Makara J. Health Res., 2016, 20(3): 73-78
doi: 10.7454/msk.v20i3.4578

The Effect of Squid Extract (*Loligo Sp.*) on TNF-α and TGF-β1 Serum Levels during Wound Healing in Streptozotocin-induced Diabetic Rats

Kadek NP Dewi[1*], Heri Kristianto[2], Mochamad R Indra[3]

1. Department of Nursing, Universitas Brawijaya, Malang 65145, Indonesia
2. Medical-surgical Nursing Specialist Nursing Program, Faculty of Medicine, Universitas Brawijaya, Malang 65145, Indonesia
3. Department of Physiology, Faculty of Medicine, Universitas Brawijaya, Malang 65145, Indonesia

[*]*E-mail: prayadnidewi@yahoo.com*

Abstract

Background: Diabetes Mellitus is a chronic disease characterised by elevated levels of blood glucose known as hyperglycaemia. Diabetes is due to impaired insulin action in the metabolism of glucose and can result in impaired wound healing. Excessive production of pro-inflammatory cytokines, an increased number of macrophages and neutrophils, and decreased levels of transforming growth factor – beta 1 (TGF-β1) serum can be characteristic of impaired wound healing. This study aims to determine the effects of squid extract on certain wound parameters such as levels of tumour necrosis factor – alpha (TNF-α), and TGF-β1 serum and the number of macrophages and neutrophils. **Methods:** This was a post-test only, randomized controlled group study that was conducted on male Wistar rats. Experimental animals were divided into 6 groups; (1) normal wound with standard diet, (2) diabetic wound with standard diet, (3) diabetic wound with chitosan supplement, (4) diabetic wound given squid extract orally once a day, (5) diabetic wound given squid extract orally twice a day, and (6) diabetic wound given squid extract orally once every two days. Levels of TNF-α and TGF-β1 serum were observed using Enzyme-Linked Immunosorbent Assay. Haematocylin and eosin staining was used to observed macrophage and neutrophil counts. All data was analysed statistically by one-way analysis of variance. **Results:** TNF-α serum levels showed a significant decrease ($p < 0.05$) in subjects that received squid extract orally once every two days. The mean levels of TGF-β1 showed no significant differences. The mean number of macrophage cells showed a significant decrease ($p < 0.05$) in all treatment groups. The mean number of neutrophil cells also showed significant decrease ($p < 0.05$) in all treatment groups. **Conclusions:** Squid extract is effective in lowering the TNF-α serum levels and the number of macrophages and neutrophils cells in Wistar rats. However, there were insignificant findings on increasing levels of TGF-β1 serum. This data suggests that squid extract is most effective during the inflammatory phase of wound healing which takes places about 2-4 days after wound creation.

Keywords: diabetic wound, squid, TNF-alpha, TGF-beta1

Introduction

Diabetes Mellitus is a chronic disease characterised by elevated levels of blood glucose known as hyperglycaemia. It is caused by the decreased ability of the body's receptors to respond to insulin (insulin resistance) or a deficiency of insulin and can lead to severe complications.[1] Indonesia has 8.5 million cases of diabetes and it affects 5.55% of the total adult population. Diabetes has a mortality rate of 2.01% and it is estimated that by 2030 there will be as many as 21.3 million people suffering from diabetes.[2,3]

Hyperglycaemia in diabetic patients may increase the formation of advanced glycation end products, which stimulates excessive production of reactive oxygen species. This reaction activates nuclear factor-kB (NF-kB) resulting in increased degradation of the extracellular matrix and secretion of cytokines thus decelerating the wound healing process.[4,5] Additionally, Chen *et al.* reported a decrease of choline in diabetic patients that may cause a reduction in acetylcholine levels. This can lead to an increase of systemic tumour necrosis factor-α (TNF-α) causing a systemic inflammatory system and a deceleration in wound healing.[6,7]

