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Acute Toxicity Test of Toman Fish Extract on Erythrocyte Number and Hemoglobin Level in Normal and Diabetic Wistar Rat Model

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Abstract:
Patients with diabetes mellitus (DM) may experience vascular impairment, wounds that emerged from dental practice require special attention in people who suffer from DM. Toman fish has the potential as an alternative medicine to accelerate the healing process in normal and diabetic wound. Erythrocyte and hemoglobin are two components of blood profile which can be used as the parameter to identify the effect of material toxicity. This study aims to test the acute toxicity of Toman fish extract at 16 mL/Kg BW dosage on the number of erythrocyte and the level of hemoglobin in normal and diabetic Wistar rat model after 14 days treatment. This is a true experimental study with post-test only and control group design. Samples were comprised of 21 male Wistar rats allocated to three different groups; normal rats given Toman fish extract (A), diabetic rats given Toman fish extract (B), and diabetic rats given BR2 feed only (C). The results showed that in group A, the number of erythrocytes was 8.67x106/mm3 and the level of hemoglobin was 17.30 g/dL. In group B, the number of erythrocytes was 7.39x106/mm3 and the level of hemoglobin was 16.32 g/dL. In group C, the erythrocyte number was 4.88x106/ mm3 and the hemoglobin level was 11.54 g/dL. It can be concluded that Toman fish extract at the dosage of 16 mL/Kg BW after 14 days administration is not toxic which was indicated by erythrocyte number and hemoglobin level in the blood of normal and diabetic Wistar rats models.

Keywords: Acute Toxicity Test; Erythrocytes; Diabetes melitus; Hemoglobin; Toman fish.

Introduction

Diabetes mellitus is a disease characterized by hyperglicemia and glucose intolerance as the result of deficiency in insulin level, activity or both level and its activity.[1] Based on International Diabetes Federation (IDF) 2019, Indonesia ranked second in Asia with the highest DM incidents confining 10.7 million people with an age range of 20-79 years old.[2] People suffering from DM may experience hypoxia due to vascular impairment that will interfere with wound healing process. Wounds emerged in dental practice for people with DM require special attention since the healing process takes longer due to prolonged inflammation and increased risk of infection.[3,4]

In recent days, communities are engrossed by the utilization of herbal medicine on top of synthetic drugs. It is believed that herbal medicine possesses minimal side effects in wound healing whether in normal or diabetic condition, that includes the use of Toman fish extract. A study by Nicodemus (2014) confirmed that per oral administration of Toman fish extract (Channa micropeltes) at the dosage of 16 mL/kg BW might accelerate incisional wound healing on the seventh day with 97.21% rate.[5] A study by Apriasari et al (2020) also revealed that Toman fish extact (Channa micropeltes) administered per orally at 16 mL/Kg BW dosage may cure diabetic wound on day 14.[6]

Natural ingredients used for herbal medicine should be investigated for its toxicity prior to the development into medicinal product. Toxicity investigation is a preliminary requirement from BPOM to evaluate the safety of natural ingredient.[7] One of the tests performed in experimental animal is the acute toxicity test that is regulated for 14 days.[8,9] Toxicity test is managed by investigating the blood profile where erythrocyte and hemoglobin may be used as the parameter to identify the effect of material toxicity.[10] An ingredient is considered to be toxic when there is a decrease in the number of erythrocyte and the level of hemoglobin. Any toxin contained within natural ingredients may promote the lysis of lipid membrane in erythrocyte thus causing its damage and depletion in circulatory system. Destruction of erythrocytes due to the toxicity of a substance can cause a decrease in hemoglobin levels.[11] Based on those respective details, the acute toxitity test of Toman fish extract at the dosage of 16 mL/Kg BW on erythrocyte number and hemoglobin level in normal and diabetic Wistar Rat model for 14 days treatment should be conducted. The allocation of normal and diabetic Wistar rat model aims to compare the influence of Toman fish extract administration that will not impair the normal condition and not to aggravate the disease.

Research Method

This study was approved by Health Research Ethics Commissions Faculty of Dentistry Universitas Lambung Mangkurat with letter No.079/KEPG-FKGULM/EC/I/2020. A true experimental model was schemed for this study with post test only and control group design that was conducted at Biochemisty and Biomolecular Laboratory Faculty of Dentistry Universitas Lambung Mangkurat Banjarmasin. Inclusion criteria was comprised of male wistar rat aged 6-8 weeks with 200-300 mg BW and weight loss less than 10%. Rats were fed with BR2 standard feed twice daily and clean water.

