

In Vitro Chitosan Hydrogel Based Tetracycline Cytotoxicity Test on Fibroblast Viability

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Abstract. Tetracycline has been widely used as a periodontal support treatment since it has broad-spectrum bone tissue penetration and it can inhibit native collagen. Chitosan hydrogel-based tetracycline is known to have strong antibacterial effects, but the cytotoxic effects on fibroblast have yet to be studied. The objective of this study is to obtain the in vitro cytotoxicity effect of chitosan hydrogel-based tetracycline on fibroblasts. Chitosan hydrogel-based tetracycline (0.5%, 0.7% and 1%) and chitosan hydrogel without tetracycline is made by dissolving chitosan powder in citric acid. Tetracycline powder is then added to the solution. 3T3 fibroblast cells were cultured in a well microplate containing RPMI-1640 media inside an incubator. The viability assay is conducted using the MTT-assay method and repeated 5 times. The colour degradation is read with a microplate reader. The mean viability of fibroblasts applied with chitosan hydrogel-based tetracycline (0.5%, 0.7% and 1%) and chitosan hydrogel is 86.5%, 85.36%, 80.99% and 90.85%, respectively. The highest mean cell viability in the group of fibroblasts applied with chitosan hydrogel-based tetracycline is in the 0.5% group. This value is not significantly different than the value in the 0.7% group, but it is significantly different than the value in the 1% group. Chitosan hydrogel-based tetracycline shows a low cytotoxic effect on 3T3 fibroblast cells.

Keywords: tetracycline, chitosan, viability, cytotoxic

1 Introduction

Periodontal disease is considered one of the most common chronic dental diseases. This term encompasses gingivitis and periodontitis, both of which are caused by plaque-associated bacterial infections that accumulate in the periodontal pockets. Periodontal disease is managed by eliminating bacterial and inflammatory products through mechanical or chemical methods [1,2]. When used as a supportive treatment, local drug delivery provides better results, especially in patients with deep periodontal pockets.

Tetracycline is widely used because of its broad-spectrum effects, bone penetrating ability, MMPs and its ability to inhibit native collagen. According to Sachdeva, 0.7% is the concentration of tetracycline acceptable by tissues [3]. Recent developments of this method focus on employing controlled-release intra-

pocket device systems that use fibres, films, gels, strips, vesicular systems, semisolids with micro- or nanoparticles, etc. Local drug delivery is more efficient since the drugs are directly applied to the bacteria. These yields fewer adverse effects and a lower chance of bacterial resistance [4].

Chitosan is a natural polysaccharide mucoadhesive formed through the N-deacetylation of chitin, which is obtained from shrimp or crab shells. Chitosan and its derivatives have proven to be efficient drug transporters on mucous and trans-mucous because of their polymer cationic character. Their positively charged cationic nature promotes interaction with mucous epithelium. This increases the distance between cells and allows macromolecule transportation through the epithelial barrier [4,5].

The bactericidal and bacteriostatic effects of chitosan have been widely studied since the 1980s. Chitosan's bacteriostatic effects are due to its interactions with ions on cell walls. Nutrition supplies for microbes are suppressed because m-RNA and protein synthesis is inhibited, as chitosan penetrates the microbe's nucleus and creates an external barrier. The molecule weight and the degree of deacetylation plays an important role and is inversely proportional to the effectiveness of microorganism suppression [6]. This effect has been confirmed by Susanto et al. (2017) in his study about the effectiveness of chitosan hydrogel-based tetracycline on some periodontal pathogens [7]. The aim of this work is to obtain the in vitro cytotoxicity effect of chitosan hydrogel-based tetracycline on fibroblasts.

2 Material and methods

The chitosan, provided by the Chemistry Department at the Universitas Sumatera Utara, was processed from shrimp shells. Tetracycline hydrochloride was purchased from PT. Mutifa. 3T3 Fibroblast cell lines were cultured by the Parasitology Laboratory at the University of Gadjah Mada. All other chemicals used in this study were of analytical grade.

The chitosan hydrogel (4% w/w) was prepared following the method formulated by Popa et al.(2013) by continuously mixing (by hand) 2 grams of chitosan with an adequate amount of citric acid (1%)⁴ Tetracycline hydrochloride (0.5%, 0.7% and 1%) were then dissolved in the 4% chitosan hydrogel. The result was a homogeneous hydrogel. The hydrogel was then mechanically cross-linked by cooling down to 4°C prior to use. Based on the study conducted by Susanto et al. (2017), the amounts of chitosan, tetracycline hydrochloride and citric acid were modified to improve the gel viscosity for local usage (Table 1) [7].

