

Impact of hypoproteic diet on liver function and thrombopoiesis in new Zealand white rabbits

Abstract

Protein under nutrition may play a role in the development and progression of liver disease. The relationship between hypoproteic diet, liver function and subsequently thrombopoiesis has not been fully documented; hence, this study sets out to determine it in normal rabbits. The broad objective of this work is to determine the relationship between a hypoproteic diet, liver function and thrombocyte indices in normal rabbits. Twenty New Zealand White Rabbits weighing 950-1050g were used. They were assigned into two groups (A and B) of ten rabbits each and fed with rabbit chow containing 30% protein for a period of four weeks. Group A animals were changed to a 4% protein feed while group B animals were maintained on 30% protein feed. Blood samples were obtained from the marginal ear vein of the Rabbits after the period of acclimatization (4weeks) and analyzed for baseline data and twice at eight weeks intervals post-acclimatization. The samples were taken into 2ml EDTA tubes and 5ml plain tubes for the determination of platelet counts, AST, alanine transferase (ALT), alkaline phosphate (ALP), plateletcrit (PCT), platelet distribution width (PDW), mean platelet volume (MPV), Total Protein estimation, Albumin estimation and thrombopoietin assay using standard protocols. It was observed that Platelet counts, MPV, Total protein and Albumin levels were significantly lower ($P \leq 0.05$) in the rabbits fed hypoproteic diet compared to the rabbits of the control group while AST, ALT, ALP and thrombopoietin levels were significantly increased ($P \leq 0.05$) in the rabbits fed hypoproteic diet compared to the rabbits of the control group. The PCT values were decreased in the experimental group compared to the control group but were not statistically significant ($P \geq 0.05$). These findings suggest that hypoproteic diet impairs liver function and induce platelet destruction leading to low platelet count and alteration of platelets indices.

Keywords: protein, thrombocytopenia, hypoproteic, diet, liver disease

Volume 3 Issue 1 - 2016

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Received: October 04, 2016 | **Published:** November 28, 2016

Abbreviations: ALT, alanine transferase; ALP, alkaline phosphate; PCT, plateletcrit; PDW, platelet distribution width; MPV, mean platelet volume; EDTA, ethylene Di-amine tetra acetic acid

Introduction

The liver plays an important role in the production of hemopoietic hormones. It acts as the primary site of synthesis of erythropoietin in the fetal stage and it is the predominant thrombopoietin producing organ for life.¹ Thrombopoietin, which is a potent cytokine produced by the liver regulates mega karyocyte and platelet production. It acts at all stages of thrombopoiesis to regulate the development and maturation of mega karyocytes and subsequent release of platelets.² Apart from its hemopoietic functions, the liver orchestrates the metabolism of proteins and amino acids. These functions of the liver are measured using the liver function test which estimates the amount of enzymes and protein synthesized by the liver and can directly be correlated with health or disease of the liver.³

Protein deficiency is often associated with liver diseases.⁴ At present, protein deficiencies, whatever its causes, is by far the single most important factor affecting the entire clinical and experimental field of liver diseases. When protein deficiency is not accompanied by a grossly appreciable calorie deficit, the most salient pathologic feature encountered in the liver consists in the abnormal accumulation of fat, this leads to the fatty liver disease. Fatty liver in protein deficiency is predominantly due to a defect in the secretion of hepatic

triglycerides.⁵ Protein deficiency results in a reduction in plasma triglycerides and phospholipids, a rise in free fatty acids, and a fatty liver, owing chiefly to the accumulation of triglycerides associated with a reduction in hepatic phospholipids this involves the retention of lipids due to impaired hepatocyte apolipoprotein secretion or beta-oxidation.⁶