Wistar rats were acclimatized in laboratoric environment for 7 days prior to the study. Rats were allocated into three distinct groups and replicated into 7 respectively based on Higgins and Kleinbaum 1985.

Formulation and Storage of Toman Fish Extract

Eighteen kilograms of Toman fish was prepared to which it was cleaned and obtained a total of 16 kg fish flesh. The flesh was then steamed at 70-80oC for 30 minutes and liquid extract was collected by squeezing while filtrating the flesh using a filter cloth. The extract was centrifuged for 15 minutes at 6000 rpm and resulted in the form of liquid

and sedimentation. The liquid was then separated from the sedimentation to be later put into a tightly closed dark glass bottle. The extract was further stored in a refrigerator at ≤ 4oC to avoid any damage due to oxidation and contamination.

Induction of Diabetes Mellitus in Wistar Rat (STZ Injection)

Diabetic rat model was obtained by injecting STZ in wistar rat at 40 mg/Kg BW dosage. The rat was lifted by placing one hand around the upper chest without being squeezed, and the thumb was positioned on its chin without pressing the neck. Needle was injected intraperitoneally at 60o on the surface of abdomen. Glucose level was examined before and three days post STZ injection using glucometer. Rat was diagnosed with diabetes when the glucose level was above 126 mg/dL along with frail condition and decreased activity.

Treatment of Experimental Animal

Sample in this study was comprised of 21 male Wistar rats. Rats were divided into three different groups, including normal rat administered with Toman fish extract (A), diabetic rat administered with Toman fish extract (B), and diabetic rat supplied with BR2 feed only (C). Toman fish extract at the dosage of 16 mL/Kg BW was administered per orally using nasogastric tube twice a day at 8 am and 4 pm for 14 days.

Euthanizing Wistar Rat using Ketamin-Xylazine

On day 15, rats in each group were euthanized and anesthetized using a mixture of ketamine and xylazine at 1:1 ratio. The mixture was injected into Wistar rat at 0.1 ml dosage.

Handling of Wistar Rat Carcasses

Euthanized rats were buried. Rat carcasses were initially cleaned and wrapped with fabrics to be buried in the depth of ±25-50cm.

Calculation of Erythrocyte and Hemoglobin

Sample was collected from the cardium to obtain a total of 3 mL blood. Further, blood sample was trasnferred into 3 mL EDTA K3 tube.

Erythrocyte number was calculated using improved neubauer counting chamber in which 500 µL of Hayem liquid was transferred using micropipette into the test tube. As much as 5 µL blood in EDTA tube was drawn using micropipette and added into the Hayem suspension. The mixture of blood and Hayem suspension was then cautiously put into the counting chamber and concealed with a cover glass. Five gridded areas arranged in 16 small field to be observed under microscope at 40 times magnification. Erythrocyte count per mm3 blood was resulted from the calculation of erythrocyte in each visible square and multiplied with 10.000.

Hemoglobin level was determined using cyanmethemoglobulin method in drabkins suspension. Drabkins suspension was collected using micropipette in a total of 5 uL and inserted into a test tube. As much as 20 mL blood from EDTA tube was drawn with micropipette to be transferred into the test tube of Drabkins suspension. The mixture of blood and Drabkins suspension was observed using spectrophotometer under 540 nm wavelength.

Data Analysis and Statistical Evaluation

Data obtained from all groups were processed using SPSS 26.0 for Windows. The scores for all groups were presented in the mean rank. Data was then analyzed using One-Way Anova parametric test with 95% confidence level (p=0.05) and processed with Post Hoc Bonferroni test.

Results

The normal count for red blood cell in Wistar rats is ranging from 7.2 to 9.6 million per mm^3 blood.[12] The result of this study announces that the number of erythrocytes in group A was 8.67 million per mm^3 blood, group B was 7.39 million per mm^3 blood, and group C was 4.88 million per mm^3 blood.

There was no statistical difference remarked between erythrocyte number in Group A and B. The number of erythrocyte in Group A and B was ranged within normal without any depletion though it had been served with Toman fish extract at 16 mL/Kg BW for 14 days. This affirms that Toman fish extract at 16 mL/Kg BW after 14 days administration is not toxic in normal and diabetic wistar rat model based on the erythrocyte number.

The normal level of hemoglobin in Wistar rat ranged between 11.1-18 g/dL.[12] This study revealed that the hemoglobin level in group A was 17.30 g/dK, group B was 16.32 g/dL, and group C was 11.54 g/dL. Hemoglobin level in all groups were spanned within the normal range of hemoglobin in Wistar rats.

