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 Dewi1*, Heri Kristianto2, Mochamad R Indra3
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. 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. 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. 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. 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. Additionally squid contains ascorbic acid, which is an effective antioxidant and scavenger for free radicals. 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. Conversely; squid also has the antigenicity properties that are capable of inducing allergic reactions.

This study aims to determine the effect of squid extract against serum levels of TNF-\(\alpha\) and transforming growth factor – \(\beta\) (TGF-\(\beta\)) 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.

**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.

**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 Elabs, 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 (α = 0.05) and was processed using SPSS version 20.0 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. Studies have found that choline can increase the regulation of insulin and stimulate acetylcholine. 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. Decreased levels of TNF-α serum indicates that the inflammatory phase can be suppressed thus accelerating diabetic wound healing. Additionally, squid contains omega-3 polyunsaturated fatty acids (PUFAs) which has the ability to reduce the levels of TNF-α serum. 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.

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. 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.

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. Conversely, other studies report that PUFAs did not have a significant effect on TGF-β1 serum upregulation. Further studies hypothesise that diabetes can increase the levels of TGF-β1 serum. 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.

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.

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 Raisyad Indra, M.S and Ns. Heri Kristanto, M.Kept. KMB. The author also wishes to thank Mr. Ardianta GP, Mr. Kusuma, Mrs. Ratna, Mr. Gestu, Mr. Dika, and Mr. Yoga for technical assistance.

References

1. Cheng AYY. The Canadian diabetes association 2013 clinical practice guidelines for the prevention and management of diabetes in Canada. Can J Diabetes. 2013;37:S1-3.
2. International Diabetes Federation. Diabetes in Indonesia-2013. (internet) [cited: 2014 April 10]. Available from: http://www.idf.org/membership.wp.indonesia.
3. Wild S, Roglic G, Green A, Sicree R, King H. Global prevalence of diabetes estimates for the year 2000 and projections for 2030. Am Diabetes Assoc. 2014;27:1047-53.
4. Graves DT, Liu R, Oates TW. Diabetes-enhanced inflammation and apoptosis-impact on periodontal pathosis. Periodontol 2000. 2007;45:128-37.

Makara J. Health Res.

December 2016 | Vol. 20 | No. 3
5. Farmer KL, Li C, Dobrowsky RT. Diabetic peripheral neuropathy: Should a chaperone accompany our therapeutic approach? Pharmacol Rev. 2012;64:880-904.

6. Chen L, Chen YM, Wang LJ, Wei J, Tan YZ, Zhou JY, et al. Higher homocysteine and lower betaine increase the risk of microangiopathy in patients with diabetes mellitus carrying the GG genotype of PEMT G774C. Diabetes Metab Res Rev. 2013;29:607-17.

7. Tellechea A, Leal E, Veves A, Carvalho E. Inflammatory Makara J. Health Res.

8. Lobmann R, Ambrosch A, Schultz G, Waldmann K, Schiweck S, Lehnert H. Expression of matrix-metalloproteinases and their inhibitors in the wound of diabetic and non-diabetic patients. Diabetologia. 2002;45:1011-6.

9. Inan ZDS, Saraydin SU. Investigation of the wound healing effects of chitosan on FGFR3 and VEGF immunolocalization in experimentally diabetic rats. Int J Biomed Mater Res. 2013;1:1-8.

10. Levine ME. The conservation principles: a model for health. In: Schafer KM, Pond JB (eds). Levine’s Conservation Model: A Framework for Nursing Practice. Philadelphia: F.A. Davis Company; 1992.

11. Leach MJ. Wound management: using Levine’s conservation model to guide practice. Ostomy Wound Manage. 2006;52:333-7.

12. Yun JH, Lee HY, Asaduzzaman AKM, Chun BS. Micronization and characterization of squid lecithin/polyethylene glycol composite using particles from gas saturated solutions (PGSS) process. J Ind Eng Chem. 2013;19:686-91.

13. Koppen A, Klein J, Erb C, Loffelholz K. Acetylcholine release and choline availability in rat hippocampus: effects of exogenous choline and nicotinamide. J Pharmacol Exp Ther. 1997;282:1139-45.

14. Icol YO, Gurun MS, Taga Y, Ulus IH. Choline increases serum insulin in rat when injected intraperitoneally and augments basal and stimulated acetylcholine release from the rat minced pancreas in vitro. Eur J Biochem. 2003;270:991-9.

15. Hirsch MJ, Growdon JH, Wurtman RJ. Relations between dietary choline or lecithin intake, serum choline levels, and various metabolic indices. Metabolism. 1978:27:953-60.

