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Diabetic Wound Healing Biosurfactants Dialkyl Alginate Cream on TNF-(I TGF-ß Expression, Reepithelization, and Collagenization

(Penyembuhan Luka Diabetes Krim Biosurfaktan Dialkil Alginat pada Ekspresi TNF-a TGF-ß, Reepitelisasi, dan Kolagenisasi)

CUT RAIHANAH, NURUL MAHYANI, KINTOKO*

30 Faculty of Pharmacy, Universitas Ahmad Dahlan, Yogyakarta, Indonesia

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1 Abstract: Diabetic wound healing is delayed by many factors, including high TNF-u expression and low TGF-P expression which can affect the formation of new epithelial tissue and collagen as the main goal of the wound healing process. One of the diabetic wound healing agent is biosurfactant dialkyl alginate where so far its use in cream form for diabetic wound has never been reported. This study aimed to determine TNF-u, TGF-P, reepithelization and the collagenization of biosurfactant dialkyl alginate cream in diabetic biopsy wounds in STZ-induced rat. Biosurfactant dialkyl alginate was made in cream form and applied to biopsy wounds on the backs of rat twice a day for 9 days. Observation of TNF-u and TGF-P expression were performed by immunohistochemical staining, while epithelial and collagen with staining HE and Mallory. The results showed that the biosurfactant dialkyl alginate cream had an activity to decrease TNF-u expression, increase TGF-P expression and reepithelization but did not have any significant activity on collagenization. These results suggest that the biosurfactant dialkyl alginate cream can accelerate the healing of diabetic wound.

1 Keywords: Diabetic wound healing, biosurfactant dialkyl alginate cream, TNF-u and TGF-P expression, reepithelization, collagenization.

Abstrak: Penyembuhan Iuka diabetes tertunda oleh banyak faktor, diantaranya ekspresi TNF-u yang tinggi dan rendahnya ekspresi TGF-P yang dapat mempengaruhi pembentukan jarmgan epitel baru dan kolagen sebagai tujuan utama dari proses penyembuhan Iuka. Salah satu agen penyembuhan Iuka diabetes adalah biosurfaktan dialkil alginat dimana sejauh ini penggunaannya dalam bentuk krim untuk Iuka diabetes belum pernah dilaporkan. Penelitian ini bertujuan untuk menentukan TNF-u, TGF-P, reepitelisasi dan kolagenisasi dari krim biosurfaktan dialkil alginat pada Iuka biopsi diabetes pada tikus yang diinduksi STZ. Biosurfaktan dialkil alginat dibuat dalam bentuk krim dan dioleskan dua kali sehari selama 9 hari pada Iuka biopsi di punggung tikus. Pengamatan ekspresi TNF-u dan TGF-P dilakukan dengan pewarnaan imunohistokimia, sedangkan epitel dan kolagen dengan pewarnaan HE dan Mallory. Hasil penelitian menunjukkan bahwa krim biosurfaktan dialkil alginat memiliki aktivitas menurunkan ekspresi TNF-u, meningkatkan ekspresi TGF-P dan reepitelisasi tetapi tidak memiliki aktivitas yang signifikan pada kolagenisasi. Hasil ini menunjukkan bahwa krim biosurfaktan dialkil alginat dapat mempercepat penyembuhan Iuka diabetes.

Kata kunci: Penyembuhan Iuka diabetes, krim biosurfaktan dialkil alginat, ekspresi TNF-u dan TGF-P, reepitelisasi, kolagenisasi.

*Corespondence aouthor hp. 082220709977
Email: kkintok077@gmail.com
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nTRODUCTION

WORLD Health Organization (WHO) defines diabetic foot ulcer as infection, destruction of internal tissue linked with nerve and various disorders of peripheral vascular disease in the lower extremities([1]). Major problem of patients with diabetic foot injuries IS .related to the failure of wound healing([2]). Diabetes Mellitus dominates 60% of the total amputation; while, after 1-3 years, 30% - 50% of patients who have experienced an amputation will have it agam on another 33pt([3]). There are several factors associated with delayed healing of diabetic foot wounds such as the reducing fibroblast migration, Increasmg apoptosis, decreasing keratinocytes, proinflammatory cytokine production and prolonged inflammation. The activation of macrophages is changed at the delay of wo7d healing. Macrophages are stimulated to Increase the production of proinflammatory cytokines such as IL-IF, IL-6, IL-12, IL-18, TNF-u, and IFN-Y by high blood sugar levels both in viv9 and in vitro. Tumor necrosis factor-u (TNF-u) is one of the potent pro-inflammatory cytokines([2]). In the cases of diabetic ulcer, TNF-u expression have been observed triple higher than non diabetic wound rats. The increasmg TNF-u expression IS associated with the inhibition of cell migration, failure fibroblast proliferation, triggered fibroblast apoptosis, and inhibition of angiogenesis resulting in the failure of diabetic wound healin18([2]).

