AN *IN-VITRO* STUDY OF EFFECTS OF *CENTELLA ASIATICA* ON PROLIFERATION RATE OF HUMAN PERIODONTAL LIGAMENT FIBROBLASTS

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Abstract

Centella asiatica is a member of a tropical plant family which is considered to be a good source of phenolic compounds. The present study was designed to assess the effect of different concentrations of aqueous extract of *Centella asiatica* on the proliferation of human periodontal ligament fibroblast in-*vitro*. Aqueous extract of *Centella asiatica* was prepared and concentrations of 2-40 µg/ml were applied to the human periodontal ligament fibroblasts culture. The viability of human periodontal ligament fibroblast was investigated by MTT cell proliferation assay. The data were subjected to statistical analysis using ANOVA and Tukey's post hoc test. The concentration of aqueous extract of *Centella asiatica* that enhances highest proliferation of human periodontal ligament fibroblast cell was 20 µg/ml. the regeneration and protective effects of *Centella asiatica* can be utilize to treat the periodontal disease.

Keywords: centella asiatica, human periodontal ligament fibroblast, proliferation.

Introduction

The periodontium consists of periodontal ligament, gingival soft tissue, cementum covering the tooth root and alveolar bone (Melcher, 1970). The periodontal ligament is a highly specialized connective tissue located between the tooth root and alveolar bone (carnes et al, 1997) and once the periodontal ligament is severely damaged by inflammation (such as periodontitis); its regeneration is well-known to be difficult. Therefore, regenerative mechanism of periodontal ligament is an important process in preserving teeth. The periodontal ligament contains a large amount of matrix macromolecules, some of which include collagenous, glycosaminoglycans, and various noncollagenous proteins (kotaro et al., 2008). Fibroblasts are predominant in the

periodontal ligament (berkovitz and shore, beertsen et al., 1974) and were thought to be responsible for the homeostasis and regeneration of the periodontal tissues (lekic and mcculloch, 1998). The objective of periodontal therapy is to control periodontal tissue inflammation and to produce predictable regeneration of periodontium lost as a result of the disease. A variety of treatment approaches to promote restoration of lost periodontal structures and repopulate the area by desirable cells have been suggested by several authors (pietruska, 2001; sculean et al., 2005). Current methods for enhancing periodontal regeneration include implantation of different types of bone substitutes, various flap procedures, guided tissue regeneration, application of enamel matrix derivative and biological preparation of the root surfaces and use of growth factors. Previous studies had provided evidence that different connective tissues-such as skin (Abraham et al., 1991), lung (fries et al., 1994), breast (Atherton et al., 1994), and gingival and periodontal ligament (hou and yaeger, 1993; dennison et al., 1994; ogata et al., 1995; giannopoulou and cimasoni, 1996) contained not only distinct types of fibroblast having unique function, but also heterogeneous subpopulations of fibroblasts within the same tissue (hassell and stanek, 1983; mcculloch and bordin, 1995;

1991; hakkinen and larjava, 1992; hou and yeager, 1993; fries et al., 1994; Irwin et al., 1994). While such cells may sometimes appear to be morphologically and phenotypically identical, they nevertheless often display different functional activities (bou-gharios et al., 1994; fries et al., 1994). Oral epithelium generally heals faster with fewer scars than skin epithelium and the development of keloid or hypertrophic scar in the oral cavity was very rare (stephens et al., 1996). Fibroblasts are the pivotal cells in wound healing, providing tensile strength to early wound, secreting cytokines into wound matrix and inducing the fibro-proliferative response, which ultimately gives rise to scar formation. Fibroblasts from different sites show heterogeneity (Schneider et al., 1977). Fibroblasts from papillary dermis and reticular dermis also showed different proliferative potency although they were from the same patch of skin (tajima and pimell, 1981). The bioactive constituent of therapeutic interest in centella asiatica is pentacyclic triterpenoid group known as asiaticoside (de lucia et al., 1997). Saponin-containing trieterpene acids and their sugar esters which are Asiatic acid, madecassic acid and the three asiaticosides: asiaticoside, asiaticoside A and asiaticoside B (sing and rastogi, 1969; brinkhaus et al., 2000). Centella asiatica