When the liver is diseased, the hematopoietic and metabolic functions of the liver were impaired. The regulation of protein metabolism is frequently disturbed. The manifestations of disturbed protein metabolism in liver disease are varied and change with disease aetiology and severity.³ The insufficient production of thrombopoietin has been proposed to be involved in the pathogenesis of thrombocytopenia in patients with liver diseases.⁷ Thrombocytopenia is a common complication in patients with chronic liver disease that has been observed in up to 76% of patients.² It can adversely affect the treatment of liver cirrhosis, limiting the ability to administer therapy and delaying surgical and diagnostic procedures because of an increased risk of bleeding.⁸ Some data support the hypothesis that protein under nutrition may play a role in the development and progression of liver disease.⁹ Nigeria, like many developing countries of the world is faced with problems of human and animal malnutrition particularly in terms of daily protein intake.¹⁰

Protein deficiency is often expressed in liver function tests. Albumin levels are low in nutritional problems, protein loss and failure of protein synthesis through extensive loss of functioning liver

tissue and some inflammatory conditions where the liver switches to making other proteins.¹¹ Elevations of liver enzymes which include ALT, AST and ALP levels have been demonstrated in Fatty liver disease which involves deficiency of dietary protein.¹² Platelet counts and platelet indices apart from Platelet distribution width (PDW) are low while PDW are high in patients with Fatty liver disease positively correlating with ALT and AST levels and negatively correlating with total platelet count.¹³ The relationship between hypoproteic diet, liver function and subsequently thrombopoiesis has not been fully established. This objective of the study was to assess if a protein rich diet will improve liver function and thrombopoiesis or protein under nutrition impairs liver function and thrombopoiesis using New Zealand White rabbits as experimental models.

Materials and methods

Animals

Twenty adult New Zealand White rabbits weighing between 950-1050g were used for this study selected for use in this study. Only apparently healthy animals with normal rectal, body temperature and baseline hematological indices were included in the study. They were procured from the small animal house of the College of Medicine and Health Sciences, Afe Babalola University, Ado-Ekiti. The animals were housed in hanging stainless-steel wire cages kept in an isolated room at a controlled temperature (23-25°C) and humidity (40-60%). Lighting was maintained on a 12hr cycle (lights on from 07.00 to 19.00hr). All experiments involving animals were approved by the committee on Animal House/Ethics at Afe Babalola University, Ado-Ekiti, Ekiti State, Nigeria (ERC/2014-06-18).

Determination of sample size

The sample size was calculated by resource equation method described by Festing, (2000). In this method a value E is calculated based on decided sample size. In this animal study 2 groups of animals were formed having 10 animals each for different interventions, therefore the total animals will be 20 (10×2). Hence E will be

$$E=20 - 2=18$$

Where E = Total number of animals- Total number of groups

The value if E should lie within 10 to 20 for optimum sample size. If a value of E is less than 10 then more animal should be included and if it is more than 20 then sample size should be decreased. E (18) in this study is within the acceptable limits (10-20). Hence, the sample size is adequate.

Rabbit feed

The rabbit feed was prepared at ABUAD farm. A mineral and vitamin mixture was prepared according to the recommendations for Rabbits of the American Institute of Nutrition [14]. The protein rich diet was formulated to 30% (w/w) protein whereas the hypoproteic diet only 4% (w/w). The source of protein was Soya bean. Except for the protein content, the two diets was identical and isocaloric, the total amount of Soya bean removed from the formulation of the hypoproteic diet was substituted for with the same mass of corn and starch.

Composition of feed

25g Hypoproteic feed (4% protein), 20.5 g of maize and 4g of starch was added to a mixer for 7mins. After thorough mixing, it was

taken to the hammer mill for grinding (2mins) to fine particle and stirred in another mixer (7mins). 0.4 g Di-calcium phosphate and 0.1 g of salt was added and mixed properly. This was transferred to a boiler and steamed for 3mins after steaming; it was transferred to the pelletizer to mould to required size of 6mm. It was then transferred to the cooler for cooling and the seiver that collects and separates the dust. The feed was bagged and sewn.