TGF-P is one of the most important factors in the wound healing process for playing a role in signaling for inflammation, angiogenesis, reepithelization, fibroblast migration, granulation tissue formation and homeostatic caution([4$]) But its expression decreases In .diabetic ulcer affecting the damage of wound healing process It is known that TNF-u expression often inhibits TGF-P activity. The increasmg expression of TGF-P and inhibiting TNF-u expression can also be a new alternative therapy In wound healing diabetes([6]) Impaired diabetic wound healing is also caused by chronic inflammation that is characterized with the decreased inflammatory cell at the early stages and the increase of polymorphonuclear neutrophil (PMN) at the final healing stages([2]) In c10rast, acute inflammation plays an Important role in the wound healing process m which neutrophils are released into the wound area to remove bacterial contamination. The faster the bacteria is removed from the wound, the faster the process of reepit1lization and collagenization for tissue repa1r([7]) as the mam goal of the wound healing process([8]). Re27thelization is characterized by the replication and migration of epithelial cells on the edge of the skin([9])• while .collagenization is the process in which collagen IS synthesized by fibroblast cells released into the wound area by fibr1ectin in the inflammatory phase([10]).

One of the diabetic wound healing agents is the biosurfactant dialkyl alginate-a carbohydrate-based biosurfactant (glycolipid) resulted by a reaction between carbohydrate (alginate) and fatty alcohol (stearic acid and isopropyl alco11. Biosurfactants have activity against biofilms in chronic wounds([11]) . The presence of biofilms may extend the inflammatory period([12]) thereby decreasing the proliferation of fibroblast cells that are responsible for the formation of collagen and epithelial cells that affect epithelial thickness In wound healing([13]). The biofilm also targets the inflammatory players such as cytokines. The presence of Staphylococccus aureus biofilms is associated with the increasmg expression of TNF -u([12]) .The study of Sambanthamoorthyet al.reported that th22iosurfactants produced from Lactobacillus had antimicrobial, anti-adhesive, and antibiofilm activity against A. baumanii,E. coli, and S. aureus [14].Banatet al.mentioned several types of biosurfactants , sources, and their effectiveness against the biofilms of several bacteria such as E. coli, Pseudomonas, Staphylococcus, Candida albicans, and others([15]).

Carbohydrate-based biosurfactants that have been studied have an activity against wound healing generally derived from rmcroorgamsm sources as reported by Guptaet al. where the healing test of carbohydrate-based biosurfactant ointment (glycolipid) produced by Bacillus licheniformis bacteria was able to improve the reepithelization and remodeling of connective tissue (collagen)[06]). Other carbohydrate-based biosurfactant compounds such as dirhamnolipid isolated from Pseudomonas aeruginosa bacteria also show wound healing activity in both burns and decubitus ulcers(17,18) Carbohydrate-based biosurfactant wound healing activity from natural sources such as dialkyl alginate 26mpounds has not been reported so far. Alginates used in wound healing are generally still in the form of carbohydrate compounds - not in the form of biosurfactant (glycolipids). The oligosaccharide guluronate present in alginate has been investigated to have antiinflammatory activity([19])• hence, it can be used to speed up the tissue repair of the wound. Alginate compounds are also often used as wound dressmgs because for being capable of spurring tissue granulation and reepitheliza28n([20]). Laurienzo mentioned that alginate dressing can accelerate the wound healing and granulation tissue formation, but this ability is constrained by the hydrophobic properties of alginate. Therefore, the structural

modifications are needed to add hydrophobic properties to alginates, one of which is by adding alkyl groups such as dialkyl alginate([21]).

