ORIGINAL ARTICLE

The Effect of Cytotoxicity of Collagen-Chitosan Hydrogel on Platelets-Rich Plasma Various Formulation for Human Primary Fibroblast

Tutut Prabantari Anindyajati[1], Sri Pramestri Lastianny[2], Ronny Martien[3], Kwartarini Murdiastuti[2]

[1] Clinical Dentistry Magister Program, Periodontology, Faculty of Dentistry, Universitas Gadjah Mada, Jl. Denta, Sleman, Yogyakarta, 55281, Indonesia
[2] Departement of Periodontology, Faculty of Dentistry, Universitas Gadjah Mada, Jl. Denta, Sleman, Yogyakarta, 55281, Indonesia
[3] Departement of Pharmaceutical technology, Faculty of Pharmacy, Universitas Gadjah Mada, Jl. Sekip Utara, Sleman, Yogyakarta, 55281, Indonesia

ABSTRACT

Introduction: Platelet-rich plasma (PRP) contains growth factors that accelerates the process of wound healing. The application of liquid PRP is tricky; therefore, it requires collagen to act as a carrier. Upon exposure to body fluids, collagen often loses its shape and size due to degradation. Collagen is synthesized with chitosan during material selection. The formulations of collagen–chitosan hydrogel in PRP that met the criteria of physical test were 25/75 and 0/100. However, to investigate the biocompatibility, MTT assay was employed to test cytotoxicity by detecting cell viability using human primary fibroblast. The aim of this study is to determine the cytotoxic effect of collagen–chitosan hydrogel formulation on PRP in human primary fibroblast. **Methods:** The study divided into three groups: collagen–chitosan hydrogel in PRP 25/75 (n=9), collagen–chitosan hydrogel in PRP 0/100 (n=9), and PRP-only (n=9). After dripping MTT into the microplates, optical density were read using ELISA reader and the viability were counted. The data that was acquired from the viability calculation was analyzed with one-way ANOVA and LSD test. **Results:** There were significant differences (p=0.007) between collagen–chitosan hydrogel 25/75 on PRP and other groups. The collagen–chitosan hydrogel 25/75 group had the highest level than collagen–chitosan hydrogel 0/100 and PRP-only. **Conclusion:** This in vitro study demonstrated hydrogels collagen–chitosan ratio of 25/75 and 0/100 on PRP had no cytotoxic effects in human primary fibroblasts.

Keywords: Chitosan, Collagen, Human Primary Fibroblast, Hydrogel, Platelet-Rich Plasma

Corresponding Author:
Kwartarini Murdiastuti, PhD
Email: kmurdiastuti@ugm.ac.id
Tel: +6281328734900

INTRODUCTION

Platelet-rich plasma (PRP) contains growth factors that supports soft tissue healing, encourages a faster healing process, and is proven to increase bone formation and density by 19% to 25% within 4 months of an injury (1). PRP contains high concentration of both platelets and fibrinogen. During the wound healing, the first cell to respond to the injury is platelet. Along with the procoagulants, platelets secret growth factors that involved in angiogenesis during the healing soft and hard tissue wounds (2). The addition of PRP into the operating area degrades the PRP inside the oral cavity (3) owing to the solubility of PRP in water, which necessitates collagen to act as a carrier (3,4).

The collagen in PRP is a safe and effective alternative because it stimulates the platelet granules to release growth factors, which increase the gelation of PRP, thereby making it easier to use. In addition, it reduces PRP clot retraction. These properties of collagen render it important to be used as a scaffold (3,4). Upon exposure to body fluids, collagen often loses its shape and size due to degradation. In the material selection process, collagen is synthesized with chitosan because it has a slower rate of degradation than collagen; therefore, chitosan is expected to increase the mechanical strength of collagen. Chitosan performs various functions such as by acting as an antibacterial agent, accelerating tissue regeneration and synthesis of fibroblasts. Furthermore, it is widely used in tissue engineering (5). Chitosan is a biomaterial that has certain properties, including hydrophilic, non-toxic, and positively charged that can promote compressive strength (6).

