ejpmr, 2018,5(7), 541-549

SJIF Impact Factor 4.897

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

FORMULATION AND EVALUATION OF 3D INJECTABLE BIODEGRADABLE HYDROGEL FOR THE TREATMENT OF PERIODONTAL DISEASES

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Article Received on 22/05/2018 **Article Revised on 12/06/2018** **Article Accepted on 01/07/2018**

ABSTRACT

Periodontitis is an inflammatory disease resulting in the destruction and loss of tooth-supporting structures (periodontal ligament and alveolar bone). **Objective:** This study is designed to formulate, and evaluate a degradable bio-adhesive hydrogel contains titrated extract of Centella Asiatica (TECA 3%, w/w) for healing and regeneration of the periodontal cells in-vitro. **Methods:** The 3D potential hydrogel formulation is composed of pectin (P) and hydroxypropylmethyl cellulose (HPMC) in various concentrations. The hydrogels were formulated in the range of 1-5%, w/w of pectin and 2 - 4% w/w of HPMC. Characterization of the hydrogels was done using rheometer to study the effect of P and HPMC concentrations on hydrogel rheology behaviour. The texture of hydrogel was characterized using texture analyzer to evaluate its syringeability, firmness, adhesiveness and cohesiveness. Fibroblast proliferation assay was done to assess the effect of the gel on the cells proliferation. Southern blot assay was also done to ascertain the effect of the gel on maintaining fibroblast telomere length during inflammation. **Results:** The experiments showed that the hydrogels containing higher P and HPMC have potential properties to deliver active compound. Incorporation of TECA in the hydrogels formulations does not affect the physical properties of the hydrogel. One of the best hydrogel formulations with high content of pectin and HPMC were selected. Cell proliferation assay was done to assess the proliferative effects of TECA on the human periodontal ligament fibroblasts (PDLFs). The parameters depicted significant activity of TECA hydrogel in enhancing the proliferation of fibroblast together with preserving their telomere length during inflammation. **Conclusion:** Due to the regenerative effects of TECA hydrogel, and the suitable gel mechanical properties achieved, the formulated bioadhesive hydrogel may be used as an adjunct for the treatment of periodontal disease and regeneration of the periodontal structure.

KEYWORDS: Hydrogel, periodontal disease, TECA, fibroblast, telomere length.

INTRODUCTION

Periodontitis is an inflammatory disease resulting in the destruction of the supporting structures of the teeth (the periodontal ligament and the alveolar bone). It results in the formation of pockets between soft tissue of the gingiva and the tooth, can eventually cause tooth loss.[1] Periodontal treatment requires not only combating and eradication of bacterial invasion, but it also requires the restoration of the lost supporting tooth structure. Experimental studies have shown that the potential of periodontal regeneration seems to be limited by the regenerative capacity of the cells involved. The regeneration of damaged periodontal tissues is mediated by various periodontal cells and is regulated by a vast array of extracellular matrix molecules.[2]

It is commonly accepted that the treatment of local diseases, e.g., infection or inflammation, is most effectively performed by the topical delivery of the required therapeutic agent to the target tissue.[3-6] The potential side-effects in administering systemic antibiotics and the inability of antiseptic mouthwashes to penetrate the periodontal pocket have fuelled interest in the sustained delivery of therapeutic agents within the periodontal pocket, thus ensuring a high effective concentration of the agent at the site of infection. The success of such treatments is intrinsically dependent on the chemical and mechanical properties of the formulation. Ideally formulations should be easily delivered into the periodontal pocket (preferably from using a syringe), show controlled release of drug into the crevicular fluid, exhibit retention within pocket for the desired period of time (without the aid of mechanical bonding to tooth surfaces), be biodegradable, non-toxic and non-irritant. Of the currently commercially available and experimental systems few show prolonged retention

within the periodontal pocket, whilst the majority must be either mechanically bonded to the tooth surface to ensure retention and/or physically removed by the dentist from the pocket at the end of therapy.[7] It is proposed that these problems may be primarily overcome by natural biodegradable formulations that are both easily introduced into the periodontal pocket and, additionally, whilst in this environment, interact with the mucin-coated epithelial and tooth surfaces by means of specific interfacial forces in a process commonly referred to as mucoadhesion.