No statistical difference was observed on hemoglobin level in group A and B. Hemoglobin level in group A and B was presented within the normal range and no depletion was observed after the administration of 16 ml/Kg BW toman fish extract for 14 days. This demonstrates that Toman fish extract at 16 mL/Kg BW dosage after 14 days administration in normal and diabetic wistar rat model is not toxic based on the hemoglobin level.

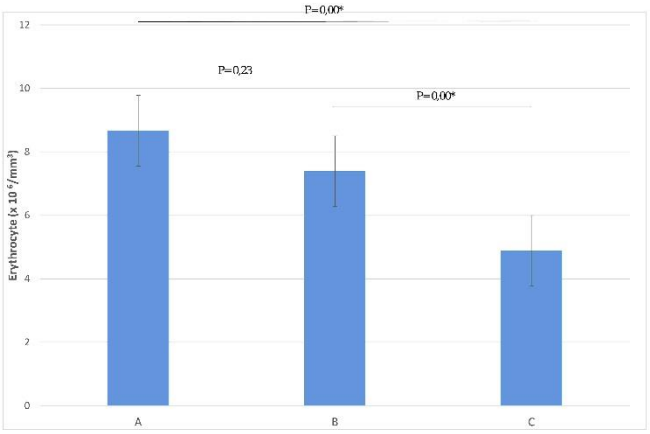

Figure 1. The number of erythrocyte in three treatment groups, with the level of significance less than 0.05 ($p<0.05$) (A: Normal wistar rat administered with Toman fish extract at 16mL/Kg BW dosage; B: Diabetic wistar rat model administered with Toman fish extract at 16 mL/Kg BW dosage; C: Diabetic wistar rat model given BR2 feed only)

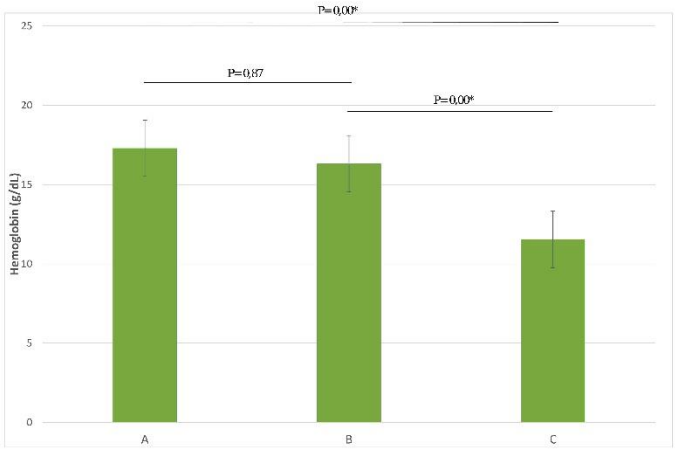

Figure 2. The level of hemoglobin in three treatment groups, with the level of significance less than 0.05 ($p<0.05$) (A: Normal wistar rat administered with Toman fish extract at 16 mL/Kg BW dosage; B: Diabetic wistar rat model administered with Toman fish extract at 16 mL/Kg BW dosage; C: Diabetic wistar rat model given BR2 feed only, $p<0.05$)

Discussion

Erythrocytes are one of the hematological components which can be used as a biomarker to evaluate the influence of substances that enter the body.[10] The number of erythrocytes in group A prevailed within the normal range because of the presence of albumin in Toman fish extract. Protein may amplify erythrocyte number and optimize the effect of erythropoietin hormone functioned to stimulate red blood cell formation (erythropoiesis) in bone marrow.[13] Erythropoietin enhances the ability to defend, proliferate and differentiate erythroid progenitor during erythropoiesis, therefore it promotes the formation of erythrocyte.[14]

The number of erythrocyte in group B was shown to be statistically different than those in group C. Eryhtrocyte number in group C was lower than normal due to hypeglicemic condition in diabetes mellitus which instigates the glycation of protein. Protein glycation is a binding process between high concentration of protein amine group and glucose aldehyde group which is also known as Maillard reaction. This reaction produces Advanced Glycation End Products (AGEs) which indicates an increase in the level of free radical and oxidative stress.[15] Oxidative stress incites peroxidation of phospholipid membrane in eryhtrocyte that will resulted in the formation of Malionadehyde (MDA). Malionadehyde may reduce the elasticity of erythrocyte to change shape under a given level of stress, therefore degrading its ability to survive.[16] Oxidative stress may also harm erythroid DNA in eryhtropoiesis causing the depletion of mature erythrocyte to be released in circulatory system.[17]

