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Formulation and Evaluation of Wound Healing of Alginate-Chitosan and Calsium Alginate-Chitosan Membran in Guinea Pigs

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Abstract: Guinea pigs wound healing has been investigated by using calcium alginate-chitosan and alginate-chitosan membrane.Membrane strength tested with a universal testing machine type: SC-2DE. Skin wound made by cutting the abdominal skin to the dermis of guinea pigs with a size of 1 x 1.5 cm.The wound was treated by adhering the membrane to the wound. Then, the wound was covered by using sterile bandage. Every 3 days the wound area was measured. The inflammation, the dryness of the wound, the presence of the pus were observed. Furthermore, it was also performed the histopathological observation of the wound that has been treated at 0 day and 12th day by using chitosan-alginate membrane and calcium chitosan-alginate membrane.The result of macroscopical observation showed that the wound healing by calcium chitosan-alginate membrane and chitosan-alginate was faster than untreated wound (control). The wound healing by calcium chitosan-alginate membrane was faster than by chitosan-alginate membrane. The result of tensile strength test showed that calcium chitosan-alginate membrane was stronger than that of chitosan-alginate membrane. The result of histophatological observation the untreated (control) showed that at the 12th day, it was found the epidermis hasn't been dense, the hair folicle hasn't been formed, and has a lot of fibroblast. But, the wound healing by calcium chitosan-alginate and chitosan-alginate was found that the epidermis was dense, the hair folicles were formed and fibroblast was less. It was found that the wound that healing by calcium chitosan-alginate the epidermis was more dense and the fibroblast was lesser.

Introduction

Poly-anionic alginate and poly-cationic chitosan, when dissolved in the right conditions, can interact with each other through the carboxyl group and amino group of the alginate and chitosan.[1,]

Poly electrolyte complex formed is expected to provide better application due to the unique structure and properties. So far, the complex poly electrolyte alginate-chitosan widely used as fibers, capsules and granules.While publications on its utilization as membrane remains limited.On the other hand, chitosan alkaline and easily soluble in acidic media are widely used for the manufacture of gel in several variations such as grain, membrane coatings, capsules and fiber.Alginate preparations have been made in the form of an ointment. The results showed that the ointment on the basis of alginate can release the drug compound, able to absorb water and does not irritate the skin.[1,2,3]

It has been made alginat-chitosan membrane, calcium alginate and calcium alginate-chitosan and test the development of the third membrane in an aqueous medium. The results showed that the increase in weight of alginate-chitosan membrane is greater than the calcium alginate-chitosan membranes. This means that the alginate-chitosan membranes have the ability to absorb fluid that is greater than the calcium alginate-chitosan membranes. Membrane calcium alginate showed a large absorption at the beginning of time but dissolves ahead of the first hour.[4,5]

The test results showed that the antibacterial activity of membrane-kiktosan alginate and calcium alginate-chitosan no growth of bacteria Escherichia coli and Staphylococcus aureus whereas calcium alginate membranes showed significant growth this bakteri.Hal on polyelectrolyte complexes in alginate-chitosan membranes and membrane calcium alginate-chitosan has antibacterial activity, whereas the membrane calcium alginate no antibacterial activity.[4,5]

Method

The tools used in this study consisted of glass tools commonly used in the laboratory, the balance of electricity (Mettler Toledo), pH meter (Hanna), glass objects, mortar and stanfer, microtome (Reichert Jung, Germany), surgical tools , oven, heating table, staining jar, chamber, cover glass, a microscope, a digital camera, bunsen, the universal testing machine type: SC-2DE.

Sodium alginate production Wako Pure Chemical, chitosan production of Sigma-Aldrich Inc., glacial acetic acid, alcohol, 0.1 M calcium chloride, distilled water, hydrochloric acid is E'Merck product, 10% sodium hydroxide, sodium chloride infusion of physiological 0.9% Bhakti PT.Widarta production, production pH indicator PT.Macherey-Nagel, formalin, xylol, paraffin, Ehrlich hematoxylin, eosin Y, Canada balsam.