Carbohydrate-based biosurfactants such as dialkyl alginate have some advantages such as readily available and renewable for its ingredients, having higher biodegradability and lower toxicity compared to petrochemical surfactants([22]). Creams have the advantage to be applied to diabetes wounds especially O/W type creams where the base is able to absorb the fluid released by the wound([23]) This type of cream is also capable of increasing the permeability of the glycolipid group active substances as reported in Rodríguez-Luna([24]). This study aims to examine the healing activity of diabetic wounds from biosurfactantsdialkyl alginate cream In vivo based on the expression of TNF-u and TGF-ß, reepithelization and collagenization parameters.

MATERIAL AND METHOD

MATERIALS. Biosurfactant dialkyl alginate was obtained from Faculty of Chemistry of Universitas Pembangunan Nasional (UPN), Yogyakarta; Animal test: male rats of Wistar strains with body weight of 150-200 g obtained from Solo, Central Java, Indonesia;
Madecassol@ cream (Corsa@), Streptozotocin injection (Nacalaitesque@), sodium citrate, hydrogen chloride , glucose GOD FS reagent (Diasys@), Ketamine hydrochloride inj ection (Generik@), formalin solution 10%, Hematoxylin and eosin stain, Mallory stain (Anilin blue, acid fuchsin, & Orange G); TNF-u Polyclonal Antibody (Bioss@), and TGF-P Polyclonal Antibody (Bioss@).

METHODS. Preparation of biosurfactant dialkyl alginate cream. Biosurfactant dialkyl alginate was formulated in cream with formula from Dipahayu et al. ([26]) modified by a trial and error method. The composition of the cream IS presented in Table l .

Table 1. Composition of the cream.

Ingredients	Amount (^{0}0)
Biosurfactant dialkyl alginate	10
Vaseline	4
Stearic acid	1
Cera alba	2
Paraffin	13
Propylene glycol	15
Propil paraben	0,05
Trietanolamin	1
Metil paraben	0,10
Aquadest ad	100

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Animal Preparation. Male rats of Wistar strains were acclimatized in individual stainless steel cage for one week prior to the induction of diabetes. Rats were fed with AD II and water ad libitum. The cage was illuminated with 12 h light/ 12 h dark cycle in laboratory condition (temperature 22 ± 2^0 C, humidity 60-70%)([27]).All rats have obtained an ethical approval from the Research Ethics Committee of the Faculty of Medicine and Health Sciences of Universitas Muhammadiyah Yogyakarta (KEP UMY) (No: 296/ EP-FKIK-UMY/V/2017).

Induction of Diabetes. The rats were fasted overnight and then their blood was taken for initial blood glucose measurement. The rats subsequently were induced with STZ 45 mg/kg of body weight. STZ was dissolved in cold citrate buffer (0. I mol/l, pH 4.5). A total of I .47 grams of sodium citrate were dissolved in 50 mL of C02-free aquadest and added a few drops of HCI to obtain pH 4.5 using pH meters. The dissolution process was carried out under cold conditions In an ice bath. Blood glucose examination was performed on day 5 after induction of ST Z (baseline). Rat with blood glucose levels above 200 mg/dL was used for the expenment([28'29]).

Measurement of Blood Glucose Level. Blood glucose measurement was performed on the fifth day post-diabetic induction at Integrated Research Development Laboratory (LPPT) Gadjah Mada University (UGM), Yogyakarta. Blood glucose levels of rats were checked by glucose oxidase method using a spectrophotometer. Days 0 and 10 during the topical treatment of blood glucose levels were rechecked. After being fasted overnight, I mL mouse blood was taken from the orbital plexus usmg a capillary Pipe and collected in an eppendorf tube. Blood flowed through the tube wall to avoid hemolysis. After 30 minutes, the blood was centrifuged at 7000 rpm for 1 5 minutes to obtain serum. The serum was separated from the blood by micropipette and determined its sugar content by the addition of GOD-FS reagents.
A total of 10 YL serum and 1000 YL of reagents was mixed and incubated for 15 min at 37 °c. Furthermore, blood glucose levels were read using a UV VIS spectrophotometer that calibrated its blank absorbance at number 0 by measuring the absorbance of the aquadest blank. The sample and standard absorbance was measured against the blank at a wavelength of 505 nm([30]). Body weights were monitored throughout the study and blood glucose levels were re-measured prior to euthanasia to ensure rats were actually in a

diabetic condition[25].