Diabetes displays a higher economic burden to the patient.[8] This substance is a polysaccharide obtained from the deacetylation process of Chitin, which is commonly found in crustaceans and insects. It can have anti-inflammatory properties and is capable of promoting

collagen fibres and accelerating the inflammation phase of wound healing.[9] The Myra Levine Nursing Model on structural integrity conservation confirms that interference in structural integrity, as a result of pathological processes, can affect the function of the body and as such nursing interventions that repair and heal the malfunction is needed.[10] The application of the Levine Model in the management of structural wound integrity includes providing good nursing interventions using wound dressings or alternative therapies such as nutritional therapy.[11]

Squid (*Loligo sp*.) extract can be used as an alternative supplement in the treatment of diabetic wounds. Lecithin, a substance found in squid, plays an important role in wound healing and is a potential source of choline. Choline may increase acetylcholine synthesis and secretion and can improve insulin regulation, which is very useful in diabetic patients.[12-14] According to the research by Hirsch *et al*., consumption of lecithin may increase levels of serum choline and in fact, be more effective than consumption of choline.[15] Additionally squid contains ascorbic acid, which is an effective antioxidant and scavenger for free radicals.[16] Squid is often a staple food of people who reside near the coast however, many are unaware of its potential health benefits. It is important that tangible scientific research is conducted to discover the possible health benefits of squid.[17] Conversely; squid also has the antigenicity properties that are capable of inducing allergic reactions.[18]

This study aims to determine the effect of squid extract against serum levels of TNF-α and transforming growth factor – β1 (TGF-β1) and the number of macrophages and neutrophils in diabetic wounds.

Methods

Research design. This study was conducted in an experimental laboratory with a post-test only, randomized control group design. Experimental animals were divided into 6 groups; 1) The negative control group (N), normal rats without diabetes and wounds treated with NaCl 0.9%; 2) The positive control group (Po), diabetic rats with wounds treated using 0.9% NaCl; 3) The standard treatment group (Ps), diabetic rats given chitosan orally and wounds treated using 0.9% NaCl; 4) Treatment group 1 (P1), diabetic rats given up to 450 mg of squid extract orally once a day, 200 g of Body Weight (BW), and wounds treated using 0.9% NaCl; 5) Treatment group 2 (P2), diabetic rats given up to 450 mg of squid extract orally twice daily, 200 g of BW, and wounds treated using 0.9% NaCl; 6) Treatment group 3 (P3); diabetic rats given up to 450 mg of squid extract orally once every two days, 200 g of BW, and wounds treated using 0.9% NaCl.

N.B. Squid extract doses were tailored to ensure any required daily restriction of cholesterol for diabetic patients was adhered to.

Experimental animals. Experimental animals used were male albino rats (*Rattus norvegicus*) exhibiting the Wistar strain. Each group consisted of 5 experimental animals, 30 subjects in total, aged 2.5 to 3 months and they weighed between 200 and 300 g. The Ethics Committee of Health Research and the Medical Faculty of the University of Brawijaya approved all treatment methods on the experimental animals.

Squid extraction (*Loligo sp.*). Almost 10 kg of fresh squid was dried in an oven at 45 °C for approximately 2 days and resulted in around 1 kg of dried squid powder. The powder was placed in an Erlenmeyer glass and was immersed in 96% ethanol for 24 hours. The ethanol solution was then boiled at a temperature between 70 and 80 °C to evaporate the residual solvent, the concentrated extract was then captured in a reservoir after it was allowed to collect for 1.5 to 2 hours. As much as 140 g of squid extract was collected and was diluted in distilled water to attain the required concentrations. The composition of the final squid extract was not independently determined, however it was assumed based on previous literature. Lastly the squid extract dosage was adjusted to the recommended cholesterol levels for diabetic patients of <200 mg per day.[19] 100 g of fresh squid can contain up to 233 mg of cholesterol, hence we used 25 g of squid that contained around 58 mg of cholesterol. This amount was then adjusted for the subject's weight and 450 mg of squid extract and 200 g of BW orally was administered.