Manufactureof Alginate-Chiitosan Membrane

The procedure of making the membrane is based on previous research (Santi, 2008). Weighed 1 gram of chitosan, then added with 25 ml of distilled water and dissolved in 5 ml of glacial acetic acid while crushed in a mortar to form a homogeneous mixture, moved into a closed erlenmeyer. Furthermore weighed 1 gram of sodium alginate and dissolved in 25 ml of distilled water in the Erlenmeyer. The second solution is left to stand for 24 hours.[4,12,14,17]

Both of the polymer solution was mixed and added 2 ml of 32% hydrochloric acid and then allowed to stand 5 minutes. Further added sodium hydroxide 10% (w / v) to obtain a pH of 5.2. The gel that is formed is placed in a porcelain cup and plate printed on a glass slide, each glass plate containing 1 gram of gel, flattened and then dried at room temperature.

In a state of semi-arid ie after draining one night, object glass plate was soaked and rinsed in distilled water until neutral pH, then dried again at room temperature. The drying time for ± 72 hours.

Manufacture of Calsium Alginate-Chitosan Membran

The procedure of making the membrane is based on previous research (Santi, 2008). The same procedure as the manufacture of alginate chitosan membrane, made a mixture of alginate and chitosan, and printed on a glass slide, and then dried at room temperature. After drying one night, the glass plate containing a mixture of alginate with chitosan gel is rinsed and soaked in distilled water until pH neutral, each glass plate is then dipped into 10 ml of 0.1 M calcium chloride solution for 10 seconds, removed and dried at room temperature for ± 72 hours, to obtain results in the form of a thin and transparent layer.[4,14,17,18]

In Vivo Procedure

Used 9 tail male guinea pigs with weigh 700- 900 grams purchased from Brastagi, North of Sumatera, Indonesia. Before being treated as guinea pigs, hamsters are adapted to the environment during the first 2 weeks. During each experiment guinea pigs reared in separate cages and fed sufficiently. For the experiment, each guinea pig was treated:

Guinea Pigs I,II and III : Control
Guinea PigsIV,V, and VI : Alginate-Chitosan

Guinea PigsVII, VIII, and IX : Calcium Alginate-Chitosan

Macroscopically Observations

Animal experiments before surgery, shaved on the right or left abdominal skin of guinea pigs, and then marked on the abdominal skin of guinea pigs with a size of 1 x 1.5 cm, then using phenobarbital anaesthetized by intramuscular injection of 60 mg / kg bw, then cut the skin of guinea pigs to layer dermis using tools that are sterilized in such a way, then the abdominal skin of guinea pigs which have been hurt photographed with a digital camera. Cleaned the wound using a sterile infusion of saline solution (0.9% NaCl), and then affixed to each membrane size of 2 x 2 cm and covered with sterile bandages and plaster. For control, untreated wounds, only closed sterile bandages. Do the turn of the membrane of every 3, 6, 9, 12 days and at the turn of the wound preparation beforehand wetted with saline infusion solution (0.9% NaCl) until the membrane can be lifted out of the wound. Then the wound was observed that the presence of inflammation, dryness of the wound, pus after 3, 6, 9, 12 days and measured the length and width of the wound. Then the skin organ immersed in 10% formalin. Furthermore, this skin organ to microscopic examination to look at the skin tissue of guinea pigs.[6,17,19]

Microscopically Observation

Guinea pig skin is taken on day 0 and day 12 after injury. The skin organ re-fixed in 10% formalin for further making preparations for returning skin tissue.

