian section using the aseptic technique. The baby's umbilical cord of length 3 cm, was collected after the mother's consent. The wharton's jelly MSCs extracted were isolated using the mixed enzymatic-explant method which was based on a modified protocol developed by Azendeh *et al* (2013). Small amount of blood vessels and outer sheath of umbilical cord were removed to allow the WJ to contact the enzymatic solution directly. It was minced into approximately 1 cm^3 pieces, washed with PBS, placed in sterile tube containing α-MEM medium with enzymatic cocktail comprising of 0.75 mg/ml Collagenase Type IV and incubated at 37°C for 30-60 minutes. After the incubation period, the WJ pieces were washed with culture media and transferred to a new sterile tube centrifuged at 300 g for 5 min. The supernatant was discarded and cell pellets suspended in a culture medium composed of á-MEM, 1% penicillin-streptomycin, 1% amphotericin B, 1% NEAA, 5% FBS, and 5ng/mL bFGF. In addition, the cells and remaining small pieces of wharton's jelly were cultured in an incubator at 37°C and 5% CO_2. The culture media was changed every 3 days till 90% confluency was reached and passed using the warm trypsination method.

Cell characterization

The MSCs phenotype cells were characterized at passage 3 in expressions of CD 105, CD 90, CD 73 and CD 45 for confirmation. Approximately 5×10^3 cells were cultured in 96 multi-well plates with 80% confluency and fixated with 10% formaldehyde for 15 minutes. The fixated cells were stained with FITC conjugated antibody for each antibody and incubated for 60 minutes at 37°C followed by an overnight incubation at 4°C. Furthermore, the stained cells were washed 3 times using PBS tween of 0.2%, which was followed by DAPI counterstained. Stained cells were visualized under inverted fluorescence microscope (BH2-RFL-T3 model fluorescence attachment, Olympus).

Secretome production using resveratrol preconditioning

A total amount of 5×10^3 cells/well of WJ-MSCs at passage 4 was cultured in multi-well plate 96 with culture media for 24 hour to obtain 70% confluency. The cells where further treated using media composed of á-MEM, 1% penicillin-streptomycin, 1% amphotericin B, 1% NEAA and 2 % FBS supplemented with various doses of resveratrol after a day. The compound was divided into 6 groups of resveratrol supplementation doses which consist of 0.1 μM, 0.2 μM, 0.4 μM, 0.5 μM, 0.8 μM and 1 μM. Also culture media with 2% and 10% FBS was used on the control group, with cells secretome collected after

48 hours of treatment. All experiment groups were conducted in 4 replications.

Cell viability

3-(4,5-dimethylthiazol-2-yl)-2,5-diphyniltetrazolium bromide assay were conducted to determine the effect of resveratrol at various dose on cell viability using Riss *et al* (2004) research analysis. After 24 hour of resveratrol preconditioning, 10 µl of 0.45mg/mL tetrazolium solution was added to each well and incubated at 37°C for 4 hours. This was followed by the addition of 100 µl solubilization solution to dissolve the formazan crystals. The plate was immediately analyzed using microplate reader at an absorbance of 570nm.

Growth factor measurement

Growth factor measurement was conducted to determine the optimal dose of resveratrol capable of inducing EGF and HGF secretion from WJ-MSCs and decreased VEGF secretion. Enzyme linked immunosorbent assay was performed according to Bioassay Technology Laboratory protocol, with the cell supernatants collected and centrifuged at 3000rpm for 20 minutes. A total amount of 40 µl supernatants were placed into ELISA well plate, with Biotinylated antibody and streptavidin HRP added, and incubated at 37°C for 60 minutes. Sample were washed 3x with buffer, with solution A and B added and incubated at 37°C for 10 minutes. Finally, stop solution was added and the plate was read immediately using microplate reader at an absorbance of 450nm.

Statistical analysis

The data of cell viability and growth factor level were presented in mean ± standard deviation, while the difference of cell viability among groups was analyzed with ANOVA and Post-hoc Tukey using SPSS software version 19.0. A statistical significant p value of $P < 0.05$ was also utilized.

RESULTS

Cell isolation and characterization

WJ-MSCs were successfully isolated using the mixed enzymatic-explant method with fibroblast like morphology appearance observed after 72 hours. The cells reached 80% confluency on day-14, while its characterization at passage 3 indicated MSCs population with positive stained for CD 105, CD 90, and CD 73 FITC conjugated antibody with negative CD 45 antibody as depicted in Fig. 1. The cell population used in this research, fulfilled The International Society for Cellular Therapy criteria to define MSC as adhere to tissue culture dish and present specific surface markers for MSCs.