Induction of diabetes mellitus. Diabetes Mellitus was induced in the subjects using an intraperitoneal, single dose injection of Streptozotocin (STZ). The subjects were fasted for 12 hours and the STZ was administered at a rate of 45 mg/kg BW in a solvent of 0.1 M citrate and had a pH of 4.5. Three days following the STZ injection, the rats' glucose levels were measured using a NESCO branded digital glucometer, the rats were considered diabetic if their blood glucose levels were above 200 mg/dL.[20]

Making the diabetic wound. Rats were anaesthetised with 50 mg/kg of Zoletil intramuscularly prior to incisions. Hair on the back area of the subjects was shaved and disinfected with a 70% alcohol solution. Full thickness incisions of 1.5 x 1.5 cm were then made.

Treating the diabetic wound. Wound treatments were performed daily for 14 days using sterile techniques and the wounds were covered with gauze to prevent infection. Oral treatments were performed using a sonde.

Measuring the levels of TNF-α and TGF-β1 serum. Following the experiment, animals were euthanised via administration of excessive chloroform. A blood sample was completed through the heart following surgery; these samples were then centrifuged to obtain blood serum. Blood serum was tested using Enzyme-Linked Immunosorbent Assay kits, manufactured by Elabscience, to obtain levels of TNF-α and TGF-β1. Absorbance values were read at a wavelength of 450 nm.

Quantification of the macrophages and neutrophils. Dermal tissue samples were taken post surgery and were immersed in a solution of 10% formalin fixative. The prepared tissue samples were examined using hematoxylin and eosin (H&E) staining. One histological slide per sample was prepared to calculate the numbers of macrophages and neutrophils, the slides were viewed under 400x magnification and the process was assisted by OlyVIA software. All quantification protocols were performed as blind trials.

Data analysis. All data collected was analysed using one-way analysis of variance (ANOVA) with a 95% confidence level ($\alpha = 0.05$) and was processed using SPSS 20 for Windows.

Results

Effect of the squid extract on the mean levels of tumor necrosis factor-α (TNF-α) serum. Mean levels of TNF-α serum are outlined in Figure 1, with the lowest levels found in the N group, equal to 85,900 pg/mL, and the highest levels found in the P1 group, equal to 339,400 pg/mL. One-way ANOVA testing showed a significant difference between the experimental groups however, post hoc tests showed that group Po and P3 were on a different subset of columns. These results therefore concluded that administration of squid extract once every two days was the most effective in lowering levels of TNF-α serum.