was claimed to have wide varieties of pharmacological effects on wound healing, disorders, atherosclerosis, fungicidal, antibacterial, antioxidant and anticancer (Sharma et al., 1985; karting, 1988; maquart et al., 1999). Asiaticoside derived from the plant centella asiatica was known to posses good wound healing activity. Enhanced healing activity has been attributed to increase collagen formation and angiogenesis (inamdar, 1996; bonte et al., 1995). Pure triterpenoids of centella asiatica have been reported to cause alteration in gene expression in human fibroblast (coldren et al., 2003, lul et al., 2004). Recently, asiaticoside has been shown to induce Type I collagen synthesis in human dermal fibroblast (lee et al., 2006).

Material and method

Preparation of extract

Centella asiatica plant was identified by the FRIM (Forest Research Institution Malaysia) given the code number KOD: SBID 024/08 (Appendix A1, A2, A3). The leaves were dried in an oven with air renewal and circulation at 40ºC. The dried leaves were then triturated in a blender until a finely granulated powder was obtained. Distilled water 1:20 w/v was added to the triturated powder under constant stirring. The mixture temperature was elevated up to 100°C. A magnetic stirrer was used to stir the mixture for 6 hours before filtration done with Whatman No.1 filter paper. The filtrate was lyophilized by using freeze dryer machine (Labconco, Kansas city, USA) (Plate 3.1) and final product was stored at 0°C and sealed with aluminum foil to avoid oxidation by the light. At the time of use, the extract was resuspended in distilled water at the desired concentration.

Cell Culture

Human periodontal ligament fibroblast (HPLF) was obtained from the American Type Culture Collection (ATCC). HPLF cells were cultured in fibroblast medium (FM) containing antibiotics (10% Fetal bovine serum (FBS), 1% of Penicillin/Streptomycin, 1% of Gentamycin). The cell culture was incubated in 5% CO_2 incubator at 37°C.

Proliferation assay

The cells were cultured in 96-well microplate with flat bottomed. 50µl of cells suspension ($2.5x10^4$ cells/well) mixed with 50µl fresh medium were added to viewed under an inverted microscope to check the percentage of the confluence. The medium was then removed from the 96-well microplate by a micropipette. *Centella asiatica* concentrations of 2, 5, 10, 20, 30 and 40 µg/ml were used in the study to treat the HPLF. Positive controls were the cells with medium and the negative controls were medium only included in experiment . The cells were then incubated for another 24 hours. At the end of incubation period, the entire medium was removed from the wells using a micropipette and then washed with 30µl phosphate buffer saline. The cells were each well. The cells were incubated in 5% CO_2, 95% O_2 incubator, at 37°C for 24 hours. Then the confluent cells were

mixed with 100µl fibroblast medium into each well. MTT cell proliferation assay was used to measure the metabolic cell activity after the culture period. 15µl of MTT dye solution (Cell Titer 96 Non-radioactive Cell Proliferation Assay kit, Promega) was added into each well and the incubation was continued for 4 hours in 5% CO_2, 95% O_2 incubator, at 37°C. After the incubation, 100µl of the solubilization solution/stop solution was added to each well. Finally, the cell optical density was measured using a microplate reader at 570nm wavelength.

Statistical analysis

All the cells count were tabulated and subjected to statistical analysis using one-way ANOVA. Post-Hoc Analysis was compared all by using Tukey's test. *P* value of less than 0.05 was considered to be significant.

Results

MTT cell proliferation assay was used to measure the cell metabolic activity after the culture periods (1, 2, 3, 7 days). Result of MTT cell proliferation assay of HPLF was expressed as average absorbance ± standard deviation. Cell cultures treated with different concentrations of *Centella asiatica* had exhibited proliferation rate activity as compared to control group which was statistically significant except at the concentration 30 and 40µg/ml.