25g 30% Protein feed 19.1g of soya, 1g of starch and 0.4g of Di-calcium phosphate was combined in a mixer (7min). This was taken to the hammer mill for grinding to fine particle (2mins) and stirred in another mixer with sequential addition of 0.1g of methionine, 0.1g of lysine, 0.1g of salt and 0.1g of enzymes and mixed properly (7min). This was transferred to a boiler and steamed for 3mins after steaming; it was transferred to the pelletizer to mould to required size of 6mm. It was then transferred to the cooler for cooling and the seiver that collects and separates the dust. The feed was bagged and sewn.

Duration of the experiment

The rabbits were acclimatized for a period of 4 weeks during which they were fed standard rabbit pellets containing 30% protein and allowed water ad libitum. Blood was collected after acclimatization for baseline data. Group specific feed were then introduced for 16 weeks during which the animals were bled midway (8 weeks post acclimatization) and after another 8 weeks for the laboratory analysis presented in this report.

Experimental design

A total of twenty rabbits were used for this experiment, which were randomly assigned into two group of ten rabbits each as follows:

Group A: Maintained on a hypoproteic feed (4%) after acclimatization

Group B: Maintained on a 30% Protein feed after acclimatization

The animals bled via the marginal ear vein after acclimatization (4weeks) and at week 12 and 20 of the experiment.

Sample collection and analysis

Blood sample was obtained from the marginal ear vein of the Rabbits into 2ml ethylene Di-amine tetra acetic acid (EDTA) tubes and 10ml plain tubes. The Samples obtained into EDTA were mixed by inversion while those in plain tubes were spun in a bucket centrifuge. The serum was aspirated and kept frozen at 4°C before analysis. Platelet counts, AST, ALT, ALP, PCT, PDW, MPV, Total Protein estimation, Albumin estimation and thrombopoietin (Enzyme Linked Immunosorbent Assay) were carried out on the samples.

Ethical considerations

Ethical approval of the experiment protocol was obtained from the ethics and research committee of the small animal house, College of Medicine and Health Sciences, Afe Babalola University, Ado-Ekiti Nigeria (ERC/2014-06-18). All procedures and techniques for the study are in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals.

Determination of platelet indices

Plateletcrit (PCT), Mean Platelet Volume (MPV), Platelet distribution width (PDW) and Platelet count were determined using Abacus® junior vet hematology auto analyser.

Determination of liver function parameters

Total Protein, Albumin, Alanine transaminase, Aspartate transaminase, Alkaline Phosphatase was determined by the colorimetric method using Randox® kits.

Determination of serum thrombopoietin

Thrombopoietin was determined by Enzyme Linked Immunosorbent Assay (MyBiosource®).

Statistical analysis

The data obtained were analyzed using Statistical Package for Social Sciences (SPSS 11.0) for Windows (SPSS, Chicago, IL). Student's t-test was employed for the comparison of the results obtained from the two groups. A p-value of equal to or less than 0.05 ($p \leq 0.05$) was considered as statistically significant.

Results

The platelet count, thrombopoietin levels, Mean Platelet Volume (MPV), Plateletcrit (PCT), Platelet Distribution Width (PDW) of group A rabbits which were fed 4% protein and group B rabbits (Control group) which were fed 30% protein is presented in Table 1. The results obtained show a significant decrease ($P \leq 0.05$) in Platelet counts of the rabbits fed hypoproteic diet which decreased from 285 ± 142.5 at baseline to 229 ± 80.2 after 16 weeks (terminal) compared to the rabbits in the control group which recorded a platelet count of 335 ± 184.3 at the end of the experiment. A decline in platelet count was accompanied by a significant rise ($P \leq 0.05$) in the thrombopoietin levels (218 ± 32.7) after 8 weeks (intermediate) and at terminal stage (226 ± 101.7) of the research in the rabbits fed hypoproteic diet compared to the rabbits of the control group which was 181 ± 81.5 at the early stages of the research, 192 ± 76.2 at intermediate stage and $187.37.4$ at the end of the study.