Centella asiatica (a herb) has been used for hundreds of years as a traditional medicine of many Asian countries to improve wound healing. It has been reported that wound and ulcer healing are enhanced *via* promoting fibroblast proliferation and collagen synthesis by *C. asiatica* extract treatment in Europe.[8] These properties are ascribed to the active ingredients, asiatic acid (AA), asiaticoside (AS), and madecassic acid (MA). Madecassol, a formulation based on the titrated extract of *Centella asiatica* (TECA), prompted the proliferation of granulation and tensile strength through improving connective tissue formation, epithelization, and angiogenesis when applied locally on wounds in rat dorsal skins.[9]

Although few adjunctive remedies are currently available for the non-surgical or surgical treatment of periodontal disease, almost all of them are made by a complicated manufacturing process, require training and skill for handling, and mostly are not cost-effective.[10,11]

In the present study, we describe the formulation and characterisation of bioadhesive containing TECA for healing of periodontal disease and regeneration of the periodontal structure in terms of mechanical and release properties. The rheological performance was also determined using rheometer to study effect of pectin and HPMC concentrations on hydrogel rheology behaviour. In addition, cell proliferation assay was performed to assess TECA effect on the proliferation rate of the periodontal ligament fibroblast. Fibroblast telomere length was estimated Southren Blot procedure to ascertain the effect of TECA gel in maintaining or protecting telomere during inflammation.

MATERIALS AND METHODS

MATERIALS

Titrated extract of *C. asiatica* (TECA composed of 40.4 % asiaticoside, 57.2% asiatic acid and madecassic acid) was obtained from Health Sources Pharmaceutical, Beijing, China. Pectin and Hydroxypropylmethyl cellulose (HPMC) were purchased from Sigma, St. Louis, USA. All other chemicals were purchased from Promega, USA.

Preparation of 3D Bioadhesive Gel Containing TECA

In the present study, silanized gel was prepared from pectin (1 - 5 % w/w) and HPMC (2 - 4% w/w). The mixture was grafted with 3-glycidoxypropyltrimethoxysilane (3-GPTMS) and blended with the appropriate weight of phosphate buffered saline (PBS, 0.03 M, pH 6.8) using mechanical stirrer. The gel was transferred onto an ointment slab and TECA particles (3% w/w) were thoroughly mixed into semi-solid formulations until homogenous. The formulation were placed in a vacuum, to ensure the removal of entrapped air, and stored at 4°C prior to analysis.

Mechanical Characterisation of Bioadhesive Formulations

Texture profile analysis (TPA) was performed using a Stable Micro Systems Texture Analyzer (TA-XT2® Texture Analyzer, Haslemere, Surrey, UK). The selected formulations were transferred into McCartney (30-ml volume, grade 2 clear glass) bottles to a fixed height (7 mm) avoiding the introduction of air into the samples and the temperature of each allowed to equilibrate to 20± 1°C by storage in an oven for 48 hours (Jones et al. 2000). In TPA mode, the hemispherical analytical probe (diameter 1 cm) was twice compressed into each sample at a defined rate (2 mm s^{-1}) to a depth of 15 mm. A delay period (15 s) was allowed between the end of the first and the beginning of the second compression. At least four replicate analyses were performed for each formulation. The following mechanical parameters were derived from the resultant force–time plots.

1. Hardness (the force required to attain a given deformation)
2. Compressibility (the work required to deform the sample during the first compression of the probes)
3. Adhesiveness (the work required to overcome the attractive forces between the surface of the sample and the surface of the probe)
4. Cohesiveness (the ratio of the area under the force-time curve produced on the second compression cycle to the corresponding area produced on the first compression cycle)

Syringeability of Bioadhesive Formulations

The syringeability of each formulation was determined using the texture analyzer. In brief, formulations were transferred into identical plastic syringes to a constant height (3 cm). The content of each syringe was fully expressed using the texture analyzer in compression mode and the resistance to expression was determined from the area under the resultant force-time plot. Increased work of syringeability was denoted by increased areas under the curves. All measurements were performed at least in quadruplicate.

Rheological Analysis of gel formulations

Flow rheology of each formulation were determined at 20 ± 0.1 °C, using a Carri-Med CSL2-100 rotational rheometer in continuous shear analysis mode using a parallel plate geometry and a fixed gap of 1 mm. The sample geometry was selected according to the consistency of the formulations. Modeling of the flow

properties of the various formulations was performed using the Power Law (Oswald-de Waele) models, as follows: $\sigma = k\gamma^n$ where σ is the shearing stress, γ is the rate of shear, k is the consistency, and n is the pseudoplastic index.