Wounding of Rats. The wound on the rats was made under the anaesthesia of ketamine (10 mg/mL of 0.4 mL i.p). The rat's hair on the right and left sides of the back was shaved and an excl,slon wound made with a 5 mm diameter usmg a punch biopsy. Furthermore, topical treatment was given in rats twice a day. This method refers to Aksoy et al. ([31]) with modifications.

Experimental Procedure. Wounded rats were grouped into 6 groups with 5 rats in each group. They were normal, negative, positive, bio, cream, and base cream group. The positive group was given by Madecassol cream that consists of Centella asiatica 1 [0]/0. Bio group was given by biosurfactant dialkyl alginate; cream group by biosurfactant dialkyl alginate cream, and base cream group by basic of cream.. Topical treatment was applied to every rat twice daily in the mornmg and afternoon for 9 days. This procedure has been referred to Kintoko et al. ([25])with modifications.

Histopathological Study. Rats of each group were sacrificed under Ketamine@ anaesthesia on day 10 post-injury for histopathological examination. The skin tissue was taken with a size of 0.5 cm from the outer edge of the wound. The skin tissue was fixed with 10 % formalin, immersed in paraffin, and sliced using microtomes with a thickness of 5gm with transversal. Furthermore, the sliced tissue was placed on the glass object to be stained. Haematoxylin eosin staining was performed to observe PMN cells, fibroblast cells and epithelial thickness; Mallory staining was performed to observe collagen; and immunohistochemistry to observe TNF-u and TGF-P expression. The coloured tissue was analysed using Olympus BX51 microscope with magnification 100x and 400x then processed using Image J application([25]).

Statistical Analysis. Data were analysed statistically using SPSS 16 with post design analysis. The significance test used the One Way Anova parametric test and non-parametric Mann-Whitney test with 95 % significance level.

RESULT AND DISCUSSION

This study used streptozotocin (STZ) as a diabetic inducer. The administration of STZ in rats resulted permanent (irreversible) pancreatic (3-cell necrosis [32]which could remove its ability to produce insulin. Laboratory experiments on STZ-induced rats of 45 mg/kgBW dose intrapentoneally was proven effective In producing the condition of diabetes([33]). Diabetic condition in which blood glucose levels above 200 mg/dL occurred in all STZ-induced groups on day 0 of observations that were significantly different (p<0,05) compared with normal group. The biosurfactant dialkyl alginate group and cream group showed higher blood glucose levels significantly (p<0,05) compared with negative group on day 0, while on day 10 blood glucose levels decreased, especially the biosurfactant dialkyl alginate group that was significantly different (p<0,05) compared with negative group. These findings due to the differences In metabolism and immunity of the rats as reported in the Kintoko et al. study ([25]). Zulkarnainalso reported a decrease in blood glucose levels after administration of low-dose ST Z due to the spontaneous reversibility of pancreatic beta cells([34]).

STZ also affected weight loss in the induction groups due to increased gluconeogenesis, glycogenolysis, and loss of tissue proteins. These weight loss percentage were higher and significantly different (p<0,05) compared with normal group. Meanwhile, the cream group showed a smaller percentage of weight loss and was significantly different (p<0,05) than negative group.Insulindeficiency after ST Z induction causmg metabolic disorders such as decreased protein levels([35]) and impaired lipid metabolism that caused low triglycerides([25]). This is also in line with the study of Zafar and Naqvi[33]in which STZ-induced animal appeared sick, polydipsy, and weight loss.

Table 2. Blood glucose levels and weight loss of rats during treatment (n = 4).

Group	Blood glucose levels day 0	Blood glucose levels day 10	Weight loss ([0]0)
Normal	84,4±12,31	63,4±8,59	3,34±1,03
Negative	426,2±46,05*	397,35±16,01*	-11,88±1,89*
DA	451,5±9,06*	255,05±117,57*	-6,82±4,10*
CDA	570,8±23,48*#	170,52±118,83#	-9,06±9,81*
BC	509,4±13,05*#	305,02±128,31*	-5,00±4,56*#
	341,6±82,02*	277,55±135,37*	-11,68±5,27*

MC : Madecassol cream that consists of Centella asŽatŽca 1 [0]0.
DA : Biosurfactant dialkyl alginate.
CDA : Cream biosurfactant dialkyl alginate.
BC : Basic of cream

Weight loss after STZ induction was also reported in the Nagarchi et al. (36).

