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Cytotoxicity Assay of Chitosan-Collagen Wound Dressing using Brine Shrimp Lethality Test Methods

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ABSTRACT

Wound dressing has a function to protect the wound area from external exposure and provide a moist state around the wound area for accelerating wound healing process. Collagen and chitosan are known as appropriate biomaterials to synthesise of wound dressing because they have anti-bacterial, anti-fungal, biodegradable and biocompatible properties. One of biocompatibility assay for a material is cytotoxicity assay using Brine Shrimp Lethality Test (BSLT) that could be applied before in Vivo assay. The aim of the research was to know the cytotoxicity level of collagen-chitosan wound dressing with variance of concentration such as K0 as control used pure chitosan, K1 used collagen 25% and chitosan 75%, K2 used collagen 50% and chitosan 50%, K3 used collagen 75% and Chitosan 25%. Skin and scales of Gabus fish (*Channa striata*) were extracted using 2% HCl solvent to obtain collagen and chitosan powder dissolved in 1% acetic acid. Furthermore, wound dressing is made by a combination of collagen-chitosan concentration according to each group (K0, K1, K2 and K3. Citotoxicity assay used Brine Shrimp Level Test (BSLT) method with concentration each sample group were 10, 50, 100, 250, 500, 750 and 1000 ppm. The results showed that each wound dressing group such as K0, K1, K2 and K3 had LC50> 1000ppm that indicated wound dressing was non-toxic.

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INTRODUCTION

Wound Healing Society, a wound is a physical damage due to the opening or damage to skin tissue that causes imbalance of the anatomical structure and normal skin function. Skin tissue damage includes loss of epithelial tissue along with connective tissue or without connective tissue (Solanki & Nagori, 2011). Wound healing process, it requires a moist atmosphere (moist state) to increase the activity of growth factors for cell growth so that there is an increase in cell migration, fibroblast proliferation and re-epithelialization of cells in the injured tissue (Andini & Prayekti, 2019). Wound dressing is one application that can be used to maintaining the humidity of the wound area. Wound dressing has been widely developed and applied by health practitioners in several developed countries. Collagen and chitosan are the main ingredients most widely used in making wound dressing. Chitosan is biomaterial which has characteristic nontoxic, environmentally friendly, biocompatible, biodegradable and antimicrobial properties (Andini & Prayekti, 2019). While collagen plays a vital role in wound healing because it forms extracellular matrix (ECM) in connective tissue (Aisyah *et al*., 2017).

One source of collagen that can be used is collagen from the skin of cork fish (*Channastriata*). Cork fish has a high protein content so that collagen can be used for wound healing because it contains high amino acid Glycine which played a role in collagen synthesis, Glutamine played a role during the inflammatory phase and proliferation of wound healing and acts as an energy source, while arginine played a role in immune function and stimulates function endothelial cells (Rahayu *et al*., 2016).

The use of wound dressing can be applied if it has been through a series of tests in vitro and in vivo.In vivo testing, it is necessary to conduct biocompatibility tests to determine biological responses including cytotoxicity, sensitization, toxicity, intracutaneous irritation, carcinogenicity, biodegradation, and reproductive or developmental (Gani, 2015). Therefore, in this study a cytotoxicity assay was performed using the Brine Shrimp Lethality Test (BSLT) method on wound dressing-based chitosan based on natural ingredients, namely collagen from cork fish (*Channa striata*) and chitosan from shrimp and crab shells. This research hopes that collagen-chitosan wound dressing can be a candidate for wound dressing based on natural ingredients that can be applied for wound healing in the future.

MATERIALS AND METHOD

Materials

This research uses several tools used for the extraction and mixing of collagen and chitosan, namely glass beaker, glass funnel, aluminum foil, filter cloth, filter paper, wound dressing molds with a thickness of 2-3 mm and gauze cloth. Whereas BSLT uses aerators, containers for the process of hatching eggs and growth of *Artemia salina*, as well as petri dishes used for BSLT observation.

The material used in this research uses collagen extracted from the scales and skin of Cork fish (*Channastriata*) using 2% HCl and 0.1 M NaOH and chitosan powder dissolved in 1% acetic acid. Cytotoxicity test with Brine Shrimp Lethally Test (BSLT) method using *Artemia salina* shrimp larvae developed in artificial sea water by mixing distilled water with sea salt with a ratio of 1: 50 (Muaja *et al*., 2013).