In vitro release of TECA from gel formulations

In vitro release of TECA from the gel formulations was performed (in triplicate) using a 37 ml Franz diffusion cell. The diameter of the donor cell was 26 mm and the dissolution medium was PBS. The diffusion cell was water jacketed at 37°C; 1.5 g of the gel was transferred to the Durapore HVLP membrane (0.45 µm) of the vessel. At pre-determined intervals, samples of dissolution fluid (5 ml) were removed, analysed using ultra-violet spectroscopy at 353 nm and an equal volume of fresh, pre-warmed dissolution fluid doublereplaced into the dissolution vessels. The mass of TECA released at each time interval was calculated following reference to a previously constructed calibration curve (concentration range 1.0–100.0 mg/ml, *r*.0.99 with zero intercept).

Cell Proliferation Assay

Cell proliferation assay was done to determine the most suitable concentration of TECA which could enhance the HPLDFs proliferation as well as to assess TECA cytotoxic effect. Human periodontal ligament fibroblast (HPDLF) was obtained from the ScienCell Research Laboratories,

Table 1. Formulations of bioadhesive periodontal gel.

Formulation	Pectin % w/w	HPMC % w/w	TECA % w/w
A	1	2	3
B	1	4	3
C	5	2	3
D	5	4	3

California, USA.

HPDLF cells were cultured in 96-well micro plate ($2.5x10^4$ cells/well) with the addition of 50µl fibroblast medium containing 10% Fetal bovine serum and antibiotics (1% Penicillin/Streptomycin, 1% Gentamycin). The cell culture was incubated in 5% CO_2 incubator at 37°C for 24 hours. The cultured cells were then treated with TECA concentration of 2, 5, 10, 20, 30 and 40 µg/ml. The cells were incubated with TECA for another 24 hours. 15µl of [3-[4,5, dimethylthiazol-2-yl]-5-[3-caboxymethoxy-phenyl]-2-[4-sulfophenyl]-2H-tetrazolium salt (MTT) dye (Promega) was added into each well and incubation was continued for a further 4 hours. The optical cell density was later checked using a micro plate reader (Packard,USA) at 570nm wavelength. The cells cultures were assessed for their optical density on the intervals of 1, 2, 3, 7 days after treatment.

Telomere Length Analysis

To induce inflammation,[12,13] PDLFs were cultured with 20ng/ml TNF-α for 14 days in a 3D gel medium in the presence or absence of optimal concentration 20 µg/ml of TECA. Telomere restriction fragment (TRF) length analysis was performed as described by using the TeloTAGGG Telomere Length Assay Kit (Roche Diagnostic GmbH, Germany) according to the protocol provided by the manufacturer. The mean telomere length was calculated using the following formula (Kimura et al. 2010): TRF = ∑(OD*i*)/ ∑(ODi/L*i*), where OD*i* is the chemiluminescent signal and OD*i*/L*i* is the length of the TRF at position *i*.

Isolation of DNA

Fibroblasts were harvested and transferred to a 1.5 ml microcentrifuge tube. For adherent cells, trypsinization was done before harvesting. Number of cells used were 5 x 10^7 cells following manufacturer's protocol (Roche diagnostics GmbH, Mannheim, Germany). The cells sample was homogenized in Cellular Lysis Buffer and 10 µl Proteinase K Solution was added, followed by 1 hr incubation at 65°C. 500 µl RNase Solution was mixed with the sample by vortexing to ensure the solution is mixed with the suspension. 6 ml Protein Precipitation Solution was added to the sample which was vortexed for 5 – 10 seconds and then placed on ice for five minutes to aid in precipitation of the protein. Then the sample was centrifuged at 26,900 x *g* at 15 to 25°C. The supernatant containing the DNA was then transferred into a new, sterile 50 ml centrifuge tube containing 0.7ml of isopropanol. The sample was centrifuged at 1370 x *g* for 5 min, then the resultant DNA pellet was re-suspended in 1ml TE Buffer at 2 to 8° C overnight.