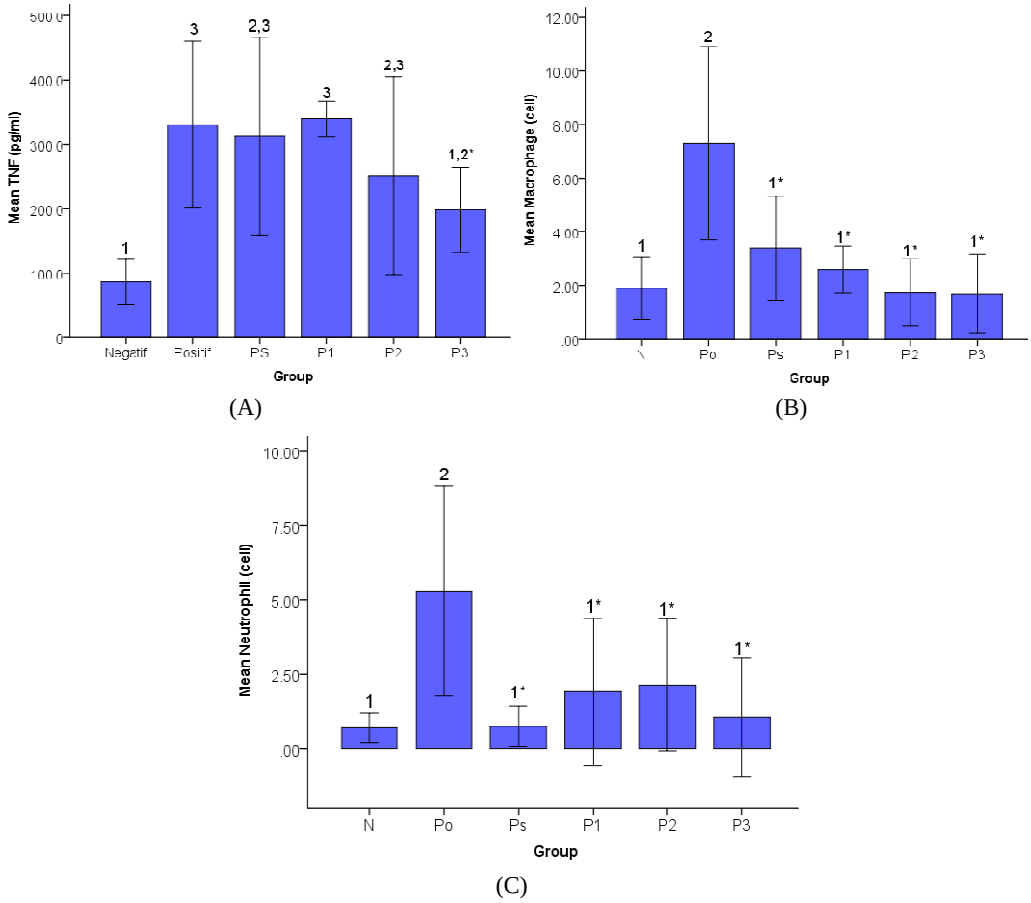

Figure 1. Effect of squid extract on (A) the levels of tnf-α serum, (B) the number of macrophages, and (C) the number of neutrophils. The animals were organised into the following groups: (N) negative control group without diabetes induction, (Po) positive control group with diabetes induction, (Ps) standard treatment group received chitosan supplement orally, (P1) treatment group i received 450 mg of squid extract orally once per day, (P2) treatment group ii received 450 mg of squid extract orally twice per day, and (P3) treatment group iii received 450 mg of squid extract once every two days. (1) Subset column 1, (2) subset column 2, and (3) subset column in post hoc tests. The results represent the mean ± SD of 5 experimental animals per group. *$p < 0.05$ compared to the positive control group

Effect of The Squid Extract On The Mean Levels of Transforming Growth Factor-β1 (TGF-β1) Serum. Outlined in Table 1, the lowest mean levels of TGF-β1 serum, equal to 265,700 pg/mL, were found in the P1 group, whilst the highest mean levels, equal to 358.20 pg/mL, were found in the N group. Kruskal-Wallis tests were then used, as the aforementioned data was not normally distributed. As such, the results confirmed that there were no significant differences between any of the experimental groups.

Effect of the squid extract on the mean of macrophage cell. Figure 1 shows the average number of macrophages found in each sample group. The N group had the lowest mean number of macrophages, equal to 1.9 cells, and the highest levels of macrophages were found in the Po group, equal to 7.3 cells. One-way ANOVA tests revealed that there were significant differences between the experimental groups. Post hoc tests showed that all treatment groups were on different subset columns to the Po group. These results suggest that squid extract and chitosan are effective in reducing the number of macrophage cells. Histological features of the skin wounds displaying macrophage cells can be seen in Figure 2.

Effect of the squid extract on the mean of neutrophils cell. The mean number of a neutrophil cells are highlighted in Figure 1. The lowest mean number of neutrophils was found in the N group, equal to 0.7 cells. The Po group had the highest mean number of neutrophils, equal to 5.3 cells. One-way ANOVA testing showed significant differences between all experimental groups. Similar to results found for macrophages, post hoc tests revealed that all treatment groups were on different subset columns to the Po group. Once again, results suggest that squid extract and chitosan are effective in reducing the number of neutrophils. Histological features of the skin wounds displaying neutrophil cells can be seen in Figure 3.