The values of MPV (4.5 ± 0.1) were significantly lower ($P \leq 0.05$) in the Group A rabbits (fed hypoproteic diet) compared to the control group (5.7 ± 0.2). The PDW values showed no significance statistically ($P \geq 0.05$) at the intermediate stage of research (14.6 ± 6.7) but declined significantly ($P \leq 0.05$) in the rabbits of placed on a hypoproteic feed (Group A) at the end stage (16.3 ± 8.15). The values of PCT were decreasing in the rabbits fed hypoproteic diet (from 0.15 to 0.10) as the research progressed but showed no statistical significance ($P \geq 0.05$) between the experimental and control group (Table 1).

The total protein, Albumin, Alanine Transaminase (ALT), Alkaline Phosphatase (ALP) and Aspartate Transaminase (AST) of Group A (4% protein) and Group B (control group) were evaluated and compared to assess liver function and is presented in Table 2. The results obtained showed a significant decrease ($P \leq 0.05$) in the levels of Total protein in the rabbits fed hypoproteic diet which decreased from 5026 at the baseline stage to 3614.76 at intermediate and 288.55 at the terminal while the rabbits of the control group maintained a steady level. Albumin levels also declined significantly ($P \leq 0.05$) in the experimental group from 23.3520.1 at the beginning of the research to 16.46.6 at the end of the research. However, the levels of Albumin increased in the control group from 2510.2 to 33.2613.3 in the control group. The results showed a steady rise in the levels of liver enzymes ALT, AST and ALP ($P \leq 0.05$) in the rabbits fed hypoproteic diet while the control group maintained a steady level from the early stages of the research to the end of the experiment.

Table 1 Platelet count, thrombopoietin levels, MPV, Mean Platelet Volume; PCT, Plateletcrit; PDW, Platelet Distribution Width; of Group A (4% protein) and Group B (control group) rabbits

		A	B	P-value
Platelet counts (unit)	Baseline	285 ± 142.5	314 ± 125.6	0.004
	Intermediate	256 ± 102.4	307 ± 184.2	0.003
	Terminal	229 ± 80.2	335 ± 184.3	0.012
Thrombopoietin (pg/mL)	Baseline	172 ± 51.6	181 ± 81.5	0.126
	Intermediate	218 ± 32.7	192 ± 76.8	0.044
	Terminal	226 ± 101.7	187 ± 37.4	0.017
MPV (fL)	Baseline	4.2 ± 0.1	5.6 ± 0.2	0.006
	Intermediate	4.4 ± 0.6	5.5 ± 0.1	0.001
	Terminal	4.5 ± 0.1	5.7 ± 0.2	0.006
PCT (%)	Baseline	0.15 ± 0.03	0.16 ± 0.02	0.374
	Intermediate	0.12 ± 0.06	0.14 ± 0.04	0.568
	Terminal	0.10 ± 0.04	0.17 ± 0.03	0.481

Table 2 Total protein, Albumin, ALT, Alanine Transaminase; ALP, Alkaline Phosphatase; AST, Aspartate Transaminase; of Group A (4% protein) and Group B (control group) rabbits

		A	B	P-value
Total Protein (g/dL)	Baseline	50.26 ± 20.1	52.0 ± 26	0.0195
	Intermediate	36.9 ± 14.76	57.3 ± 45.8	0.053
	Terminal	28.5 ± 8.55	54.4 ± 38.1	0.025
Albumin (g/dL)	Baseline	23.35 ± 11.7	25.46 ± 10.2	0.005
	Intermediate	19.6 ± 6.9	31.46 ± 18.9	0.0018
	Terminal	16.4 ± 6.6	33.26 ± 13.3	0.0007
ALT (iu/L)	Baseline	15.0 ± 7.5	12.0 ± 7.2	0.006
	Intermediate	33.4 ± 10	10.4 ± 3.1	0.0002
	Terminal	32.1 ± 12.84	16.1 ± 8.1	0.0002
ALP (iu/L)	Baseline	15.23 ± 3.0	12.4 ± 4.34	0.001
	Intermediate	29.4 ± 7.2	18.5 ± 9.3	0.003
	Terminal	35.82 ± 10.7	22.3 ± 11.2	0.008
AST (iu/L)	Baseline	20.0 ± 10	19.2 ± 7.7	0.166
	Intermediate	25.8 ± 10.32	18.8 ± 5.6	0.004
	Terminal	30.4 ± 18.2	19.0 ± 14.3	0.011