Southern Blot Procedure

1.5 µg of DNA obtained from the cells sample was digested with the restriction enzymes *Hin*fI (10U) and *Rsa*I (10U) (Roche Diagnostics GmbH, Mannheim, Germany), the DNA fragments were separated by gel electrophoresis (Sigma-Aldrich, St. Louis, MO, USA), which was run at 5 V/cm in 1X Tris-acetate-ethylene diamine tetra acetic acid buffer (TeloTAGGG Telomere Length assay kit; Roche Diagnostics GmbH, Mannheim, Germany). The DNA was transferred to a positively charged nylon membrane, according to the manufacturer's protocols. Thereafter, membranes were hybridized at 65°C with the digoxigenin (DIG)-labelled telomeric probe overnight in 5 x SSC, 0.1% Sarkosyl, 0.02% SDS and 1% blocking reagent (Roche, GmbH, Mannheim, Germany). The membranes were washed three times at room temperature in 2× SSC, 0.1% SDS each for 15 min and once in 2× SSC for 15 min. The DIG-labeled probe was detected by the DIG luminescent detection procedure (Roche, GmbH, Mannheim, Germany) and exposed on X-ray film. The mean telomere length was calculated using the following formula (Kimura et al. 2010): TRF = ∑(OD*i*)/ ∑(ODi/L*i*), where OD*i* is the chemiluminescent signal and OD*i*/L*i* is the length of the TRF at position *i*.

RESULTS AND DISCUSSION

Mechanical Characterisation of Bioadhesive Formulations

In this study, the mechanical properties of the formulations for the treatment of periodontal disease have been determined. Texture profile analysis (TPA) defines the mechanical parameters in terms of hardness, compressibility, and adhesiveness, properties that will affect the ease of product application into, and retention within, the periodontal pocket, respectively. TPA also allows an estimation of the extent of structural reformation following product administration (cohesiveness), a factor which will influence product performance. Therefore, in this regard, TPA is an applicable technique for the characterization of formulations designed for application to the periodontal pocket. Increased product hardness, compressibility are associated with increased concentrations of pectin and HPMC in the formulation (Table 2). Each of these parameters describes the resistance of each formulation to compression, and therefore, reflects alterations in product viscosity. When the semi-solid nature of the product was increased, formulation cohesiveness decreased. Decreased product cohesiveness associated with increased concentrations of the polymers was a function of increased product viscosity. The work required to expel each sample from a syringe is presented in Table 3. The work to expel the gel increased significantly as the concentrations and viscosity of each polymeric component were increased.

Table 2: Mechanical properties of gel formulations

Formulation	Hardness mean (± S.D)	Adhesiveness mean (± S.D)	Compressibility mean (± S.D)	Cohesiveness mean (± S.D)
A	2.01 ± 0.06	3.89 ± 0.11	16.81 ± 0.11	0.82 ± 0.03
B	2.05 ± 0.03	4.11 ± 0.01	16.87 ± 0.32	0.81± 0.01
C	2.11 ± 0.01	4.32 ± 0.25	17.25 ± 0.65	0.76 ± 0.01
D	2.55 ± 0.52	4.45 ± 0.15	17.46 ± 0.85	0.74 ± 0.02

Table 3: Work required to expel each formulation from a syringe

Formulation	Work (N. mm)
A	98.01±3.34
B	106.75±3.80
C	113.16±3.79
D	131.05±3.28

Flow Rheology

All of the binary interactive systems were pseudoplastic with limited thixotropy. The decreases in the non-Newtonian viscosity as a function of increasing shear rate were most appropriately mathematically modeled using a Power law (Oswald-de Waele) model, from which the consistency (*k*) and pseudoplastic index (*n*) were determined (Table 4). Increasing the concentration of either the polymer within formulation enhanced the consistency and reduced the pseudoplastic index. This infers that greater polymer-polymer interaction occurred whenever the % ratio of P to HPMC was greater. In addition to the gel consistency, the pseudoplastic index (*n*), a measure of the ease of shear thinning, was derived from the experimental flow data. Formulation displayed low pseudoplastic index values would therefore be suitable for application using an extrusion applicator, facilitating spreading of the host epithelium.[14]

Table 4: Oswald-de Waele Parameters for gel formulations.

