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Protein Malnutrition in BALB/c Mice: An Experimental Model Resembling Clinical Scenario

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Abstract

Objectives The study aims to develop a stable malnourished experimental mice model resembling the human population for future experimental studies.

Materials and Methodology At weaning, female BALB/c mice are separated into two groups: one receiving a low protein diet (LPD, 10% protein) and the other receiving a commercially available normal pellet diet (ND, 18% protein). Model development and stability were assessed using body mass index (BMI), biochemical parameters such as glucose, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, total protein, albumin, total cholesterol, calcium, and phosphorus using serum samples at the 12th and 15th weeks of the study, antioxidant assay, and liver histopathology observation. Antioxidant assay and histopathology observation using liver tissue sample excised after euthanasia.

Results LPD mice are categorized under grade I malnutrition based on the body weight change with respect to ND as per the principles of Gomez's classification of malnutrition. A significant long-term decrease in BMI of the malnourished group indicates the development of the stable malnourished model. Elevated serum enzyme levels in the 15th week and decreased antioxidant activity suggest liver injury and oxidative stress at the cellular level in the malnourished group. Histopathology alterations in the liver tissue further strengthen these observations reported in the human population of malnutrition.

Conclusion This study confirms the development of a stable malnourished experimental model using a LPD (10% protein). This model may be used to study the role of malnutrition in the pathophysiology of any disease, drug action, and its kinetics in the future.

Keywords
- antioxidant assay
- BALB/c
- BMI
- low protein diet
- mice model
- protein malnutrition

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Introduction

Malnutrition is recognized as one of the most severe global health challenges, with each country suffering from one or more forms of malnutrition.[1] World Health Organization (WHO) states that malnutrition is the deficiency or excess of nutrition causing an imbalance in the nutrient utilization in the body.[2] Its consequences have spiked up due to the recent coronavirus disease 2019 pandemic. Recently, WHO reported that undernutrition is responsible for approximately 45% of deaths among children younger than 5 years.[3–5] Clinical features of malnutrition depend on the degree of nutritional deficiency, and the etiology of these conditions has less clarity.[6,7]

Protein malnutrition is the main category of malnutrition. Food habits play a vital role in assuring an individual's health status. According to the Indian consumer market 2020, only one-third of the food budget goes toward food with high protein. In contrast, a significant portion of the budget is taken up by processed food.[8] According to UNICEF, in India, a quarter of women of reproductive age are undernourished, with a body mass index (BMI) of less than 18.5 kg/m^2. This leads to the birth of malnourished children, which triggers the malnutrition cycle.[9,10]

Liver is the vital organ responsible for maintaining nutritional and energy balance in a healthy individual. It is a primary metabolizing organ for all the molecules and drugs entering the body. As a result, it is more susceptible to various complications. Protein malnutrition plays a vital role in developing liver diseases. It is a significant global challenge for health care systems.[11–13] Decreased protein content in the diet may cause hepatic impairment, characterized by altered alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), electrolytes, and lipid profile.[14]

To understand the complexity of malnutrition, there is a need for extensive research on malnutrition conditions. Further, to conduct such research, there is a need for a stable, long-term, and clinically relevant malnourished model. There are experimental models using various animals,[15] but these models either involve diet restriction and starvation or are short-term models designed for a specific experiment.[16–18] Recently, a study using a low protein diet (LPD) was validated.[19] The validated diet was used for a concise period of observation and would not resemble the clinical condition where the exposure is chronic/continuous.

Our study aims to develop a stable, long-term, and clinically relevant malnourished experimental mice model using an LPD, which shall avoid restricted diet/starvation with consideration to the welfare of mice as per animal ethics. A mice model that shows variation/alteration in the body weight, BMI, and biochemical parameters such as ALT, AST, and ALP supported by changes in the liver histopathology compared with that of normal (healthy mice) as observed in clinical scenario (human population) is considered as a stable model.

Materials and Methodology

Animals

Healthy female BALB/c mice at weaning were selected and housed at Nitte University Centre for Animal Research and Experimentation, NGSM Institute of Pharmaceutical Sciences (NGSMIPS), Nitte (Deemed to be University) Mangaluru under controlled standard housing conditions (12-hour dark and 12-hour light cycle, 22 ± 2°C temperature). Mice had free access to designated food and purified drinking water. Experimental procedures were approved by the Institutional Animal Ethics Committee (IAEC, NGSMIPS, NITTE [Deemed to be University], India) with approval no: NGSMIPS/IAEC/MAY-2020/185.