Discussion

This study was conducted to determine the effects of hypoproteic diet (low protein) on liver function and thrombopoiesis in New Zealand White Rabbits. The main objective of this study was to determine the relationship between a hypoproteic diet, liver function and thrombocyte indices in normal rabbits. From the results, platelet count in the rabbits fed hypoproteic diet (4% protein) decreased significantly ($P \leq 0.05$) when compared with the platelet count in the rabbits fed the

control diet (30% protein). The decrease in platelet count of the rabbits fed hypoproteic diet can result from decreased platelet production, increased platelet consumption or sequestration.¹⁵ Multiple factors can contribute to the development of thrombocytopenia including splenic platelet sequestration, immunological processes and reduced levels or activity of the hematopoietic growth factor thrombopoietin.¹⁶

Liver disease usually causes persistent thrombocytopenia resulting from direct toxic marrow suppression and splenic sequestration.¹⁷ The patho-physiology of thrombocytopenia in liver disease has long been associated with the concept of hypersplenism, where portal hypertension was thought to cause pooling and sequestration of all corpuscular elements of the blood, predominantly thrombocytes in the enlarged spleen.¹⁸ In this study, the spleen of the rabbits fed low protein diet was enlarged compared to the rabbits of the control group, this suggests that protein deficiency induced an impaired liver function which in turn resulted in hypersplenism and subsequent sequestration of platelets that ultimately led to lower platelet counts in rabbits fed the hypoproteic diet when compared with rabbits fed control diet.

However, values from both the experimental and control diets were all within the normal range of platelets as reported by other research.^{19,20} In these reports, thrombocytopenia are often drug induced or due to infections such as hepatitis which is expected to be more severe than that of thrombocytopenia caused by liver disease of protein deficiency. It was observed that the mean platelet volume (MPV) of rabbits on 4% protein was lower ($P \leq 0.05$) than those of the rabbits on the control feed at the terminal stage of this research. This is inconsistent with the report of Giovanetti et al.²¹ which noted an inverse relation between platelet count and MPV values. However the MPV values of this study is in agreement with the findings of Omran et al.²² who reported a reduction in platelet count and MPV values in patients with liver disease. The leukocyte infiltration observed in the histology of the liver (plate 1) of the animals placed on a hypoproteic diet is suggestive of a progression towards liver disease.

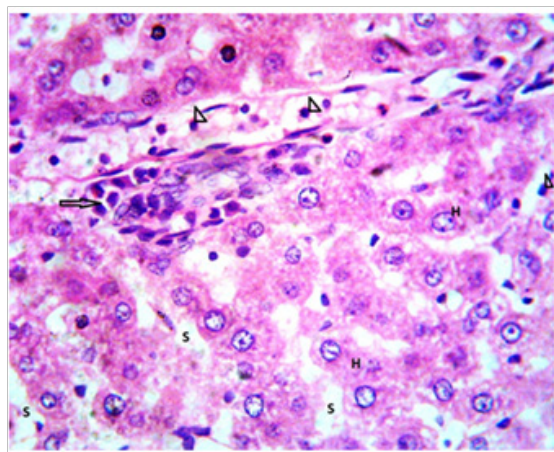


Plate 1 Micrograph of liver section stained with H&E showing Group A section with mild periportal leukocytes infiltrations (arrow head, arrow) the hepatocytes (H), Sinusoids (S) appear distinct and unremarkable.