Formulation	mean (± s.d.) consistency(*k*) (Pa.sn)	mean (± s.d.) pseudoplastic index (n)
A	609.2 ± 3.9	0.21 ± 0.01
B	993.1 ± 25.8	0.25 ± 0.01
C	1637.3 ± 88.8	0.34 ± 0.04
D	2414.3 ± 106.7	0.41 ± 0.03

Release of TECA from Gel Formulations

The time required for 10% ($t_{10\%}$) and 30% ($t_{30\%}$) release of TECA were calculated and statistically compared for each formulation. The results in Table 5 show that increasing the concentration of the polymers significantly increased $t_{10\%}$ and $t_{30\%}$. Decreased TECA release from formulations could be described as the corresponding increase in product viscosities that are associated with increased polymer concentrations.

Table 5: The time (min) required for the release of 10 and 30% of the original mass of TECA from the formulations.

Formulations	$t_{10\%}$	$t_{30\%}$
A	168.12±2.97	564.34±9.46
B	249.85±9.87	1022.80±71.80
C	241.64±8.90	1211.12±62.42
D	335.66±11.41	1406.1±144.22

Cell Proliferation Assay

MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) Cell proliferation assay was done after the culture periods (1, 2, 3 & 7 days) to test the effect of different concentrations of TECA hydrogel on the HPDLF growth. The mean optical densities were calculated with 95% confidence intervals. Repeated measures ANOVA between group analysis with regard to time was applied ($p < 0.001$). Assumptions of normality, homogeneity of variances and compound symmetry were checked and fulfilled. Cell viability increased in samples treated with TECA hydrogel.

Human periodontal ligament fibroblasts proliferation rate was significantly enhanced with the concentration 20µg/ml of TECA extract after 2 days treatment interval (Figure 1). In the present study, it was found that lower concentrations of TECA were required to enhance the regeneration of PDLF compared to previous study done on the skin fibroblasts where the concentration was up to 40µg/ml.[15] Samples treated with concentrations 2, 5 and 10 µg/ml showed less optical densities. However, these concentrations (2, 5 and 10 µg/ml) significantly enhanced cells proliferation compared to the control samples. The decrease in optical densities after the 2nd day of treatment can be explained by the depletion of TECA active ingredient over time, hence, the ingredient was utilised by the cultured cells.[16-20]

Enhancing proliferation of oral fibroblast is considered to be a crucial step to reinstate the lost tissues of the periodontium due to the inevitable inflammatory periodontal destruction in disease.[21,22] Ensuring continuous and progressive cell growth during inflammation would maintain the connective tissue production,[23] hence, minimise the consequent changes in the periodontal tissues architecture.[24-27]

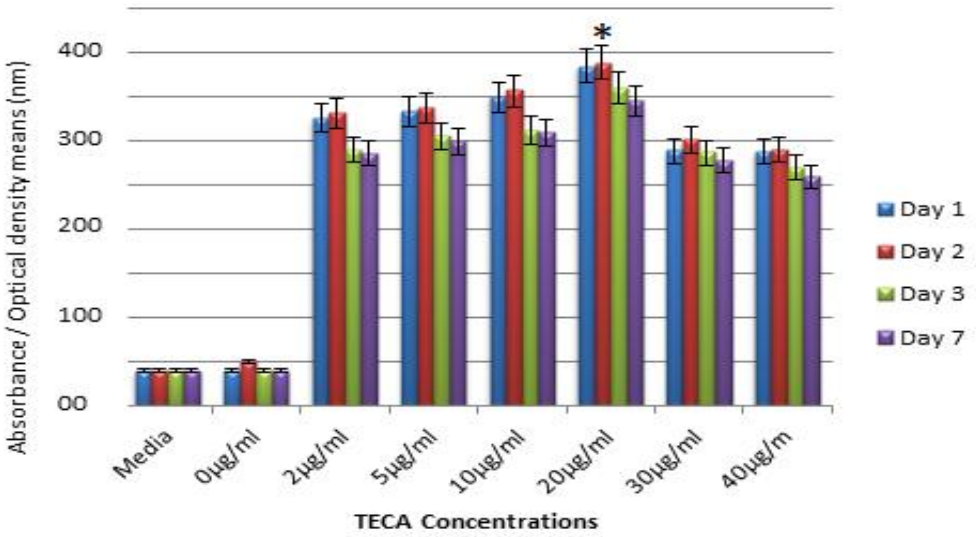

Figure 1: Effect of Different Concentrations of TECA Hydrogel on PDLF Proliferation.