To obtain 1 x 10⁴ cell/well density, 3T3 fibroblast cell lines were cultured in microplates containing 1640 media from Rosewell Park Memorial Institute and incubated for 24 hours. After the media were washed with PBS and refreshed, 25 µl of each experimental hydrogel was then added to the microplates. This was followed by a 5% CO₂ incubation process at 37°C for 24 hours.

Table 1. Composition of the chitosan hydrogel based tetracycline 0.5%,0.7% and 1% and chitosan hydrogel without tetracycline.

Hydrogel group	Material		
	Chitosan	Tetracycline	Citric acid 1%
Tetracycline 0.5%	2 gr	0,25 gr	50 ml
Tetracycline 0,7%	2 gr	0,35 gr	50 ml
Tetracycline 1%	2 gr	0,5 gr	50 ml
Chitosan hydrogel	2 gr	-	50 ml

After 24 hours, the solution in the wells was flicked off and 50 µl of MTT dye was added to each well. The microplates were then packed and left at room temperature for an entire night. A total of 130 wells were used; the control and blank groups comprised 5 wells and the experimental hydrogel groups comprised 25 wells. The absorbency level was measured using a microplate reader at a wavelength of 595 nm. The percentage of cell viability was calculated using the following formula:

$$\%Cell\ viability = \frac{(Absorbance\ value\ of\ test\ compound - Absorbance\ value\ of\ blank)}{(Absorbance\ value\ of\ control - Absorbance\ value\ of\ blank)} \times 100$$

The percentage of cell inhibition was calculated by subtracting the percentage of cell viability from 100. The Saphiro-Wilk normality test was conducted on the viability results, followed by the ANOVA test. The results were considered statistically significant when the p value was < 0.05.

3 Result

The absorbency level results in Table 2 were obtained from the microplate reader. Each of the mean values were then used to calculate the viability of the 3T3 fibroblast cells using the aforementioned formula (Table 3).

Table 3 shows that the highest mean of cell viability is with chitosan hydrogel. However, the highest mean of cell viability in the chitosan hydrogel-based tetracycline group is a concentration of 0.5 %.

The viability percentage of each experimental group was measured by the Saphiro-Wilk normality test, resulting in normal distributions with significance levels greater than 0.05 (Table 4). The ANOVA test was then conducted to determine if there were any significant differences in each group's viability percentages (Table 5).

Table 2. Absorbance result of each hydrogel groups on 3T3 fibroblast cells.

Groups	Mean, \pm SD	Min-Max
Cell Control	0.625, \pm 0.024	0.591-0.635
Blank Control	0.08, \pm 0.05	0.079-0.087
Chitosan	0.577, \pm 0.004	0.575-0.586
hydrogel	0.575, \pm 0.005	0.567-0.582
	0.575, \pm 0.006	0.567-0.582
	0.573, \pm 0.003	0.568-0.575
	0.576, \pm 0.004	0.572-0.584
Tetracycline	0.553, \pm 0.009	0.522-0.561
0.50%	0.55, \pm 0.005	0.544-0.557
	0.553, \pm 0.003	0.552-0.561
	0.551, \pm 0.003	0.552-0.559
	0.551, \pm 0.004	0.549-0.558
Tetracycline	0.546, \pm 0.017	0.517-0.546
0.70%	0.543, \pm 0.003	0.552-0.547
	0.547, \pm 0.002	0.544-0.549
	0.545, \pm 0.006	0.539-0.554
	0.545, \pm 0.005	0.539-0.551
Tetracycline	0.521, \pm 0.007	0.509-0.528
1.00%	0.52, \pm 0.004	0.514-0.523
	0.523, \pm 0.004	0.524-0.528
	0.521, \pm 0.006	0.512-0.524
	0.522, \pm 0.005	0.513-0.526

Table 3. Viability and inhibition percentage of Chitosan Hydrogel based Tetracycline

	% Viability Mean, \pm SD	% Inhibition Mean, \pm SD	Min-Max
Chitosan hydrogel	90.85 \pm 0.55	1.15 \pm 0.55	90-91
Tetracycline 0.5%	86.53 \pm 0.55	13.47 \pm 0.55	86-87
Tetracycline 0.7%	85.36 \pm 0.55	14.64 \pm 0.55	85-86
Tetracycline 1%	80.99 \pm 0.55	19.01 \pm 0.55	78-81