Table 1 : Resveratrol induce growth factors secretion from WJ-MSCs at dosage dependent manner.

Group	VEGF-A (pg/mL)	HGF (pg/mL)	EGF (pg/mL)
0.1 µM	65 ± 5.02	130 ± 11.2	76.67±15.67
0.2 µM	490 ± 21.1	120 ± 13.2	41.6 ± 8.6
0.4 µM	50 ± 6.21	90 ± 11.7	21.67 ± 4.3
0.5 µM	65 ± 8.8	73.33 ± 11	5 ± 1.3
0.8 µM	545 ± 25	250 ± 8	5 ± 3.3
1 µM	565 ± 32.26	210 ± 10	8.3 ± 3.3
FBS 2%	295 ± 22	243.33 ± 11.3	15 ± 3.5
FBS 10%	335 ± 21.5	200 ± 12	25 ± 5.25

Cell viability

3-(4,5-dimethylthiazol-2-yl)-2,5-diphyniltetrazolium bromide assay displayed highest cell viability in cells treated with 0.5 µM resveratrol (112.12 ± 2.51%), followed by 0.4 µM (98.17 ± 2.01%), 0.1 µM (98.02 ± 2.11%), 0.8 µM (97.75 ± 2.61%), 0.2 µM (97.57 ± 2.57%), 1 µM (92.33 ± 2.76%), and FBS 2% (88.01 ± 2.81%). Relatively lower cell viability was observed in 1 µM resveratrol and FBS 2% group with a significant difference between 0.5 µM resveratrol group with 1 µM and FBS 2% group, P 0.007 and P 0.001 respectively (Fig. 2).

Growth factor level

Enzyme linked immunosorbent assay showed various levels of EGF, HGF and VEGF in each supernatant (Fig. 3). High level of HGF was found on 0.8 µM (250 ± 8 pg/mL), 1 µM (565 ± 32.26 pg/mL), and FBS 10% groups (200 ± 12 pg/mL), while the lowest amount of HGF was found in the 0.5 µM group (73.33 ± 11 pg/mL). The highest concentration of EGF was in the 0.1 µM group (76.67 ± 15.67 pg/mL), while low level was in the control group. The lowest concentration of VEGF was 0.4 µM (50 ± 6.21 pg/mL), while 0.8 µM (545 ± 25 pg/mL), 1 µM (565 ± 32.26 pg/mL), FBS 2% (295 ± 22 pg/mL) and FBS 10% (335 ± 21.5 pg/mL) presented high amount of VEGF (Fig. 3, Table 1).

DISCUSSION

In 2014, Garzon *et al* (2014) reported differentiated capacity of WJ-MSCs into corneal epithelial cells, thereby, making it the candidate source of extraocular MSCs in term of ocular surface regeneration. However, this is the first study conducted to observe the effect of resveratrol on cell viability and growth factor secretion from WJ-MSCs. The cell viability was observed in all resveratrol groups compared to the 2% FBS group. Resveratrol at 0.5 µM dose exerted the highest viability among all groups, which decreased at 1 µM dose. A study conducted by Peltz *et al* (2012) revealed that 0.1 µM resveratrol promotes MSCs self-renewal by inhibiting cellular