MTT Cell Proliferation Assay Shows the Resultant Optical Density in Each Concentration
at Each Time Interval. *$p < 0.001$.

Fibroblast Telomere Length / DNA Damage Response (DDR)

The effect of TECA hydrogel on telomere during inflammatory senescence was analysed by assessing the telomere length using TeloTAGGG Telomere Length Assay (Figure 2). The means of telomere length in kilo base pair (Kbp) were obtained as depicted in Table 5. One-way ANOVA test was applied followed by post-hoc comparison Tukey test.

The results indicated that there was a significant difference among the tested samples (F-stat (df) =357.34 (2,24), $p < 0.001$). The mean telomere length was shorter in non-treated "PDLF + TNFα (without TECA)" samples as compared to the treated "PDLF + TNFα + TECA" samples. Although sample "PDLF+ TNFα + TECA" was undergoing inflammatory process (due to the presence of TNFα), the telomere length in this sample was not shortened remarkably as compared to "PDFL + TECA" samples (Table 5; Figure 3). This implies that TECA gel preserved or protected the telomere during inflammatory senescence.

Table 5: Means of Telomere Length of Cells in the Hydrogel with or Without TECA

Variables	Telomere length (Kbp) Mean (SD)	F statistic[a] (df)	p-value
PDLF + TECA	22.08 (0.61)	357.34 (2, 24)	<0.001
PDLF + TNFα (without TECA)	15.67 (0.53)		
PDLF + TNFα + TECA	20.36 (0.42)		

One-way ANOVA test was applied. F-stat (df) =357.34 (2,24), p<0.001.

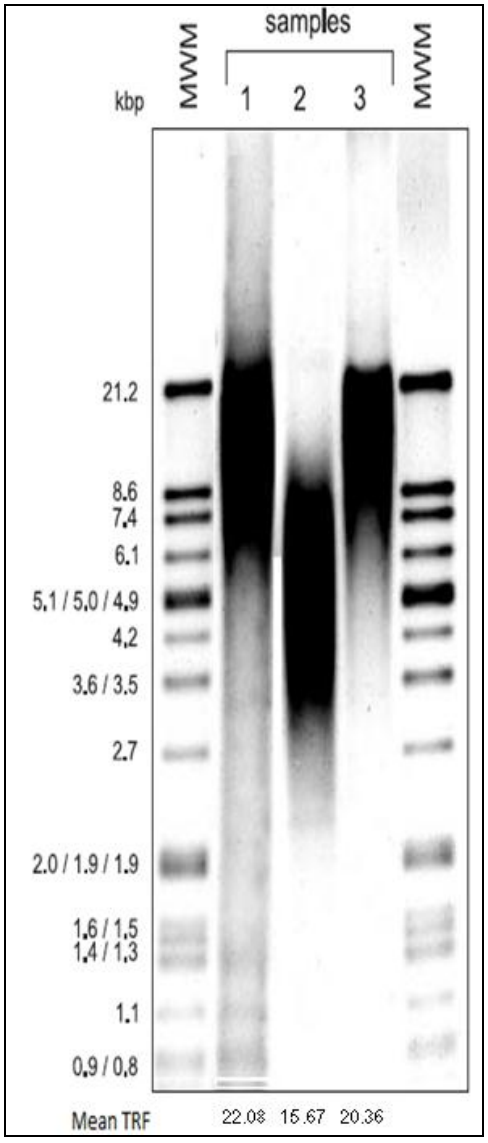

Figure 2: Chemiluminescent Detection of Telomere Restriction Fragment (TRF) Lengths. After exposure of the blot to an X-ray film, an estimate of the mean TRF length can be obtained by comparing the mean size of the smear to the molecular weight marker (MWM). Sample 1: PDLF + TECA; Sample 2: PDLF + TNFα (without TECA); Sample 3: PDLF + TNFα + TECA); Kbp: kilo base pair; PDLF: Periodontal ligament fibroblast; TECA: Titrated extract of *Centella asiatica*; TNF-α: Tumour necrosis factor-alpha.