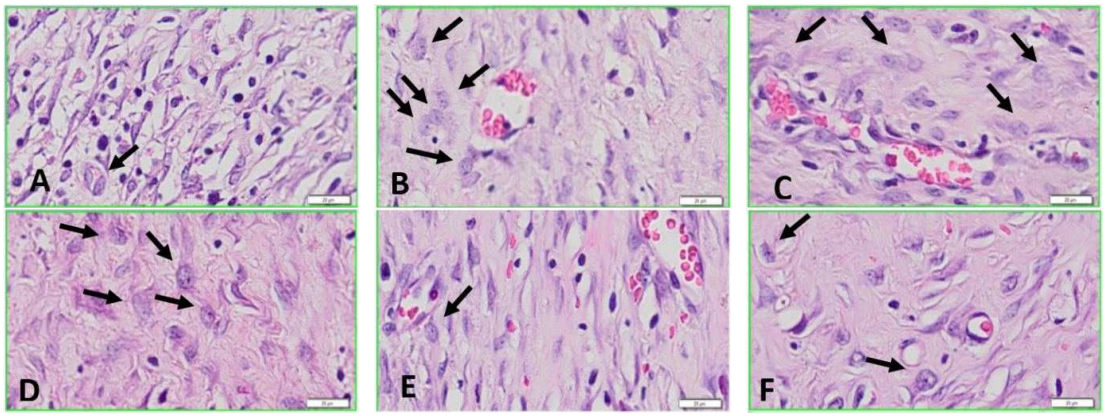

Figure 2. Histological features of animal subjects dermal wounds showing macrophage cells. the negative control group (A) had less macrophage cells (arrowhead) when compared with the positive control group (B). The Standard treatment group (C) had fewer macrophage cells compared to the positive control group. Likewise, there are fewer macrophage cells displayed in treatment groups I (D), II (E), and III (F) when compared with the positive control group (H&E staining, 400x magnification)

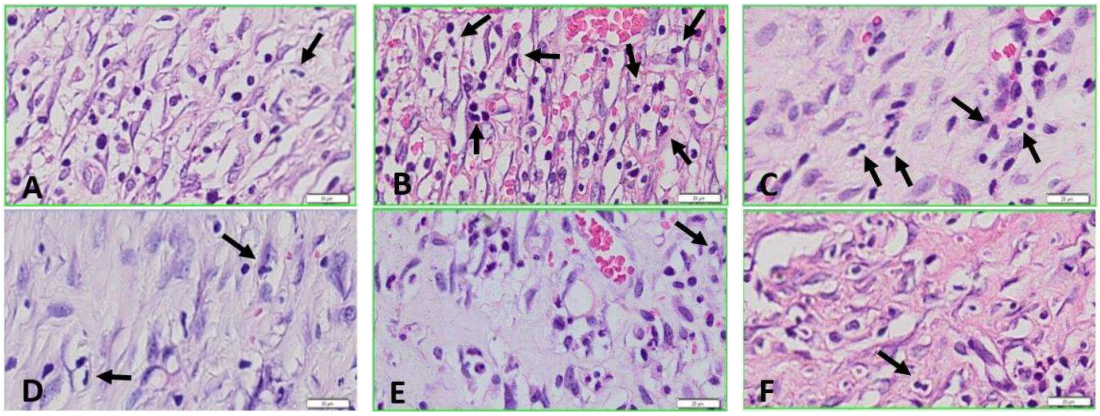

Figure 3. Histological features of animal subjects dermal wounds showing neutrophil cells. There appears to be more neutrophil cells in the positive control group (B) when compared to the negative control group (A). The standard treatment group (C) had fewer neutrophil cells when compared to the positive control group. Similarly, treatment groups I (D), II (E), and III (F) displayed fewer neutrophil cells than the positive control group (H&E staining, 400x magnification)

Discussion

Results suggest that squid extract administered once every two days (225 mg/day) is the most effective in decreasing levels of TNF-α serum. The decreased levels of TNF-α serum are likely due to the content of lecithin in squid, which can increase levels of choline in the body.[13] Studies have found that choline can increase the regulation of insulin and stimulate acetylcholine.[14,21] Acetylcholine plays an important role in the activation of α7 subunit-containing nicotinic acetylcholine receptors (α7nAChR).