The obs[5]vation number of PMN cells on day 10 showed a significant difference between negative group and normal group in which in negative group it still had PMN cells while in normal group was gone. The topical application of base cream was able to shorten the inflammatory period significantly ($p<0,05$) compared to negative group but not as effective as the cream group and madecassol group that shortened the inflammatory period as a normal group. The topical application of blosurfactant dlalkyl alginate showed no significant activity ($p>0,05$) in shortening the inflammatory period compared to negative group.

Table 3. Number of PMN cells from skin tissue of rats (n = 4).

Group	Number ofPMN cells (5 fields of view)
Normal	0,0±0,0
Negative	3,0±2,16*
MC	0,0±0,0#
DA	0,5±1,0
CDA	0,0±0,0#
BC	0,5±0,58#

The inflammatory period of the bi[17]rfactant dialkyl alginate, cream, and base cream did not show any significant difference ($p>O,()5$) compared to the positive control group (madecassol). In addition, the period of inflammation between topical application of cream and biosurfactant dialkyl alginate showed no significant differenc[4] ($p>O,05$).Diabetes caused an extended period of inflammation in the wound healing process([37]) characterized by presence of polymorphonuclear neutrophil (PMN) cells at the end of inflammatory phase. PMN is very important at the early stages of the inflammatory phase and includes the major ce[32]that are recruited into the wound area. However, at the end of the inflammatory phase, these cells must be apoptosis and cleared fr[29] the wound area by macrophages that indlcatmg the end of the inflammatory phase to begin the next wound healing phase. Failure of apoptotic process and clearance of PMN cells from this wound area led to chronic inflammation that caused wound was difficult to be healed as in diabetic wounds([38]). In this study there was zero (0) on the number of PMN cells. It did not mean the absen[11] of an inflammatory response, but indicated that the inflammatory phase of the wound healing process on the day 10 was over. The abundant number of PMN cel[10]ould be found on day I -3 postwound, after that the role of PMN cells in the wound healing process was replaced by macrophages from day 4-7 post-wound([39]).

Deficiency or insulin resistance in diabetes

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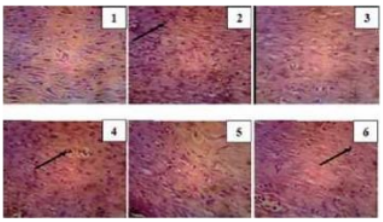

Figure 1. Histopatholokv or PM:N cell with Hematoxylin & Eosin (HE) staining (4001 magnification).

(1) ₌normal, (2) = negative, (3) = MC, (4) -2 DA, (5) — CDA, (6) - BC.

resulted in impaired keratinocyte migration which affected irregular reepithelization processes([A0]). The ilTegularities were histologically seen in the epithelial layer of the diabetic wound which was thinner than the normal wound as observed in the negative group. This result was in line with the research of Lan et al-which reported that diabetic wound has delayed epithelialization compared with normal wound([41]) . Meanwhile, diabetic wound treated with madecassol, biosurfactant dialkyl alginate, and biosurfactant dialkyl alginate cream showed significantly thicker epithelial layer than the negative group. The capability of reepithelization of biosurfactant dialkyl alginate cream was in line with research conducted by

Gupta et al. in which the surfactant tested in rats with

Table 4. Epithelial thickness from skin tissue of rats (n = 4).

Group	Epithelial thickness (um)
Normal	75,15±22,35
Negative	28,1±5,61*
MC	77,7±8,94#
DA	77,2±4,87#
CDA	78,92±9,23#
BC	54,3±3,12#,α,β,γ

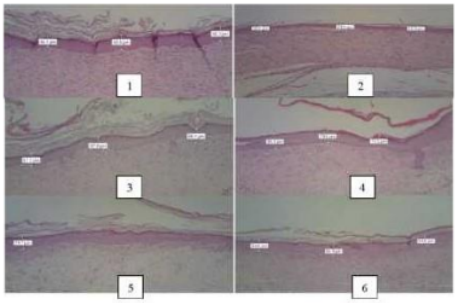

Figure 2. Histopathology of epithelial thickness with Hema-
of epithelial thickness with Hematoxylin & Eosin (HE) staining (4001 magnification).
(1) — normal, (2) — negative, (3) — MC, (4) = DA, (5) — CDA.