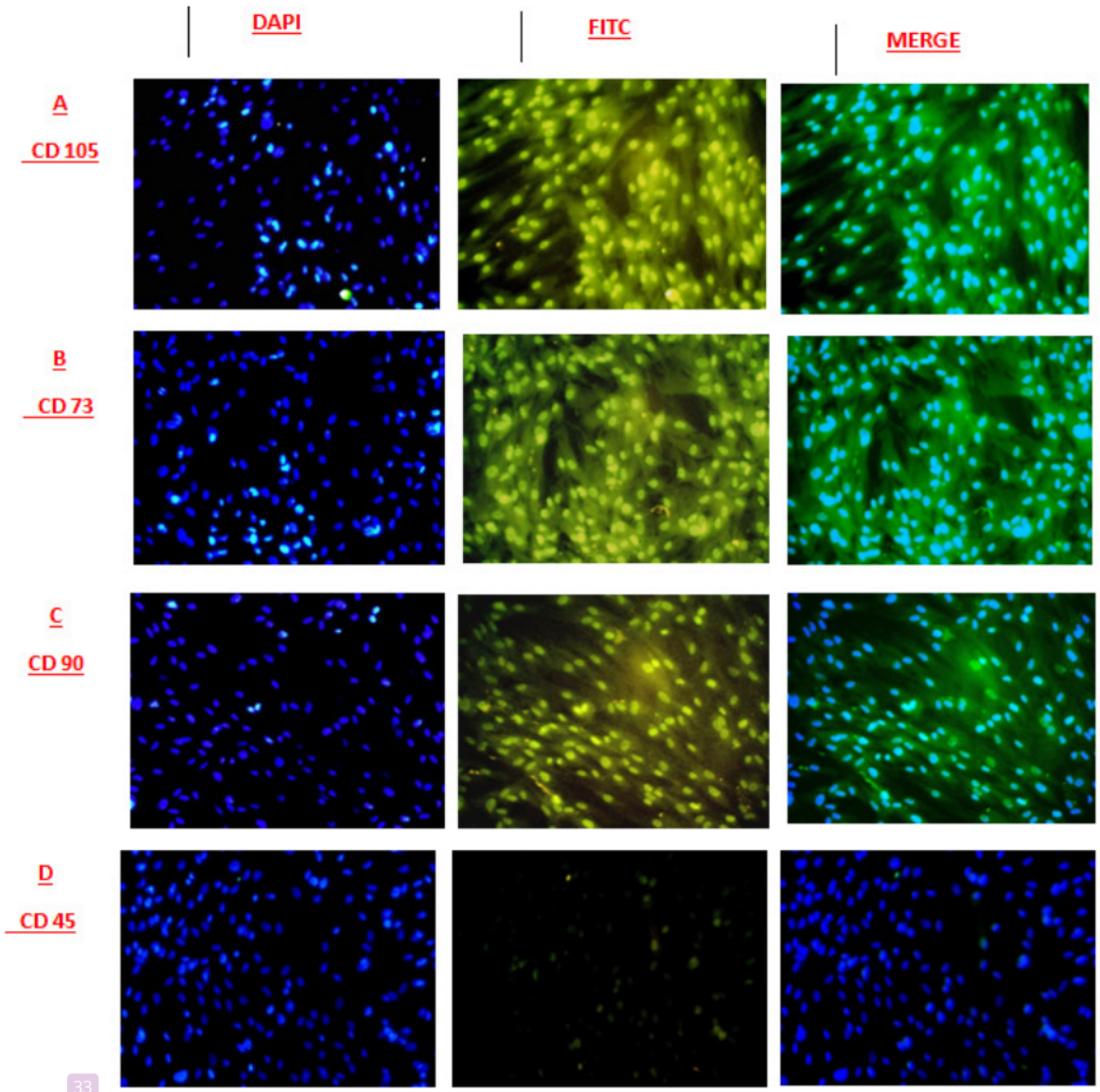

Fig. 1 : The cells were stained with specific antibody for MSCs. (A) cells were positive for CD 105 FITC conjugated antibody with the left section, stained using DAPI, the with FITC labelled CD 105, while the right merged with the previous two sections. The cells were stained with specific antibody for MSCs. (B) cells were positive for CD 73 FITC conjugated antibody with the left section stained using DAPI, the middle with FITC labeled CD 73, while the right was merged with the previous two sections. (C) Cells were positive for CD 90 FITC conjugated antibody. In the left section, they were stained with DAPI, FITC labelled CD 90 in the middle and merged with the previous two sections. (D) Cells were negative for CD 45 FITC conjugated antibody, stained with DAPI in the left section, FITC labelled CD 45 in the middle and merged with the previous two section in the right (Inverted Fluorescence Microscope, 200x magnification).

senescence, whereas at 0.5 µM and above inhibits cell proliferation. At 1 µM resveratrol, its minimal effect on cell self-renewal exerts inhibitory effect after prolonged exposure which was accompanied with increased senescence rate (Peltz *et al*, 2012). However, it is reported that resveratrol has dual effects as anticancer (pro-apoptotic) and pro-survival effect which is yet to be fully elucidated. In term of pro-survival effect, resveratrol is augmented to stimulate Sirtulin (SIRT) 1, which deacetylate and destabilize p53 activity, leading to delayed apoptosis and prolonged cell survival (Yoon *et al*, 2014; Peltz *et al*, 2012). Yoon *et al* (2014) revealed that resveratrol at a concentration of 0.1 and 1 µM mediated SIRT-1 activation by suppressing SOX-2 acetylation and