Preparation of Skin Tissue

skin that had been taken were fixed in 10% formalin solution for 2 days, then washed with a solution of alcohol 70% v/v repeatedly or allowed to stand for 1 day. Then dehydrated in alcohol-rise starts by soaking in alcohol 70% v/v for 30 minutes, then in alcohol 80% v/v, 90% v/v, 96% v/v, and absolute alcohol during each 24 hours.Then the skin organ clarified in pure xylol 2x30 minutes and put in toluol solution of paraffin has melted in the oven for 60 minutes. The next row leather organs were put into pure paraffin I, II, III respectively 60 minutes.Skin organ inserted into a mold containing molten paraffin and allowed to harden. Paraffin block containing the skin organ 6μm thick sliced - 10 μm using a microtome and then the slices are placed in a slide that has been smeared with albumin and a few drops of distilled water, then placed on a table attached to the heater until the tissue slide.Then the tissue put in xylol solution for 15 minutes. Once the tissue is dipped successively into absolute alcohol, 96%, 90%, 80%, 70%, and distilled water. Then the staining of the tissue by placing it in erhlich hematoxylin solution for 3-7 seconds and then washed with running water approximately 10 minutes.Then dipped in distilled water, alcohol 30%, 50% and 70%. Once it is done again staining by putting it in 0.5% eosin solution for 3 minutes, followed by immersion in 70% alcohol, 80%, 90%, 96% and absolute alcohol, then dried with absorbent paper, then the tissue is spilled with Canada balsam and covered with a glass lid. The tissue preparative observed under a microscope with a magnification eyepiece magnification 10 times and lots 10 and 40 times.[7]

Tensile Strength Test

Membrane tensile strength testing is done at room temperature with the universal testing machine tool type: SC-2DE, with a heavy load of 100 kgf, and the speed of the engine drag (cross-head) 10 mm/min. Samples (membrane calcium alginate-chitosan and alginate-chitosan) is placed on the second clamp (grip) whose position perpendicular to the drag tool. Pull the engine switch and the switch is turned on chart recorder together. The tensile strength of the membrane can be seen from the Load and Stroke has. Rated load (kgf) represent tensile strength at break, whereas stroke (mm / min) shows the tensile strength at break. Rated load and stroke are usually inversely.[2,11,19]

Result

Alginate-Chitosan Membran

Alginate-chitosan membranes can be made by mixing a solution of alginic acid and chitosan acetate solution in water that has been allowed to stand 24 hours at pH 5.3 and then printed on a glass plate and dried at

room temperature for 72 hours resulted in a fairly thick membrane, thickness measuring ± 0.1 mm, not easily torn, yellowish white. Its surface is shaped like a homogenous fiber braid.

Calcium Alginate-Chitosan Membran

Calcium alginate-chitosan membrane is made by dipping a membrane of alginate-chitosan in 0.1 M calcium chloride solution for 10 seconds produces a thick membrane, measuring ± 0.2 mm thickness, not easily torn and yellow. The membrane was brittle and looks stronger than the membrane of alginate-chitosan.[15,16,19]

Comparison of Membrane Calcium Alginate-Chitosan and Alginate-Chitosan

Table 1. Comparison of Tensile Strength (Load), Strength Strain (Stroke), and Added Long Membrane Calcium Alginate-Chitosan and Alginate-Chitosan

Type of membrans	Load (kgf)	Stroke (mm/minute)	The Length (%)
Alginate-Chitosan	0.235	9.345	18.182
Calcium alginate-Chitosan	0.385	13.925	33.182

Based on the data owned by the mechanical strength of the membrane calcium alginate-chitosan and chitosan alginate above and Appendix 3, it can be concluded that the calcium alginate-chitosan membrane has a tensile strength that is better than alginate-chitosan membranes. One of the properties of sodium alginate is when mixed with calcium chloride solution quickly formed calcium alginate gel that does not dissolve in water. The bond between the calcium alginate is a chelate bond between the calcium ions with the carboxylate anion in block G-G through inter-chain mechanism that causes the polymer chains increasingly tight.[9,12,13]

Wound healing

Macroscopically Observed

Control

Guinea pigs in the control treatment I, II, and III and table 2 can be seen the wound area ratio average decline slowly. On day 3-6 has been no drought injury, on the 9th day looks a little dry wounds, while the average wound area decreased seen on day 6 (Table 2). On the 3rd day seen reddened wound inflammation in guinea pigs II.