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excision wounds showed rapid reepithelization([16]).

Diabetic wound healing disorders were caused by increased apoptosis of fibroblasts and collagen deposition disorder0[2]). This was seen in the negative group in which the number of fibroblasts cells was significantly (p less than the nonnal group, as did the density of collagen.

Table 5. Number of fibroblasts and collagen density from skin tissue of rats (n = 4).

Group	Number Of fibroblasts	[illegible]
Normal	96,5±7,85	49,87±3,68
Negative	62,5±17,67*	41,28±4,71*
MC	64±4,32*	56,96±1,00*,#
DA	50±6,98*,α	48,50±5,35
CDA	50,5±7,94*,α	48,90±3,08α
BC	44,75±6,65*,α	49,71±6,55

Collagen density

49.87+3.68

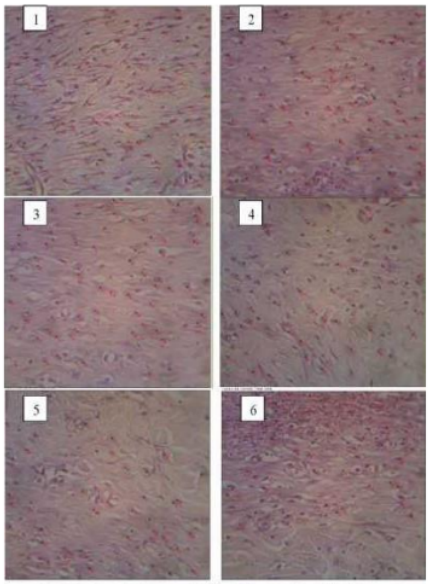

Figure 3. Histopathology of fibroblast cell with Hematoxylin & Eosin (HE) staining (400x magnification).
(1) - nonnal, (2) - negative, (3) - MC, (4) - DA, (5) - CIDA, (6)-BC.

The collagen density of the topically administered group was also seen not to increase collagen density significantly compared with the negative group except for the madecassol group which collagen density was higher and significantly different from the negative group. This result was in line with the study of Wu et al. which reported that Centella

asiatica extract in madecassol was able to Increase collagen synthesis([43]). The decrease of fibroblasts and collagen cells in biosurfactant dialkyl alginate and biosurfactant dialkyl alginate cream according to Tajima et al.vvhich examined the effects of alginate on fibroblast cell proliferation and collagen expression found that alginate was able to suppress the number of fibroblast cells and decrease collagen synthesis by

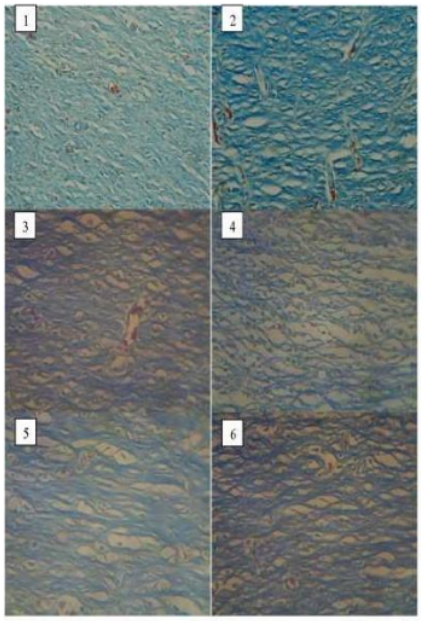

Figure 4. Histopathology of collagen density with Mallory staining (4001 magnification). (1) - normal, (2) = negative, (3) - MC, (4) = DA, (5) -CDA,

half the control group([44]).

Expression of TNF-awas higher significantly ($p<0,05$) in negative group compared than normal group. It similar to result reported by Xu et al([2]). Meanwhile, the topical application of biosurfactant dialkyl alginate cream showed decreasing of 'INF -u expression and increasmg of TGF-13 expression significantly ($p<0,05$).This result was similar to data submitted by DeClue and Shot-nick that high expression of TN[12] inhibited TGF-(Ð activity([6]).