The number of erythrocyte in Group B remained within normal as the result of albumin in Toman fish extract. Albumin prevents glycation in diabetic Wistar rat where the reaction between protein and glucose occured at high concentration to produce AGEs. The production of this compound indicates the increase of free radical and oxidative stress. It modifies the structure and function of protein, therefore prompting the disruption of erythrocyte

protein as well as depletion in erythrocyte number.[15,18] Albumin in Toman fish will bind glucose via Maillard reaction which interferes with the binding of erythrocyte protein to glucose and hinders the glycation reaction together with structure changes in erythrocyte protein molecule. Further, albumin also prevent the disruption and downturn of erythrocyte number.[19,20] Albumin as well serves as antioxidant with the presence of sulfyhidril group (-SH) and operates as radical scavanger to bind metal ion involved in ROS production to reduce the formation of oxidative stress.[21,22] Cell damage due to oxidative stress will be less visible and depletion in erythrocyte number will be downregulated.[23]

Hemoglobin is an essential protein that also composed 90% weight of erythrocyte.[18] This protein is also responsible as one of the hematological parameters to evaluate material toxicity.[10]

Group A was reported with normal level of hemoglobin as the result of adequate protein intake. Toman fish contains high level of albumin when compared to other Channidae families.[24] Protein is a nutritional compound with an essential role for the formation of hemoglobin in our body.[25] It serves as a significant component for ion absorption, storage and transport.[26] Iron is one of the protein compounds which plays a significant role in hemoglobin synthesis. Most of hemoglobin synthesis takes place in erythroid cell of bone marrow and liver. The synthesis covers eight stages that are aminolevulinic acid (ALA) formation, profobilinogen (PBG) formation, hydroxymetibilane formation, uroporfirinogen II (UPG) formation, coprophorphyrinogen II (CPG), protophorphyrinogen (PPG), protophorphyrin IX formation, and hemoglobin formation. The last stage of hemoglobin synthesis utilizes iron to bind with protophorphyrin IX and later produce hemoglobin molecules.[27]

Hemoglobin level in group B and C demonstrates a significant diferrence statistically, yet it remained within the normal span. Diabetic condition induce hyperglicemia that will promote glucose penetration into erythrocyte membrane and bind hemoglobin to form HbA1c (glycated hemoglobin). HbA1c indicates an increase in glucose level within the blood of patient who suffers from diabetes mellitus.[28,29] It is resulted from the binding between amine group and hemoglobin via protein glycation that will promote changes in protein structure and function, and leads to hemoglobin destruction.[15,18] Albumin content in Toman fish extract may protect other protein from glycation process. It swiftly advances to the glycation process in which the amine group of albumin will bind to the aldehyde group of glucose through Maillard reaction.[30] This will reduce the amount of glucose that binds with other protein including hemoglobin, therefore it lowers the rate of glycation reaction and lessen protein breakdown in haemoglobin.[19,20] Glycation process is known to be downregulated with the presence of high albumin level that impedes with HbA1c formation.[31]

Different reaction was observed in group C without the administration of Toman fish extract. Hemoglobin level in group C was lower than those in group B because hyperglicemic condition in DM fosters the glycation reaction between glucose and hemoglobin protein at high concentration. This modifies the structure and function of protein that further disrupts protein compound in haemoglobin.[15] Additionally, hemoglobin destruction may also be prompted by the presence of oxidative stress.[18] Hemoglobin in diabetic rat was still within the normal range because the diabetic effect has yet been prolonged as the study was conducted for 14 days when compared to the 28-day research which the hemoglobin level was below normal.[32] This delineates that the difference in the duration of the study has affected the severity of diabetes mellitus.

Conclusions

The erythrocyte number and hemoglobin level in normal and diabetic Wistar rat administered with 16 ml/Kg BW dosage for 14 days are within the normal range. This illustrates that Toman fish extract at 16 mL/Kg BW dosage is not toxic after 14 days administration based on the number of erythrocyte and the level of hemoglobin in the blood of normal and diabetic Wistar Rat model. The development of herbal medicine using Toman fish extract as its ingredient may be advanced to sub-acute and chronic toxicity investigation. Toman fish extract is anticipated to be an alternative that may accelerate wound healing process particularly for post extraction dental practice.

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