A study has stated that collagen–chitosan hydrogel is

more resistant to enzymatic degradation, denaturation, and has a high compressive strength. This result further suggests that collagen–chitosan hydrogel has the potential to act as a scaffold to support cell therapy and vascularity of damaged tissues (7). Hydrogels can act as humectants, thus maintaining water content in wounds, accelerating healing process, and preventing irritation. They can be easily removed without pain. Hui and Lerouge (8) reported that hydrogel formulation is determined by the ratio of collagen–chitosan concentration. The formulations used in their study were 100/0, 75/25, 50/50, 25/75, and 0/100. Adding chitosan to the hydrogel greatly increases its mechanical properties, and as the ratio of collagen in the hydrogel increases, its gelation kinetics slows down along with a decrease in its mechanical properties. According to previous studies, the formulation of collagen–chitosan hydrogel in PRP did not affect its pH but affected the viscosity and solubility; therefore, the formulation ratios that met the criteria were 25/75 and 0/100.

Before the application of the material, its feasibility was determined by the biocompatibility test. Biocompatibility is generally determined by employing toxicological principles, which provide information regarding the potential toxicity of a material for clinical applications (9). Cytotoxicity test is conducted to investigate the biocompatibility of a material (10), and it can be conducted through in vitro testing using cell culture (11). Fibroblasts are considered to be dominant in the connective tissues of healthy gingiva, and they secrete collagen fiber and extracellular substances (12,13,14) In most cases, 3-(4,5-dimethylthiazoyl-2-yl)-2,5- 10 diphenyltetrazolium bromide (MTT) is widely used in cytotoxicity testing of a substance as a dye. On the MTT test, MTT tetrazolium salt is absorbed actively into the cell through the permeable membrane of the cell and decompose by mitochondrial reaction into formazan crystals. This product unable to penetrate the permeable membrane of the cell and accumulates in the cell. Capability of a cell to decompose MTT indicates that there is activity and mitochondrial integrity which is defined as a measure of the viability of a cell. Multiwell Scanning Spectrophotometer (ELISA readers) which can measure samples with a high degree of precision. The aim of this study is to determine the cytotoxic effect of collagen–chitosan hydrogel formulation on PRP in human primary fibroblast. The hypothesis in this study is Collagen-chitosan hydrogel formulation on PRP had no cytotoxic effects in human primary fibroblasts.

MATERIALS AND METHODS

Samples

This research was a laboratory experiment approved by the Ethics Committee, Faculty of Dentistry, Universitas Gadjah Mada, Indonesia (registration number 00110/KKEP/FKG-UGM/EC/2019). Parameters that observed in this study is the amount of living human primary fibroblasts on the cell cultures after the treatment has been given. Treatment groups this study is divided based on collagen-chitosan hydrogel 25/75 on PRP (n=9), collagen-chitosan hydrogel 0/100 on PRP (n=9), and PRP-only (n=9). The sample consisted of peripheral blood collected from volunteers who met the following inclusion criteria: platelet count in the normal range (confirmed by routine blood tests) and not diagnosed with any bleeding disorder, aged 25-65 years old. Human primary fibroblasts were collected from the Dermatology and Venereology Laboratory, Faculty of Medicine, Universitas Gadjah Mada.

Collagen–chitosan hydrogel preparation

The hydrogel was prepared by mixing pharmaceutical grade chitosan powder (shrimp shell) and collagen powder (fish collagen). Chitosan was then added into a glass beaker containing 100 mL of water and placed on a magnetic stirrer at a speed of 3,000 rpm and stirred until it was dispersed. Thereafter, 1% acetic acid was added into the mixture. Hydroxypropyl Methylcellulose (HPMC) and collagen were prepared by weighing them on a digital scale. The collagen powder was added into chitosan and mixed at a speed of 3,000 rpm until it became homogeneous. The Hydroxypropyl Methylcellulose powder was added into the solution and mixed at 3,000 rpm using a homogenizer until it became homogeneous. Collagen, chitosan and HPMC was homogeneous, the mixture was left to rest for 15 min at room temperature, and then stored inside a cooling machine for 18 h to 24 h at 4 °C. All collagen–chitosan hydrogel formulations (25/75 and 0/100) underwent the same procedure. The collagen-chitosan hydrogel formulation: 25/75 : 0.5 g collagen + 1.5 g chitosan + 2 g HPMC; 0/100 : 2 g chitosan + 2 g HPMC