The difference between the PCT levels of the experimental and control group was not statistically significant ($P \geq 0.05$) however the plateletcrit of the rabbits fed hypoproteic diet was lower than those of the control diet. This agrees with the reduced platelet count observed in this study compared with rabbits of the control diet. Xianghong et al.²³ reported a decrease the platelet count, plateletcrit and an increase MPV in patients suffering from liver cirrhosis. Costa et al.²⁴ also

observed a decrease in platelet count and plateletcrit of patients with chronic liver disease but found no significant difference in their mean platelet volumes.

The platelet distribution width (PDW) of the rabbits fed hypoproteic diet show no major changes from the beginning of the research to the terminal. Rabbits in the control group had a higher PDW ($P \leq 0.05$) than the rabbits fed 4% protein. It has been reported that platelets with increased number differ in size, therefore affecting platelet distribution width.²⁵ In this research, Platelet count in the rabbits fed hypoproteic diet was lower than the values of the control rabbits therefore it is expected that the PDW of rabbits in the experimental group will not be affected. Streiff et al.²⁶ reported a low platelet count and a significant difference in MPV but not PDW among patients with liver disease associated with thrombocytopenia.

The ALT and AST levels significantly increased ($P \leq 0.05$) compared to the values obtained from rabbits fed the control diet. The increase in this value could be as a result of liver disease caused by protein deficiency.⁵ It has been demonstrated that Liver enzymes are usually increased while platelet counts are low in liver diseases.²⁷ This is in agreement with this study, the platelet counts of the rabbits on hypoproteic diet was considerably lower than those fed control diet. Similar observations have been observed by other researchers which fed low protein diet to rabbits.¹³ Abdel-Wareth AA et al.²⁸ also reported a significant increase in ALT and AST levels and a decrease in platelet count in rabbits fed low protein diet. The ALT and AST values suggest hepatic insufficiency in rabbits fed hypoproteic diet.

The ALP levels also increased ($P \leq 0.05$) compared to the values obtained from the control rabbits. Elevations of ALP levels have been demonstrated in Fatty liver disease which involves deficiency of dietary protein.¹² ALP levels are usually increased while platelet counts are low in liver diseases.²⁷ In this research, platelet counts of the rabbits fed hypoproteic diet is considerably lower than those fed control diet. In contrast the research done by Oboh et al.¹³ the ALP levels was considerably decreased. However another research reported an increase in ALP levels with increase in protein in patients with liver disease.²⁹ The ALP values of the rabbits fed Hypoproteic diet suggests a development of an impaired hepatic function.

Total protein and Albumin levels decreased significantly ($P \leq 0.05$) compared to the values obtained from the rabbits fed control diet. In liver disease, circulating proteins synthesized by the liver including albumin are frequently decreased.¹⁸ It has been reported that decrease in total protein and albumin levels signify decrease in protein metabolism.³⁰ The decrease in protein metabolism may be due to an abnormal liver function caused by protein deficiency. When the liver is diseased, the regulation of protein metabolism is frequently disturbed, total protein, albumin and platelet counts are usually low.³ Platelet count has been demonstrated to be low in rabbits fed hypoproteic diet along with a decrease in total protein and Albumin indicating protein deficiency and liver disease.²⁷ This further suggests that the liver function of the rabbits in the experimental group have been impaired due to low protein diet.

Thrombopoietin levels between the rabbits fed hypoproteic diet and control diet were compared. The levels of thrombopoietin in the rabbits fed hypoproteic diet were increasing as the research progressed ($P \leq 0.05$) while the levels were fairly similar in the rabbits fed control diet (30% protein). Thrombopoietin is predominantly produced in the liver and constitutively expressed by hepatocytes.¹⁸ The increase in the production of thrombopoietin may be due to the

fact that platelet counts were decreasing in the rabbits fed hypoproteic diet. The compensatory mechanism of thrombopoietin producing sites in producing more thrombopoietin in the advent of platelet loss could be the reason for more production. Although the liver is the major site of production of thrombopoietin, other sites such as kidney and bone marrow stroma also produce thrombopoietin but this is insufficient enough to maintain a normal level of platelet count in liver disease.³¹ In the findings of this study, although thrombopoietin levels were increasing, platelet count was decreasing in the rabbits of the experimental group.