Table 2. Observations on Controls Conditions

Days	Inflammation			Wound Humidity			Pus		
	Guinea Pigs			Guinea Pigs			Guinea Pigs		
	I	II	III	I	II	III	I	II	III
0	-	-	-	-	-	-	-	-	-
3	-	++	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-
9	-	-	-	+	+	+	-	-	-
12	-	-	-	++	+	+	-	-	-

Explanation

- : there is no
+ : a litle
++ : much
+++ : huge

Table 3. Effect on Wound Healing Time Of Guinea Pigs Controls (n = 3)

Time of Observation (Days)	Wound Ratio	Standard Deviation	Standard error
0	1.000	0.0000000	0.0000000
3	1.000	0.0000000	0.0000000
6	0.871	0.0536936	0.0310000
9	0.713	0.0702377	0.0405518
12	0.178	0.0190526	0.0110000

Explanation:
Wound Ratio : Extensive Injuries Late
Early Wound Areas

Alginate-Chitosan Membrane

In the treatment of experimental animals IV, V and VI using alginate-chitosan membrane can be wound area ratio decreased after the 3rd day. Drought cuts can already be seen on the 3rd day, unlike controls that can be seen after the 9th day and wound increasingly dry seen until the 12th day. It shows that the administration of alginate-chitosan membrane drought injuries occur more quickly compared to controls. This is due to the alginate membranes have a strong ability to absorb fluids (exudates) from injury, while chitosan is antibacterial.[4,10,19]

Table 4. Observation of Wound Conditions on Membrane Preparations Alginate-Chitosan

Days	Inflammation			Wound Humidity			Pus		
	Guinea Pigs			Guinea Pigs			Guinea Pigs		
	IV	V	VI	IV	V	VI	IV	V	VI
0	-	-	-	-	-	-	-	-	-
3	-	++	-	+	-	+	-	-	-
6	-	-	-	++	+	+	-	-	-
9	-	-	-	++	++	++	-	-	-
12	-	-	-	+++	+++	+++	-	-	-

Explanation :
- : there is no
+ : a litle
++ : much
+++ : huge

Table 5. Effect on Wound Healing Time Of Alginate-Chitosan (n = 3)

Time (Days)	Ratio of Healing	Standard deviation	Standard error
0	1.000	0.0000000	0.0000000
3	0.913	0.0808290	0.0466667
6	0.719	0.0265016	0.0153007
9	0.511	0.0386825	0.0223333
12	0.035	0.0080829	0.0046667

Keterangan :
Ratio : Extensive Injuries Late
Early Wound Area

Calcium Alginate-Chitosan Membrane

In the treatment of experimental animals VII, VIII and IX are treated using calcium alginate-chitosan membrane can be wound area ratio average decline began on day 3 and the ratio of the wound will be decreased on day 12. On the 9th day, there are still attached to the skin tissue of alginate-chitosan membrane, whereas in calcium alginate-chitosan membrane there are no tissue attached to the membrane so that the formation of the epidermis is not disturbed at the turn of the membrane. Faster wound healing occurs due to the chelate bond between the calcium ions with the carboxylate anion at GG block that causes the polymer chains so that the closer the calcium alginate-chitosan membranes are not easily degraded compared with alginate-chitosan is more easily degraded in the skin.[14,17]

Alginate can form a gel in the presence of divalent cations such as Ca 2+, Mn 2+, Cu 2+ and Zn 2+, wherein the crosslinking occurs because of the chelate complexes between divalent ions with carboxylate ions of blocks GG.