Methods

This research is an experimental study using four research groups based on variations in collagen and chitosan content in wound dressing, namely K0 as a control with pure chitosan content, K1 with 25% collagen content and 75% chitosan, K2 with 50% collagen-50% chitosan content, K3 with 75% collagen content and 25% chitosan. Cork fish collagen extract was obtained by maceration of skin and scales of Cork fish with 2% HCl solvent for 48 hours, then filtering to get the filtrate, then the filtrate was neutralized with 1 M NaOH until collagen fibers appeared.

In the next step, collagen deposition and filtration are carried out. Furthermore, synthesis of composites by mixing collagen-chitosan according to the concentration of the group (K0, K1, K2 and K3), then put into a wound dressing mold that has a thickness of about 2-3 mm, then dried (drying) for 72 hours until the composite appears dry (Andini & Prayekti, 2019).

Cytotoxicity assay with BSLT method was done by incubating 1 gram of *Artemia salina* eggs into 500 ml artificial sea water and given lighting and aeration for 48 hours. After A. salina hatch, the test solution and wound dressing are prepared which are dissolved in 1% DMSO. Variations in the concentration of the test solution are 10, 50, 100, 250, 500, 750 and 1000 ppm. Each test solution was put into a petri dish and 10 larvae of *A. salina* shrimp were added, then incubated for 24 hours and counting the number of dead larvae. The LC50 assessment can be determined by the probit analysis method (Julfitriyani *et al.*, 2016). Calculation of the percentage of *A. salina* larvae mortality uses the following formula (Meyer *et al.*, 1982)

$$\% \text{ Mortality } = \frac{\text{Total} \quad \text{Mortal}}{\text{Total } A.Salina} \text{ x } 100\%$$

RESULT AND DISCUSSION

Collagen extraction has been widely carried out using vary sources of fish waste, from skin and scale (Praba *et al.*, 2019)to fish bone (Leliani *et al.*, 2019). This study was used skin and scale of snake head fish (*Channastriata*) as source of collagen. Obtained collagen was combinedwith chitosan for wound dressing. As a wound dressing, need to consider its component function to ensure benefit. Chitosan utilization was intended to decreased bacterial and fungal contamination in wound dressing (Andini and Prayekti, 2019). Meanwhile, collagen as a main component have several use as a wound healing (Andini, 2016)). Since wound dressing addressed to treat wound then its component toxicity needs to evaluated. Collagen toxicity assay from fish bone (Leliani, Hasnah, Seniwati, & Sartika, 2019)was showed an LC50 value of 8760 ug/mL. Based on those result, fish bone has lower toxicity according to toxic criteria (Meyer *et al.*, 1982).

This study gives no toxic result for 1000 ppm of snakehead collagen. For a better application further needs to determine LC50 value. Brine Shrimp Lethality Test (BSLT) method is used to study general sample toxicity using shrimp larvae *Artemiasalina* as a test subject. This methodcan be used as preliminary test for cytotoxicity test. The cytotoxicity test for each collagen combination in this study showed at table 1, table 2, table 3, and table 4, while statistics test using SPSS showed at table 5.

According to data at table 1, table 2, table 3 and table 4, it shows descriptively no significant mortality on *Artemia salina* being tested on each collagen concentration at KO, K1, K2, and K3 groups. At K1 groups on variation concentration 500 ppm and 1000 ppm recorded mortality percentage in the amount of 10%. While K2 group on variation concentration 750 ppm have 10% mortality percentage. Despite of it, BSLT test count result indicates that wound dressing of collagen – chitosan wasnontoxic because LC50 > 1000 ppm. Data interpretation of cytotoxicity test can categorized in to three categories (Meyer et al., 1982) according to value of its LC50. Toxicity categories shown at table 6.