16. Ilamparithi C, Shering A, Raj Brito, Kavimani S. A study about antioxidant and anticancer activity of activity of methanolic extract of squid, sepia brevimana and sepia inermis. Int J Pharma World Res. 2011:2:1-10.

17. Kurniawan, Lestari S, Rachmawati SH. Hidrolisis protein undenatured whey protein. Int J Diabetes Metabolism. 2013;1799-1804.

18. Rahman AMA, Helleur RJ, Jeebhay MF, Lopata AL. Characterization of seafood protein causing allergic diseases. In: Pereira C (eds). Allergic diseases – highlights in the clinic, mechanisms, and treatment. InTech. 2012:107-40.

19. American Diabetes Association. Nutrition recommendations and interventions for diabetes: A position statement of the American diabetes association. Diabetes Care. 2007;30:S48-65.

20. Zangiabadi N, Sheibani V, Asadi-Shekaari M, Shabani M, Jafari M, Asadi AR, et al. Effects of melatonin in prevention of neuropathy in STZ-Induced diabetic rats. Am J Pharmacol Toxicol. 2011;6:59-67.

21. Kumar N, Jadhao SB, Chandan NK, Rana RS. Dietary choline, betaine and lecithin mitigates endosulfan-induced stress in Labeco rohita fingerlings. Fish Physiol Biochem. 2011;38:989-1000.

22. Ulloa L. The cholinergic anti-inflammatory pathway meets microRNA. Cell Res. 2013;23:1249-50.

23. Li Q, Brendemuhl JH, Jeong KC, Badinga L. Effects of dietary omega-3 polyunsaturated fatty acids on growth and immune response of weanling pigs. J Anim Sci Technol. 2014;56:1-7.

24. Niu Z, Zhou J, Ji W, Li H, Bao D, Yang H. The association of psychological stress related cytokines (TNF Alpha, IFN-Gamma) with essential hypertension in Ningxia Hui autonomous region. Open J Endocr Metab Dis. 2013;3:276-282.

25. Ebaid H, Ahmed OM, Mahmoud AM, Ahmed RR. Limiting prolonged inflammation during proliferation and remodeling phases of wound healing in Streptozotocin-Induced diabetic rats supplemented with camel undenatured whey protein. BMC Immunol. 2013:14:1-13.

26. Araniz I, Mengibar M, Harris R, Panos I, Miralles B, Acosta N, et al. Functional characterization of chitin and chitosan. Curr Chem Biol. 2009;3:203-30.

27. Pendima DM, Menda JP, Reddy T, Deepika R, Sastry TP. Preparation and characterization of wound healing composites of chitosan, aloe vera and calendula officinalis – a comparative study. Am J Phytomedicine Clin Ther. 2014;2:61-76.

28. Ling TY, Huang YH, Lai MC, Huang SS, Huang JS. Fatty acids modulate transforming growth Factor-β activity and plasma clearance. FASEB J. 2003;17:1559-61.

29. Sustrova T, Rozikova V, Kompdra T, Sladek Z. The effect of rats diet to production pro-inflammatory and anti-inflammatory cytokines. Mendel Net. 2014:435-8.

30. Huseynova GR, Azizova GI, Efendiyev AM. Quantitative changes in serum IL-8, TNF-α and TGF-β1 levels depending on compensation stage in Type 2 Diabetic patients. Int J Diabetes Metabolism. 2009;17:59-6.

31. Ibrahim S, Rashed L. Estimation of transforming growth Factor-Beta 1 as a marker of renal injury in Type II Diabetes Mellitus. Saudi Med J. 2007;28:519-23.

32. Schumann J. Modulation of macrophage response mechanisms against persistent pathogens by PUFAs. Formatex. 2013;1799-1804.

33. Williams-Bey Y, Boularan C, Vural A, Huang N-N, Hwang I-Y, Shan-Shi C, et al. Omega-3 free fatty acids suppress macrophage inflammasome activation by inhibiting NF-kB activation and enhancing autophagy. PLOS ONE. 2014;9:1-8.

34. Boussetta T, Raad H, Letteron P, Gougerot-Pocidalo MA, Marie JC, Driss F, et al. Punicic acid a conjugated linolenic acid inhibits tnfa-induced neutrophil hyperactivation and protects from experimental colon inflammation in rats. PLOS ONE. 2009;4:1-12.

35. Calder PC. n-3 polyunsaturated fatty acids, inflammation, and inflammatory diseases. Am J Clin Nutr. 2006;83 suppl: 150S-19S.