Cells treated with concentrations 2 and 5µg/ml had shown the highest proliferation after Day 1 and the lowest proliferation after Day 7 (Figure 1). HPLF showed the highest proliferation when treated with

concentrations 10 and 20µg/ml after Day 2 of treatment. The highest HPLF proliferation was obtained when the cells stimulated with 20µg/ml at absorbance concentration. The viability of the HPLF after Day 3 and Day 7 treatment with concentration 20µg/ml of extract has enhanced the cell growth with absorbance average 0.362±0.008 and 0.344±0.014 average 0.389±0.004 after Day 2 treatment which was higher than Day 1 reading for the same respectively. However, it was less than the Day 2 treatment. Concentrations 30 and 40µg/ml showed the lowest cell proliferation rate compared to the other *Centella asiatica* concentrations.

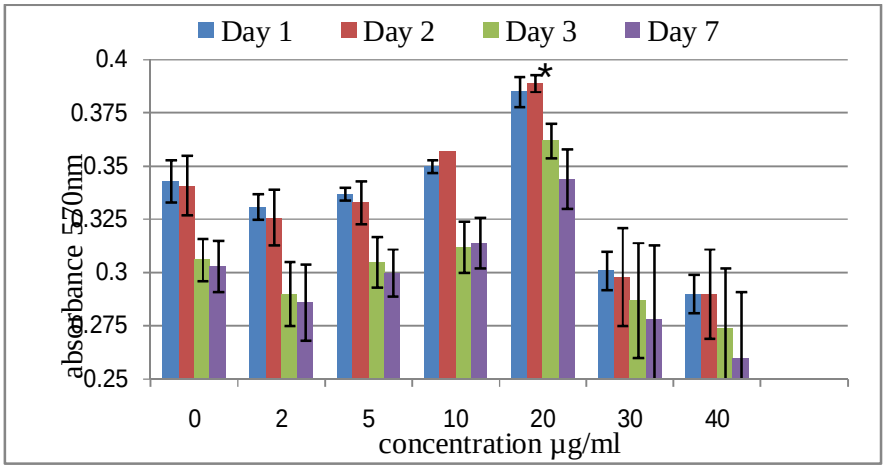

Figure 1: MTT Cell Proliferation Rate of HPLFs over Time

Overall the cell viability Day 7 treatment was the least compared to the other period of treatment (1, 2, 3 days) (Table 1).

Table 1: Equality Test of MTT Means between Concentrations by Days

OD	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Power
Day 1	.018	6	.003	58.251	0.001	0.470	0.961
Day 2	.021	6	.003	16.484	<0.001	0.615	0.876

Day 3	.014	6	.002	7.527	0.001	0.468	0.763
Day 7	.013	6	.002	4.906	0.007	0.481	0.678

Table 2, showed the HPDLF proliferation rates vs. days when stimulated with concentrations 2, 5, 10, 20, 30 and 40µg/ml of *centella asiatica* aqueous extract.

Concentration	**Day 1**	**Day 2**	**Day 3**	**Day 7**
0	0.343 ± 0.010	0.341 ± 0.014	0.306 ± 0.010	0.303 ± 0.012
2	0.331 ± 0.006	0.326 ± 0.013	0.290 ± 0.015	0.286 ± 0.018
5	0.337 ± 0.003	0.333 ± 0.010	0.305 ± 0.012	0.300 ± 0.011
10	0.350 ± 0.003	0.357 ± 0.000	0.312 ± 0.012	0.314 ± 0.012
20	**0.385 ± 0.007**	**0.389 ± 0.004**	**0.362 ± 0.008**	**0.344 ± 0.014**
30	0.301 ± 0.009	0.298 ± 0.023	0.287 ± 0.027	0.278 ± 0.035
40	0.290 ± 0.009	0.290 ± 0.021	0.274 ± 0.028	0.260 ± 0.031

Table 2: Optical Density of HPDLF Growth Rate vs. Day

Discussion

MTT (3- 4, 5-Dimethylthiazol-2-yl -2, 5-diphenyltetrazolium bromide) is a yallow, water-soluble tetrazolium salt. The reductionof MTT by the succinate dehydrogenase system of mitochondria in metabolically active cells yields a water-insoluble purple formazon product. Since the amount of formazon produced is directly proportional to to number of active cells in the culture, MTT and other tetrazolium salts have been used in cellular proliferation and cytotoxicity assays (Mosmann, 1983; Hussain et al., 1993).