Platelet-rich plasma preparation

A total of 100 mL of each donor's blood was taken from the mediana cubital vein using wing needle, and each 9 mL of blood was stored inside of vacutainer tubes, each containing 1 ml of 3.8 % sodium citrate. Each tube was then centrifuged for 10 min at 1,200 rpm. The first centrifugation (EBA 20 Hettich) produced two separate layers: the upper layer consisted of platelet-poor plasma (PPP), and the lower layer consisted of red blood cells. Platelet-poor plasma was separated with a three-way stopcock (OneMed). The second centrifugation was performed at 3,500 rpm for 10 min. This second centrifugation created two separate layers: the upper two-third layer was of PPP and the one-third bottom layer consisted of PRP. The obtained PRP was then inserted into a syringe, then mixed until it became a homogeneous PRP. The collagen–chitosan hydrogel formulation was added to PRP with a ratio of 1:1 (15).

Cytotoxicity test with Microculture Tetrazolium Salt (MTT) assay

Human primary fibroblasts were obtained from the extracted healthy premolar teeth due to orthodontic

indications. The extracted teeth were then rinsed with a saline solution from apical to coronal areas to avoid contamination. Periodontal ligament cells were taken from the teeth's root surface by using blade No. 15. The ligaments were taken from the teeth's root surface, specifically the middle third area. Thereafter, the ligament cells were cultured using Dulbecco's Modification of Eagle's Medium in a culture flask added with 10 % FBS, 2 % streptomycin and penicillin, and fungizone. Human primary fibroblasts were extracted from the culture flask and then divided into a 96-well microplate, with each well containing four groups of 2.5 x 10^3 human primary fibroblasts. The next day, the human primary fibroblast culture medium was replaced with a new medium and was left undisturbed until it reached an 80 % confluence. The test material was then inserted into the plate, and the cell culture was incubated in each treatment at the temperature of 37 °C for 24 h. The Microculture Tetrazolium Salt assay in cell culture was conducted by calculating the value of optical density that was previously calculated using the multiwell scanning spectrophotometer (ELISA reader). On the MTT test, the value of Optical Density (OD) obtained from calculation of the amount of formazan crystal that formed using a plate reader at a wavelength of 570 nm. Human primary fibroblast viability (%) determined by comparing the value of OD of each sample with the average of OD control. The cytotoxicity of a material can be measured based on cell viability relative to controls as following formula:

$$\text{Cell viability (\%)} = \frac{\text{Optical Density sample}}{\text{Optical Density control}} \times 100\%$$

The classified level of cytotoxicity of a material based on the relationship between cell viability (%) in the sample group compared to the control group as follows (16):
Cell viability > 90% Non-cytotoxic material
Cell viability 60-90% Slightly cytotoxic material
Cell viability 30-59% Moderately cytotoxic material
Cell viability ≤30% Very cytotoxic material

Statistical analysis
Comparisons for collagen–chitosan hydrogel 25/75, collagen–chitosan hydrogel 0/100 and PRP-only were performed by one-way ANOVA. For all of the tests, a statistically significant result represented by the p-value of <0.05. Then followed by the Least Significant Difference test with α=0.05 (LSD0.05). The data were analyzed using the SPSS program version 22.0. Values for measurements are presented as values of mean ± SD.