Thrombocytopenia associated with liver disease is usually attributed to reduced thrombopoietin production. In a research by Giannini et al.¹¹ thrombopoietin was reduced in patients with liver disease leading to thrombocytopenia. However, it was reported by Emmons et al.³² that thrombopoietin levels are characteristically high in humans and experimental animals with insufficient platelet production secondary to megakaryocytic hypoplasia. It was also reported by Kuter³³ that thrombopoietin levels are inversely proportional to the rate of platelet production.

The liver sections of Group A and B rabbits were compared, the liver section of the rabbits fed hypoproteic showed mild leukocyte infiltrations (Plate 2). Reimers et al.⁹ reported a hepatocyte ballooning, liver fibrosis and fat accumulation in advanced protein deficiency. The animals in the experimental group were fed hypoproteic diet for three months and it is expected that the liver histology will not show an extensive liver damage like in protein deficiency of several years. The ALT, AST, ALP, Total protein levels and Albumin levels in the rabbits fed hypoproteic diet suggests a development of an impairment of liver function.

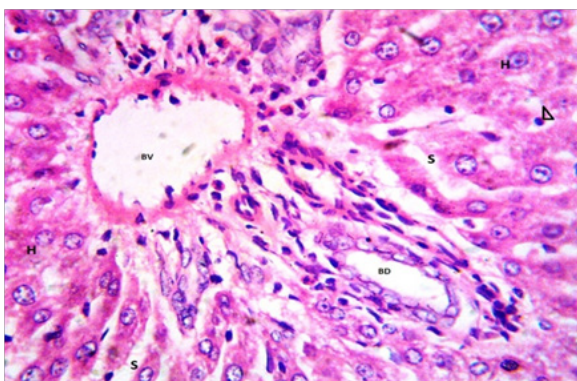


Plate 2 Micrograph of liver section stained with H&E showing Group B composed of the portal triad (circle) which is made up of blood vessel (BV), bile duct (BD) and lymphatics. The hepatocytes (H) are disposed in sheet and are separated by the sinusoids (S). Section is free from collections and marked inflammatory infiltrations.

Protein deficiency is associated with liver disease and the principal cause of protein deficiency is decreased dietary intake.⁴ This study suggests a development in impairment of liver function leading to thrombocytopenia which in turn stimulated an increase in thrombopoietin production in other production sites but was insufficient enough to cause an increase in platelet count to comparable values in rabbits placed on protein rich feed.

Conclusion

Hypoproteic diet has an impact on liver function by impairing or negatively affecting the liver function which is expressed in the liver function tests by the increase in liver enzymes. Apart from

impairing the liver function, hypoproteic diet also negatively affected platelet count, plateletcrit and mean platelet volume while platelet distribution width was not affected. In this study, it is concluded that protein deficiency does not impair thrombopoiesis but rather induces an increase in the production of thrombopoietin.

From the findings of this study, the relationship between hypoproteic diet, liver function and thrombopoiesis could be established. It was observed that protein deficiency impairs liver function which results in hypersplenism and causes sequestration of thrombocytes therefore lowering platelet counts. The low platelet count stimulates an increase in the production of thrombopoietin from other thrombopoietin production sites such as the kidney and bone marrow stroma which is still insufficient to induce a normalization of the platelet count. Therefore when liver function is impaired, platelet counts and indices are negatively affected due to destruction while there is an increase in thrombopoiesis.

Acknowledgements

None.

Conflict of interest

The author declares no conflict of interest.

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