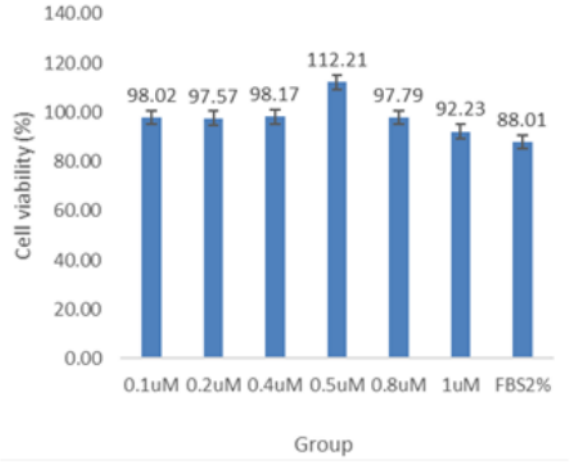

Fig. 2 : Resveratrol maintained viability of WJ-MSCs.

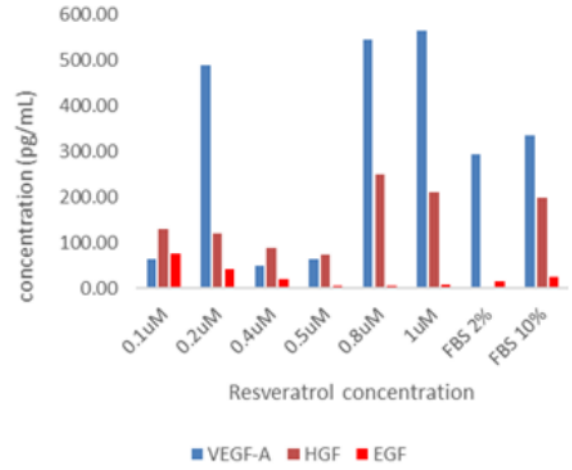

Fig. 3 : Growth factors level in WJ-MSCs secretome with resveratrol induction at various dosages.

therefore maintained self-renewal, multi-potency and differentiation potential to osteogenic and adipogenic lineages of bone marrow MSCs. Furthermore, their data also showed that resveratrol increased cellular senescence of late passage of MSCs (Yoon *et al*, 2014; Yoon *et al*, 2015).

Stem cells secretome play important role in many different biological processes such as the production of extracellular matrix, antiapoptotic, antifibrotic, angiogenic, and immunomodulatory. The composition and effect of secretome varies with cell origin and methods of manufacture (Yao *et al*, 2013; Mattar *et al*, 2015, Screven *et al*, 2014). In this study, 0.1 µM dose of resveratrol was optimal to the extraction of WJ-MSCs secretome, which is composed of high level of EGF and HGF, decreases the level of VEGF, and extends the concentration of resveratrol and duration of treatment exerted on cell fate differential effects. Lei *et al* (2016) showed that 50 µM of resveratrol maintained cell viability on oxidative stressed induced AD-MSCs, which promoted paracrine effect of MSCs on insulin secretion of rat insulinoma cell (INS-1) (Lei *et al*, 2016). Seong *et al* (2015) revealed that conditioned media from retinal pigment epithelial cells treated with resveratrol has the ability to inhibit human umbilical vein endothelial cell (HUVECs) tube formation. Resveratrol at 50 µM concentration suppressed VEGF secretion in hypoxic retinal pigment epithelial (RPE) cells via inhibition of CXC-chemokine receptor 4 (Seong *et al*, 2015). According to Zhang *et al* (2016), resveratrol inhibit hypoxia inducible factor (HIF)-1á accumulation and VEGF secretion from cobalt chloride ($COCl_2$) which induced hypoxic RPE cells. This study revealed the optimal dose of resveratrol at 50 µM to decrease VEGF expression via SIRT-1 pathway (Zhang *et al*, 2016). However, at 0.1 µM dose, the resveratrol was optimal conditioned to decrease VEGF secretion in normoxic culture. Conversely, the highest level of HGF was obtained on 0.8 µM resveratrol group, while the VEGF level remained high in this group. Furthermore, the resveratrol failed to induce EGF secretion at dose of 0.5 µM and 1 µM, but at higher levels of VEGF secretion at 0.2 µM, 0.8 µM, and 1 µM dose compared to the control groups. However, the mechanism of resveratrol on VEGF upregulation was not explored in this study.