RESULTS

Microculture Tetrazolium Salt assay was conducted to determine the effect and cytotoxicity of various formulations of the collagen–chitosan hydrogel in PRP toward human primary fibroblast. Cytotoxic effects were determined by calculating the human primary fibroblast viability (%). The calculated mean and standard deviation of human primary fibroblast viability after the exposure of collagen–chitosan hydrogel with PRP for 24 h are presented in Table I.

Table I: The mean value and standard deviation of human primary fibroblast viability after exposure to the soak solution of collagen chitosan hydrogel on PRP for 24 hours.

Sample Group	n	Optical Density Sample	Optical Density Control	Mean ± SD (%)
Collagen/Chitosan 25/75 on PRP	9	0.306	0.243	125.92 ± 17.4
Collagen/Chitosan 0/100 on PRP	9	0.298	0.243	122.63 ± 29.7
PRP	9	0.258	0.243	106.17 ± 19.9

The results showed that human primary fibroblast viability after the exposure of collagen–chitosan hydrogel of 25/75 group on PRP is greater than the other formulations, i.e., 0/100 on PRP, and PRP-only. The data was normally distributed and homogenous, as seen from the results of normality and homogeneity tests ($p > 0.05$).

The data obtained from one-way ANOVA test indicated a p-value of <0.05, thereby suggesting a significant difference in the human primary fibroblast viability (p=0.007) among the three sample groups. The result from LSD 0.05 test demonstrated significant differences ($p < 0.05$) in human primary fibroblast viability between the group with the collagen–chitosan hydrogel formulation of 25/75 on PRP and the other two groups. In contrast, the difference between collagen–chitosan hydrogel formulation of 0/100 on PRP and PRP-only was not significant ($p > 0.05$; Table II).

Table II: LSD0.05 post-hoc test of human primary fibroblast viability after exposure to the soak solution of collagen chitosan hydrogel on PRP for 24 hours.

	25/75 on PRP	0/100 on PRP	PRP
25/75 on PRP	-	0.017	0.003
0/100 on PRP	-	-	0.452
PRP	-	-	-

DISCUSSION

The effect cytotoxicity of various collagen–chitosan hydrogel formulations on PRP toward human primary fibroblast had mean values of the average human primary fibroblast viability in the three sample groups: collagen–chitosan hydrogel formulations of 0/100 on PRP, 25/75 on PRP, and PRP-only. Meric et al. (16) classified cytotoxicity material levels based on the relationship between cell viability (%) on the sample and control groups, which read as follows: if the cell viability is >90 %, then it is non-cytotoxic material; if the cell viability ranged from 60 % to 90 %, then it is

a slightly cytotoxic material; if the cell viability ranged from 30 % to 59 %, then the material is considered as cytotoxic; and if the cell viability is ≤30 %, then it is highly cytotoxic material. On an average, the three sample groups had >90 % cell viability, which means that the three groups did not cause any cytotoxic effect on human primary fibroblasts because average viability of the three sample groups shows more than 90% (16).

PRP is an autologous product with no known adverse effects (24). Collagen and chitosan have a quality of nontoxic material (23). The cytotoxic potential of a chemical compound is determined by the amount of dose administered to the cell. There is a relationship between the chemical compound dose and the response of exposed tissue cells, which is the basis of toxicology, further suggesting that higher the administered dose, greater is the risk of cytotoxic effects on the exposed tissue cells. Tissue cells have a certain threshold for a chemical compound to prevent the dose received by the cell from exceeding the acceptable threshold because it may cause cytotoxic effects as characterized by cell death (17). Thus, the ratios of collagen–chitosan hydrogel on PRP (0/100 and 25/75) and PRP only used in the present study are safe,

The results obtained from one-way ANOVA demonstrated a significant difference ($p<0.05$) in human primary fibroblast viability among the three groups. The results determined that there were differences in the effects of cytotoxicity among the three groups of collagen–chitosan hydrogels on PRP. Moreover, collagen–chitosan hydrogels on PRP with higher cell viability indicated that they have the least cytotoxic effect as compared to the other groups, thus rendering them safer for use.