Tabel 1. Collagen toxicity assay result using *Artemia salina* at K0 groups

Consentration (ppm)	Log10 (Consentration)	Total Mortality	Total	Mortality (%)	Probit
10	1.00	0	10	0%	-
50	1.70	0	10	0%	-
100	2.00	0	10	0%	-
250	2.40	0	10	0%	-
500	2.70	0	10	0%	-
750	2.88	0	10	0%	-
1000	3.00	0	10	0%	-

Tabel 2. Collagen toxicity assay result using *Artemia salina* at K1 groups

Consentration (ppm)	Log10 (Consentration)	Total Mortality	Total	Mortality (%)	Probit
10	1.00	0	10	0	-
50	1.70	0	10	0	-
100	2.00	0	10	0	-
250	2.40	0	10	0	-
500	2.70	1	10	10	3.72
750	2.88	0	10	0	-
1000	3.00	1	10	10	3.72

Tabel 3. Collagen toxicity assay result using *Artemia salina* at K2 groups

Consentration (ppm)	Log10 (Consentration)	Total Mortality	Total	Mortality (%)	Probit
10	1.00	0	10	0	-
50	1.70	0	10	0	-
100	2.00	0	10	0	-
250	2.40	0	10	0	-
500	2.70	0	10	0	-
750	2.88	0	10	0	-
1000	3.00	0	10	0	-

Tabel 4. Collagen toxicity assay result using *Artemia salina* at K3 groups

Consentration (ppm)	Log10 (Consentration)	Total Mortality	Total	Mortality (%)	Probit
10	1.00	0	10	0	-
50	1.70	0	10	0	-
100	2.00	0	10	0	-
250	2.40	0	10	0	-
500	2.70	0	10	0	-
750	2.88	1	10	10	3.72
1000	3.00	0	10	0	-

Tabel 5. Statistic test result for collagen cytotoxicity test using BSLT methods

No.	Groups	LC50	Criteria
1	K0	> 1000	Not Toxic
2	K1	> 1000	Not Toxic
3	K2	> 1000	Not Toxic
4	K3	> 1000	Not Toxic

Table 6. Toxicity categoriesaccording to LC50 value

Catagories	LC_{50} (ppm)
Highly Toxic	<30 ppm
Toxic	30-1000 ppm
Non toxic	> 1000 ppm

(Subekti, 2014)

This study result shown that collagen as composite of wound dressing collagen-chitosan have a quality of nontoxic material. As a composite, collagen and chitosan properties giving promising candidate as a wound dressing. Modern wound dressing has been develop since last twenty years as wound treatment because its efficacy in killing germ and prevent recurrent infection (Gito & Rochmawati, 2018). Polymer become main aspect in developing modern wound dressing, in which already provided in a form of hydrocolloid, hydrogel, film, foam, and alginate (Borda *et al.*, 2016). Wound dressing can be synthesised naturally by combining material which posses bioactivity properties, or even combining material which posses accelerating healing properties. Wound dressing application not only limited to heal a wound but also used as disease therapy like diabetic foot ulcer (Moura *et al.*, 2013).

This research made a wound dressing using a mixture of chitosan and collagen material sourced from snakehead fish. Chitosan has the benefit of accelerating wound healing, stimulating the immune response, an antimicrobial effect and non toxic properties. While collagen is a protein that is considered ideal in tissue engineering and dressing. Based on the healing time and mechanism, chitosan has a healing time of 21-23 days, with a natural mechanism of increasing collagen deposition and skin cell poliferation, while collagen has a healing time of ± 21 days to increase cell poliferation and accelerate wound contraction. Collagen is the main protein in the extracellular matrix which plays an important role in the healing phase. Collagen will undergo enzymatic degradation in the body with the help of the enzymes collagenase, gelatinase and metalloproteinase, thus collagen becomes the right biomaterial developed for dressing (Nasution & Sriwidodo, 2019). Collagen derived from the skin of Sangkuriang Catfish (*Clarias gariepinus* var. Sangkuriang) when given topically to the skin of wistar strain induced IIb burns, was able to reduce the total number of macrophages in the surrounding area on the 10th day significantly, increasing the total number of fibroblasts and collagen on the 10th day significantly (Aisyah *et al.*, 2017) (Andini, 2016). Therefore, the combination of chitosan and fish-based collagen will have a better effectiveness in wound healing and can be applied as a wound dressing in the future (Nasution & Sriwidodo, 2019).

CONCLUSION

Based on cytotoxicity test using Brine Shrimp Lethality Test on chitosan-collagen wound dressing shown that in all categories were not toxic due to LC50>1000.

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