The stem cells therapy characteristics is carried out through the release of various amounts of cytokines, growth factors and bioactive molecules which act as paracrine and autocrine effects under the concept of secretome (Lavoie *et al*, 2013; Makridakis *et al*, 2013). Stem cells secretome showed great potential in a variety of clinical applications as a cell-free option for regenerative medical therapies, while ocular surface pathologies are excellent objectives (Phinney *et al*, 2017; Katsuda *et al*, 2013; Oh *et al*, 2009). In recent years, it has been the emerging interest to treat different pathologies including ocular surface diseases (Joe *et al*, 2010; Sevan *et al*, 2016). Mesenchymal stem cells have gained very interesting expectations in the treatment of dry eye and ocular surface regeneration through its immunomodulatory and regeneration potential (Lee *et al*, 2015; Ljubimov *et al*, 2015). In this study, the effect of resveratrol in reducing VEGF secretion from WJ-MSCs was explored, with increase in HGF and EGF levels. This study proposed that using extraembryonic tissue extract eye drop in amniotic membrane extract and umbilical cord blood has lots of advantageous for ocular surface healing and regeneration. *In vitro* and *in vivo* studies revealed

that it increased proliferation and differentiation of corneal epithelial cells, enhances wound healing, and inhibits corneal neovascularization (Baradaran-Raffi *et al*, 2017; Shay *et al*, 2011). However, umbilical cord contains active matrix component responsible for exerting greater anti-inflammatory effect than amniotic membrane extract (Tighe *et al*, 2017; Dudok *et al*, 2015). Hepatocyte growth factor, EGF, and bFGF plays a crucial role in ocular surface regeneration, and amalgamates to facilitate migration, adhesion, and differentiation of corneal epithelium, while halting the apoptosis process. Epidermal growth factor together with HGF promotes limbal stem cells regeneration which results in corneal epithelial regeneration. However, HGF itself is a potent mediator for conjunctival goblet cells regeneration. In addition, secreted anti-inflammatory cytokine such as IL-10 and immunomodulatory cytokine eg HGF, decreases ocular surface inflammation. As the inflammation subsides, it degrades the cytokines of TGF-1 and TGF-2, while inhibiting tissue fibrosis. These crosstalk mechanisms combine to promote ocular surface wound healing (Dua *et al*, 2010; Turner *et al*, 2014). MSCs are known as activators of angiogenesis and secrete VEGF (Lee *et al*, 2013; Anderson *et al*, 2016), with an antagonist effect on corneal angiogenesis which upregulates the thrombospondin-1 and downregulates matrix metalloproteinase-2. Furthermore, a model was developed to decrease VEGF secretion from WJ-MSCs, with the assumption that the anti-angiogenic properties of MSCs secretome, has the ability to halt corneal neovascullariztion (Oh *et al*, 2009). Transplantation of MSCs in experimental DED models improved tear volume and stability, the number of goblet cells and epithelial recovery (Beyazyýldýz *et al*, 2014; Weng *et al*, 2012). Villatoro *et al* (2015) demonstrated that periglandular transplantation of MSCs was an effective route to restore lacrimal gland function while increasing the tear secretion and restoring clinical signs of the disease in dogs using refractory DED (Villatoro *et al*, 2015; Konala *et al*, 2016). In addition, the topical instillation of the secretome in ocular diseases has shown significant improvement in corneal wound healing, which attenuates the corneal inflammation by inhibition the proinflammatory cytokines and infiltrating the inflammatory cells (Bermudez *et al*, 2015; Kim *et al*, 2013).

The future advantages of using secretome in DED is to prevent the undesirable effects related to the use of stem cells which reduced the teratoma formation issue, immunogenic reaction, enables the use of allogene, and the transmission of infectious agents (Makridakis *et al*, 2013; Phinney *et al*, 2017; Tran *et al*, 2015; Su *et al*, 2015). In this research, stem cells secretome is considered a pharmaceutically manufactured topical eye drop for regenerative therapy in DED. As limitation, other mitogenic growth factors and immunomodulatory cytokines are yet to be explored in this study. Further exploration and in vivo study in animal model of DED are mandatory.

CONCLUSION

In conclusion, 0.1 µM resveratrol was the optimal dose used to increase EGF and HGF secretion from WJ-MSCs, while decreasing the VEGF level. The growth composition within WJ-MSCs is a candidate for cell-free based regenerative therapy in DED.

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