Table 4. Normality test result

% Viability	Significance
Chitosan hydrogel	0.635
Tetracycline 0.5 %	0.814
Tetracycline 0.7%	0.777
Tetracycline 1%	0.201

Saphiro-Wilk Normality test
p>0.05

Table 5. Comparison of chitosan hydrogel based tetracycline cytotoxic effect on fibroblast cells

	Tetracycline 0.5%	Tetracycline 0.7%	Tetracycline 1%	Chitosan hydrogel
Tetracycline 0.5%	-	0.007	0.000*	0.000*
Tetracycline 0.7%	0.007	-	0.000*	0.000*
Tetracycline 1%	0.000*	0.000*	-	0.000*
Chitosan hydrogel	0.000*	0.000*	0.000*	-

ANOVA test

*Significant p<0.05

In the chitosan hydrogel-based tetracycline group, the highest cell viability mean was found in the 0.5% tetracycline group. There was no significant difference in the 0.7% tetracycline group, but there was a significant difference in the 1% tetracycline group.

4 Discussion

This study concluded that all concentrations of hydrogel showed no cytotoxicity effect on 3T3 fibroblast cells according to ISO 1099-395. This states that viability percentages above 80% are considered non-cytotoxic. ISO 1099-395 further states that viability percentages between 80% to 60% are considered weak, viability percentages between 60% to 40% are considered moderate and viability percentages below 40% are considered strong [8]. The highest viability percentage (90.85%) was found in the 4% chitosan hydrogel group, while the lowest viability percentage (80.99%) was found in the 1% tetracycline group. Grobler et al. and Zhang et al. both concluded that the cytotoxic effect of chitosan will increase along with the concentration level [9,10]. Arancibia et al. reported that any chitosan concentration higher than 5 mg/ml may cause cytotoxicity on human gingiva fibroblasts [11].

Chitosan has been widely used in the pharmaceutical field due to its low toxicity. Chitosan is biodegradable, mucoadhesive and non-toxic. It also has high bio-

compatibility and low solubility in neutral environments [12]. Its biodegradability could prevent accumulation and allow for self-sustaining release.

Due to its advantages, chitosan has been widely used as drug carrier or a wound healing agent. Hydrogel is known as a three-dimensional hydrophilic polymer that can absorb and retain levels of water, or any biological fluid, up to one-thousand times heavier than its dry weight. Hydrogel may have a chemical stability that degrades and dissolves over time. This 'aging' process of hydrogel could be inhibited by the cross-linking method [13,14].

Hydrogel has good biocompatibility because of its low surface tension, which allows it to be used as an absorbent solvent for cell adhesions. Hydrogel also has a high-water content, and it can stimulate several types of tissue. Additionally, its soft character allows for low irritation and mechanical friction on living tissues [15,16,17]. Having all these qualities, hydrogel is suited for use as a macromolecular vehicle in medical applications [18].

Tetracycline is one of the most popular antibiotics for treating anaerobic bacterial infections [19]. Tetracycline can be used to support conventional therapies that may be ineffective on certain patients. Combination treatments are known to be more effective than standalone treatments [20]. Although it is believed to be safe, a recent study shows that tetracycline inhibits certain proteins and cell proliferation by binding to 30S ribosomal sub-units. It can also alter the integrity of mammalian cell membranes, resulting in macromolecular dysfunction, cellular lysis and cellular mortality [21,22,23]. An inhibitive effect of tetracycline on protein translation, which is managed by mitochondrial DNA on mammalian cells, has been reported. This inhibition will trigger the imbalance of mitochondrial protein, which will lead to interference with growth, oxygen consumption and fertility [24,25].

Various viability percentages achieved in this study might result from chitosan's controlled drug-release character. Based on the study done by Zhang et al., it was found that chitosan has a sustained-release ability that lasts for 5 days [26]. Zhang et al. also reported the characteristics of low toxicity towards 3T3 fibroblast cells and high biocompatibility when chitosan was combined with tetracycline.

Chitosan hydrogel-based tetracycline (0.5%, 0.7% and 1%) shows high viability and non-cytotoxic effects on 3T3 fibroblast cells. Chitosan hydrogel 4% shows good biocompatibility and no toxicity on 3T3 fibroblast cells.

5 Conclusions

Chitosan hydrogel-based tetracycline shows a low cytotoxic effect on 3T3 fibroblast cells.

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