The normal process of wound healing at the beginning of [5]the incidence Was found proinflammatory cytokines IL-I (3, IL-6 and TNF-a produced by macrophages.Polymorphonuclear and macrophages were recruited by these cell molecules to the wound

base. The combination of TNF-(I with IL-I (3 and IL6, stimulated the acute phase response(6).The final phase of wound healing under normal circumstances was found angiogenesis, reepithelization,rebuilding extracellular matrix fibers, and TGF-P that produced by fibroblasts. The presence of TNF-a this phasewasalmost non-existent. TGF-13 was secreted by platelets, keratinocytes, local macrophages, and fibroblasts. The expression of TGF-[3 during normal wound healing reached peak in a few hours and on the fifth day after the wound would increase again(6).

Table 6. Percentage of TNF-u and TGF-þ expression from skin tissue of rats (n = 4).

Group	Expression (/6)	
	TNF-(I	TGF-B
Normal	5,308	50, 19 + 7,43
Negative	20-88 7,07	23,67 ±
MC		26,32 4,88
DA	19,30 ± 3,18#	
CDA	20,63+ 334	30 ±
BC	28,29 + 9,73	19,35+ 3,93

Results were expressed as median±SD.
*p< 0,05when compared nith normal group.
"pc: 0.05 when compared "ith negative group.
"pc: compared with MC group. #p<0,05 when compared with DA group.
'p< 0,05when compared with CDA group.

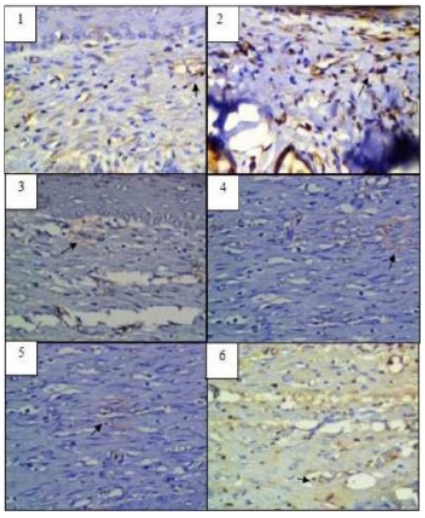

Figure 5. Microphotography of TNF-a expression with immunohistochemical staining (4001 magnification), (1) - normal, (2) - negative, (3) - MC, (4) - DA, (5) - CIDA, (6) -BC.

TGF-[Ð growth factor decreased in diabetic wound whereas this factor would induce the proliferation of cylinocyte and fibroblasts. Topical application Of growth factors claimed to successfully accelerate the process of wound healing diabet[12] The presence of persistent chemokine production in the final phase of diabetic wound healing was closely related to the recruitment of macrophages. This macrophage would continue to produce inflammatory cytokines one of them TNF-a(6). Controlling TNF-(I can improve wound closure and angiogenesis in diabetic wounds(2).

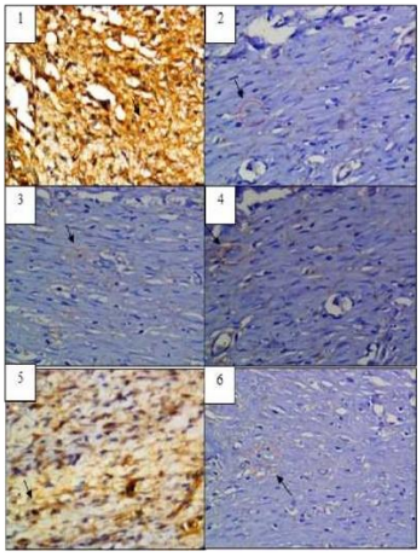

Figure 6. Microphotography of TGF-þ expression with immunohistochemieal staining (400x magnification). (1) = normal, (2) = negative, (3) — MC, (4) — DA, (5) = CDA, (6) - BC.

CONCLUSION

The biosurfactant dialkyl alginate cream has the healing activity of diabetic wounds by influencing expression ofTNF-a and TGF indirectly and able to Increase epithelial thickness in reepithelization process but not increasing the number of fibroblast cells and collagen density on the process of collagenization.

RECOMMENDATION

These results suggest that the biosurfactant dialkyl alginate cream can accelerate the healing of diabetic wound. Research needs to be done with models of diabetic wounded animals infected with bacteria for

further wound healing activities.

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