α7nAChR activation induces microRNA-124 which can inhibit TNF-alpha-converting enzyme (TACE) mRNA. Inhibition of TACE mRNA leads to an inhibition of the production of TNF-α.[22] Decreased levels of TNF-α serum indicates that the inflammatory phase can be suppressed thus accelerating diabetic wound healing.[7,8] Additionally, squid contains omega-3 polyunsaturated fatty acids (PUFAs) which has the ability to reduce the levels of TNF-α serum.[23] Ascorbic acid in the squid acts as an effective antioxidant for scavengers of free radicals and this can inhibit the secretion of excessive pro-inflammatory cytokines.[16]

Results suggest that squid extract given once per day (450 mg/day) and twice per day (900 mg/day) was unable to lower the levels of TNF-α serum. However, the frequency of the feeding needle (probe) may interfere with results. Additionally, breakages of the probe during feeding may cause trauma to the digestive tract, this can stress the rats and result in an elevation of TNF-α serum levels.[24,25] The synthetic chitosan used in this trial was obtained from a prepared commercial product. No calculations about molecular weight or other influential factors such as time of dosage were conducted, which may have resulted in lower TNF-α serum levels.[26,27]

Squid extract alone cannot increase levels of TGF-β1 serum. Research has found that PUFAs can increase the levels of TGF-β1 serum by inhibiting α2 microglobulin, which plays a role in plasma clearance and catabolism of TGF-β1.[28] Conversely, other studies report that PUFAs did not have a significant effect on TGF-β1 serum upregulation.[29] Further studies hypothesise that diabetes can increase the levels of TGF-β1 serum.[30,31] These differing theories may attribute to less accurate results of TGF-β1 serum levels in this study.

Squid extract can be effective in reducing the number of macrophages and neutrophils found in wounds on diabetic rats. This study found that squid extract was as effective as chitosan, however further research is needed to examine the use of chitosan in a more pure form and to measure its molecular weight. Docosahexaenoic acid, a portion of PUFAs, can also reduce the number of macrophage cells and inflammation by inhibiting the activity of NF-kB. Additionally, PUFAs can inhibit secretion of M1 macrophages, which play a role in stimulating pro-inflammatory cytokines, and stimulate secretion of M2 macrophages, which stimulate anti-inflammatory cytokines.[32,33]

Omega-3 PUFAs can decrease the number of neutrophils through the inhibition of eicosanoids. Eicosanoids are signalling molecules of pro-inflammatory cytokines and are composed of 20 oxidised carbon fatty acids. Eicosanoids consist of LTB4 and PGE2 and act as a chemo-attractant and a strong activator of neutrophils kuat.[34,35]

Conclusions

Squid extract successfully accelerated wound healing during the inflammatory phase by lowering the levels of TNF-α serum, the number of macrophages, and the number of the neutrophils in diabetic rats. However, squid extract administered orally had little effect in increasing levels of serum TGF-β1 or stimulating the proliferation phase of wound healing. As all treatment modalities showed similar effectiveness it is recommended that a regime of squid extract once every second day be considered, as it is the most cost effective and safe method.

Conflict of Interest Statement

The authors declare no conflicts of interest.

Acknowledgement

This work was funded by PT Indofood Sukses Makmur Tbk, via the program sponsorship Indofood Riset Nugraha 2014/2015. The author would like to thank to the research supervisors Prof. Moch Rasjad Indra, M.S and Ns. Heri Kristanto, M.Kep. KMB. The author also wishes to thank Mr. Ardianta GP, Mr. Kusuma, Mrs. Ratna, Mr. Gestu, Mr. Dika, and Mr. Yoga for technical assistance.

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