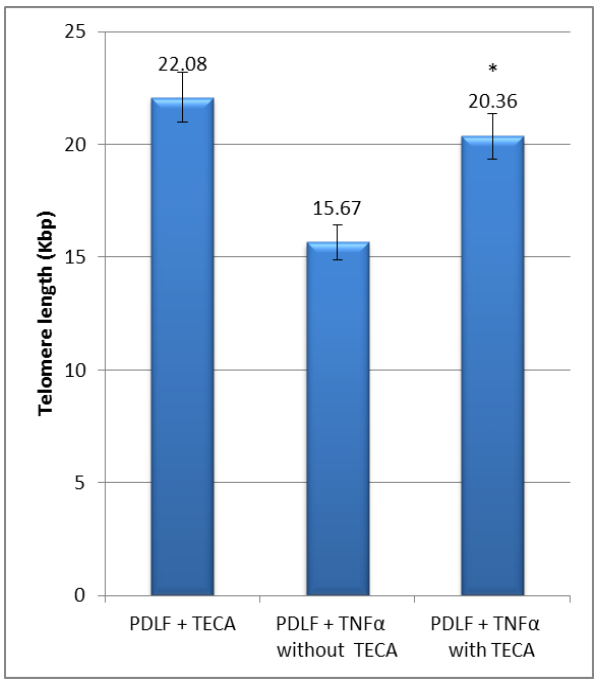

Figure 3: The Effect of TECA Hydrogel on Fibroblast Telomere Length.

One-way ANOVA test was applied. F-stat (df) =357.34 (2,24), * p<0.001. Kbp: kilo base pair; PDLF: Periodontal ligament fibroblast; TECA: Titrated extract of *Centella asiatica*; TNF-α: Tumour necrosis factor-alpha

Telomeres shorten as a result of cell replication and chronic inflammation, hence, it is thought that telomeres would serve as a replicometer for senescence.[28-31] In this connection, telomere length can be a good indicator of measuring the longevity of life biologically. However, induction of premature inflammatory senescence by stressful agents is thought to result from accelerated telomere shortening.[32-36]

Coluzzi et al., (2017) reported that protecting telomere length may prevent oxidative damage from reaching levels that threaten cell survival.[37] Hence; this would be beneficial in reducing the number of senescent cells (premature cells cycle arrest during inflammation) and protect the telomere from dysfunction and instability, subsequently preserving cells' viability and minimise tissue destruction.[38-40]

In the current study, periodontal ligament fibroblast cultures had been used, where the cell samples provoked by TNF-α in order to induce inflammation, and in

addition, cell also revealed telomere length shortening. These results clearly indicate the association between inflammation and telomere length. This can be explained by the fact that telomere shortening which probably was due to incomplete DNA replication of the chromosome ends (accompanied by premature senescence, i.e., cells cycle arrest) which occurred during inflammation.[41] It has been found that telomere shortening eventually causes chromosome instability, leading to the activation of DNA damage response pathway followed by p53-dependent cell cycle arrest and senescence.[42-45]

CONCLUSION

The design and development of topical formulation designed for prolonged application to the oral cavity presents a special challenge to the pharmaceutical formulator. Several desirable formulation attributes may be defined, including ease of removal of product from the container, favourable spreading characteristic over, and prolonged adhesion to the treated mucosal epithelium and reformation of the structural characteristics of the formulations following application. The choice of candidate formulations for clinical application will require a compromise between low hardness and compressibility (to ensure ease of product removal from the container and ease of application), maximal adhesiveness (to ensure good retention within the mouth and high cohesiveness (to ensure complete structural recovery following application). In this study, the gel has been characterized by a unique rheological behaviour and possesses the appropriate pharmacological properties to serve as an intra-pocket gel delivery system for periodontal therapy. TECA hydrogel was also shown to enhance the proliferation of PDLF cells together with maintaining their telomere length, which is a very important step to maintain the cell survival and reduce tissue destruction during inflammation. Therefore, the 3D TECA hydrogel may serve as a promising adjunct scaffold in periodontal surgical procedures. Long term clinical evaluation of TECA hydrogel is ongoing.

CONFLICTS OF INTERESTS

No conflict of interest existed

ACKNOWLEDGMENT

The study was supported by the Ministry of Science, Technology and Innovation Grant (MOSTI Sf01.01.09), Research Acculturation Grant Scheme (RAGS 119/2013) and UITM DANA (RIF 751/2012).

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