Chitosan has characteristic nontoxic, biocompatible, biodegradable and antimicrobial, while the collagen in wound healing process plays a vital role, it forms extracellular matrix (ECM) in connective tissue (23). The addition of chitosan can improve cell viability because chitosan is resistant to chemical and enzymatic changes, thereby acting as an adhesive with a positive charge (20). Collagen is one of the important biomaterials used in tissue engineering since it has a high biocompatibility. It is used as the basis for the cell culture system. The addition of collagen has a high tensile strength, can act as absorbents, and activate platelets (19). Therefore, combination of chitosan and collagen will have a better effectiveness in wound healing process (23).

The LSD test results exhibited some significant differences ($p < 0.05$) between collagen–chitosan hydrogel 25/75 on PRP and the other two groups, further suggesting that the collagen–chitosan hydrogel of 25/75 on PRP is safer to use than the collagen–chitosan hydrogel 0/100 on PRP and PRP-only. The spread of cells increased as the collagen added to chitosan due to collagen consequently encourages the cells to multiply more than what occurs in pure chitosan hydrogels (0/100). As the concentration of collagen increases, the cell concentration increases (18). Hui and Lerouge (8) postulated that increasing collagen ratio in collagen-chitosan hydrogels will lead to more cell viability as compared to pure chitosan hydrogel.

During a wound healing process, particularly in the proliferation stage, fibroblast has an important role. The characteristic of the proliferation stage is marked by the migration of fibroblast into the wound. Fibroblast has a major role in the synthesis, remodeling, and deposition of extracellular matrix (ECM). Fibroblasts then proliferate and create matrix proteins namely proteoglycan and collagen. Soon afterwards, these matrix proteins facilitate the production of the new ECM, which supports cell growth crucial for the repair process. Collagen is a strong chemoattractant to fibroblasts. In addition, it has been shown that the smaller fractions of collagen, for instance smaller peptide and α chain digested from collagen demonstrated some chemotactic properties. It is possible the regions from the collagen chains that contain amino acid sequences are capable of inducing fibroblasts (22). Pati et al. (21) mentioned in their study that type 1 collagen sourced from fish can increase the proliferation of fibroblasts. Collagen from fish is non-toxic and can be used as a potential candidate for a collagen-based bone graft. Chitosan is an excellent medium for cell growth, has bacteriostatic properties, and has analgesic effects 15, and increases collagen synthesis by fibroblasts during the wound healing process and supports tissue regeneration with anti-bacterial and anti-inflammatory properties (26). Collagen-chitosan PRP hydrogel increases fibroblast proliferation because chitosan can increase the basic Fibroblast Growth Factor (bFGF) by protecting it from degradation caused by heat or enzymes (25).

The LSD test of collagen–chitosan hydrogel 0/100 on PRP and PRP-only indicated no significant difference ($p > 0.05$). However, according to the cytotoxicity classification, both formulations were not cytotoxic, thus rendering them safe for use (16). Chitosan has a toxic effect on cell viability. This happens because chitosan can suppress cell apoptosis by denatures cell proteins in the cell membrane which causes changes in permeability cell. The cell membrane cannot maintain its components in the cell and disrupting the flow of materials that enters and leaves the cell, therefore causing the cell to die. The positive charge of the amino group in chitosan has the ability to interact with the negative charge of the cell membrane. Then it is absorbed to form a layer that inhibit the action of enzymes, so that the cell do not have enough substance to develop and cause the cell to die. The amines contained in chitosan have toxic properties for cells, but the appearance of the toxic response affected by the dosage and the concentration of the chemicals materials given (27).

CONCLUSION

This in vitro study showed that the exposure of collagen–chitosan hydrogels 25/75 on PRP better than the other two groups and hydrogels collagen–chitosan ratio of 25/75 and 0/100 on PRP had no cytotoxic effects in human primary fibroblasts. Further studies are necessary regarding the effect of collagen–chitosan hydrogel on homologous PRP for fibroblast cytotoxicity.

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