Table 6. Observation of WoundConditions on Membrane Preparations Calcium Alginate-Chitosan

Days	Inflammation			Wound Humidity			Pus		
	Guinea Pigs			Guinea Pigs			Guinea Pigs		
	VII	VIII	IX	VII	VIII	IX	VII	VIII	IX
0	-	-	-	-	-	-	-	-	-
3	-	-	-	+	+	+	-	-	-
6	-	-	-	+	++	++	-	-	-
9	-	-	-	++	+++	+++	-	-	-
12	-	-	-	+++	+++	+++	-	-	-

Explanation:

- : there is no
+ : a litle
++ : much
+++ : huge

Table 7. Wound Healing Effect Against Time on Marmots Membrane Calcium Alginate-Chitosan (n = 3)

Time (Days)	Ratio of Wound Healing	Standard deviation	Standard error
0	1.000	0.0000000	0.0000000
3	0.837	0.0496521	0.0286667
6	0.521	0.0607069	0.0350492
9	0.353	0.1619452	0.0140119
12	0.021	0.0092376	0.0053333

Explanation :

Ratio : $\frac{\text{Extensive Injuries Late}}{\text{Early Wound Area}}$

Table 8. Wound Ratio of Average Size Control, Alginate-Chitosan and Calcium Alginate-Chitosan

Sample	Time (Days)				
	0	3	6	9	12
Control	1.000	1.000	0.871	0.713	0.178
Alginate-Chitosan	1.000	0.913	0.720	0.511	0.035
Calciumalginate-Chitosan	1.000	0.837	0.521	0.353	0.021

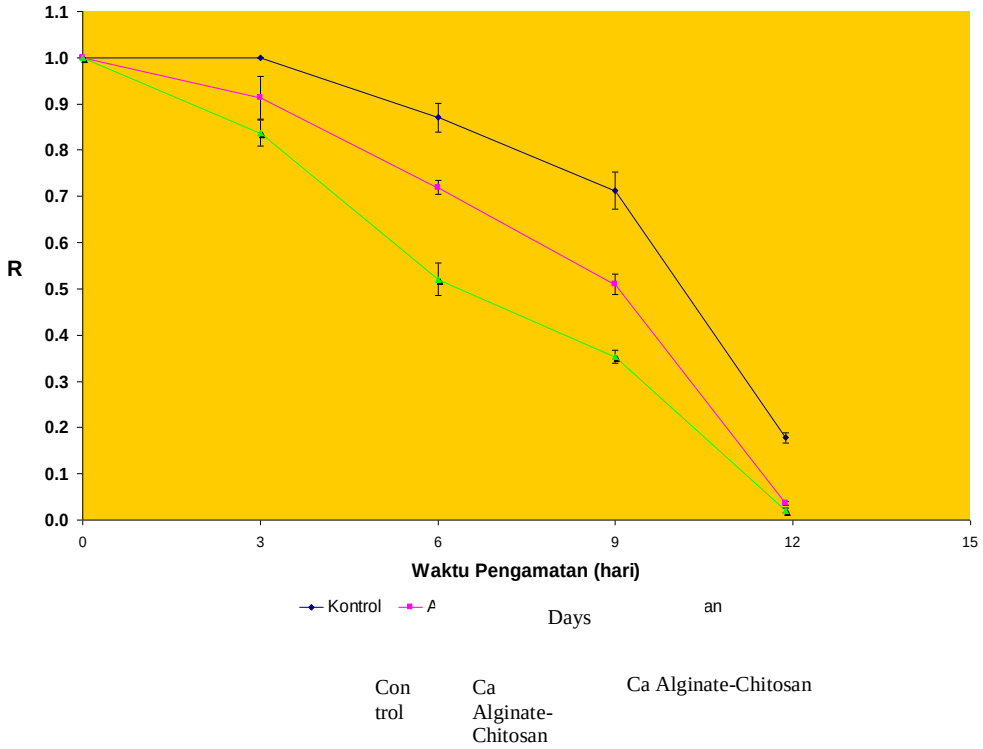

Figure 1, Effect of Alginate-chitosan membranes and Calcium Alginate-chitosan against Wound Area Ratio Marmot (mean ± SEM)

Base on the graph it can be seen the difference in the effectiveness of any comprehensive treatment of wounds in animals. The ratio of the smallest wound area is a calcium alginate-chitosan on the 12th day is 0,021 whereas in alginate-chitosan is 0.035.

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