The pilot study was conducted to assess the proliferative effect of *Centella asiatica* on the human periodontal ligament fibroblasts using MTT cell proliferation assay. The

results obtained after 1, 2, 3 and 7 days showed that aqueous extract of *Centella asiatica* had significantly enhanced the proliferation of human periodontal ligament fibroblast when stimulated with concentration 20µg/ml ($P < 0.001$). In this 40µg/ml. (Coldren *et al*., 2003). Previous studies showed that oral fibroblasts and dermal fibroblasts had selective differences in cellular behavior and in their responses to growth factors, which seems to contribute to the differences in wound healing. Generally agreed that oral mucosa heals faster with fewer scars than skin (Lee and Eun, 1999). It was also noted that, there was an enhanced rate of proliferation process of periodontal ligament fibroblast. Fibroblast growth factor-2($FGF_{2)}$ level was increased in the state of cell proliferation (Cheng *et al*., 2004). Fibroblast growth factor-2($FGF_{2)}$ is involved in the control of a variety of processes such as proliferation, migration and cell survival (Debiais *et al*., 1998; Raballo *et al*., 2000). The level of FGF_2 was increased as there was a proliferation condition and the increased level of the growth factor might be due to effect of the *Centella asiatica* (Coldren *et al*., 2003, Cheng *et al*., 2004). *In vitro* study suggested that FGF2 induces progenitor cells to undergo additional mitoses study, it was found that lower concentrations of *Centella asiatica* were required to enhance the proliferation of HPLF compared to previous study done on the skin fibroblasts where the concentration was up to (Cavanagh *et al*., 1997). Cells normally differentiate after they have undergone their last mitotic cycle, FGF2 may also influence the timing of cell differentiation. Debiais *et al*. (1998) found that the progenitor cells ALP activity decreased upon treatment with FGF2. Coldren *et al*. (2003) and Cheng *et al*. (2004) also found that, the level of basic fibroblast growth factor and FGF2 gene expression were elevated when the tissue or the human fibroblast were treated with *Centella asiatica*. In this study the 20µg/ml extract concentration induced the highest proliferation rate and associated with a minimal reduction in the ALP activity and this is in agreement with other studies done by (Debiais *et al*., 1998; Raballo *et al*., 2000). The reduction in the cells in this study could be due to ALP activity elevated level of FGF_2 associated with proliferation condition (Debiais *et al*., 1998; Raballo *et al*., 2000). FGF2 also enhances and modulates the production of the cell extracellular matrix which may influence the feedback regulation during

wound healing and regeneration processes in periodontal tissues (Shimabukuro *et al*., 2008). FGF-2 enhances and induces unique expression of osteopontin (OPN), which may play a role different from the other bone-related proteins during the process of periodontal induce the optimal condition for the cells proliferation and regeneration by its increasing the FGF-2 level and the subsequent effect on the OPN. OPN is an important anti-apoptotic factor in many circumstances. OPN blocks the activation-induced cell death of macrophages and T cells as well as fibroblasts and endothelial cells exposed to harmful stimuli (Denhardt *et al*., 2001; Standal *et al*., 2004). In this study, it is tissue regeneration (Terashima *et al*., 2008). Cells in the remaining periodontal tissues need optimal conditions if they are to perform their functions in the regeneration process (Emecen *et al*., 2009). In the present study, it is postulated that *Centella asiatica* might assumed that the elevated FGF2 expression in the proliferation cell will also be accompanied by elevated induction of opteopontin which then protect and prolong the cells survival of the aging HPLF.

In conclusion the aqueous extract of *centella asiatica* at 20µg/ml enhances highest proliferation rate of the human periodontal ligament fibroblast cell line.

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