In Vivo Study

Selected female BALB/c mice were separated into two sets: (1) normal diet (ND) (receiving commercially available standard pellet diet containing 18% protein from VRK Nutritional Solutions, Maharashtra, India) and (2) LPD (receiving patented diet combination by NUCARE, NGSMIPS containing 10% protein) (►**Table 1**).[20,21] Body weight and morphometric parameters were analyzed weekly to confirm the development of a stable malnourished mice model. Blood was sampled for biochemical analysis on the 12th and 15th weeks after the initiation of the study ($n = 6$ in each set).

Blood and Tissue Sampling

On weeks 12 and 15 of the study, blood was sampled under the influence of isoflurane anesthesia. Collected samples were allowed to stand for an hour at room temperature and later centrifuged at 3,000 rpm for 5 minutes to separate serum for further analysis. On week 15, after blood sampling, mice from both sets were euthanized using isoflurane anesthesia. Liver tissue was excised and washed; one portion was stored at −20°C for antioxidant analysis, and the rest was stored in 10% formalin solution for histopathological examination.

Biochemical Analysis

Biochemical analysis of the stored serum sample was performed in a semiautomated analyzer using Aspen biochemical kits. Glucose (mg/dL), AST (U/L), ALT (U/L), ALP (U/L), total

Table 1 Composition of LPD[20,21]

S. No.	Ingredients (LPD, 10% protein)	Percentage
1	Normal diet	44.44
2	Corn oil	2.42
3	Sucrose	6.04
4	Wheat bran	3.02
5	Vitamin mix	0.60
6	Mineral mix	2.11
7	Maize starch	41.36

Abbreviation: LPD, low protein diet.

protein (TP) (g/dL), albumin (g/dL), total cholesterol (TC) (mg/dL), calcium (mg/dL), and phosphorus (mg/dL) levels in serum were analyzed.

Body Mass Index

BMI of normal and malnourished animals was calculated using the following formula[19]:

$$\text{BMI} = \frac{\text{Weight (g)}}{\text{Nose to anal length (cm}^2\text{)}}$$

Antioxidant Assay

Five percent tissue homogenate was prepared using 0.25 M phosphate buffer and centrifuged at 10,000 rpm for 20 minutes to separate the supernatant. The separated supernatant of tissue homogenate was used to conduct the antioxidant assays. Catalase was carried out as reported by Aebi (1974),[22] superoxide dismutase (SOD) as per the procedure written by Kakkar et al (1984),[23] and glutathione (GSH) as per the protocol reported by Ellman (1958).[24,25]

Histopathology Observations

Liver tissue sample stored in 10% formalin was used to make paraffin-embedded sections. The sections were used to prepare slides and stained using hematoxylin and eosin dyes and observed under various magnifications.

Statistical Analysis

Data expressed as mean ± standard error of the mean. The differences between groups were analyzed using Student's *t*-test (nonparametric analysis) for two independent samples using GraphPad Prism Software version 8.0.1, and a *p*-value < 0.05 was considered significant.

Results

In Vivo Study

At the beginning of the study, both ND and LPD groups showed similar body weights. Later, the ND group showed a gradual increasing body weight until the completion of the study. The LPD group also showed an increase in body weight, but the growth rate was considerably less than the ND group. Though the body weight gain was less than the ND group, the mice receiving LPD were healthy and did not exhibit adverse health conditions (►**Fig. 1A**). The mice in the LPD were considered malnourished based on their body weight compared with the ND mice. They were categorized into various categories of malnutrition with respect to the ND group as per the principles of Gomez's classification of malnutrition. Initially, LPD mice showed grade I (mild malnutrition) and moved to grade II (moderate malnutrition); by the end of the 12th week, the model began to stabilize and was completely stabilized by the 15th week showing mild malnutrition (►**Fig. 1B**). Mice that are susceptible show grade III (severe malnutrition) without gaining weight from the initial point; such mice tend to show mortality in a few weeks as their body would fail to cope with the complications of malnutrition.

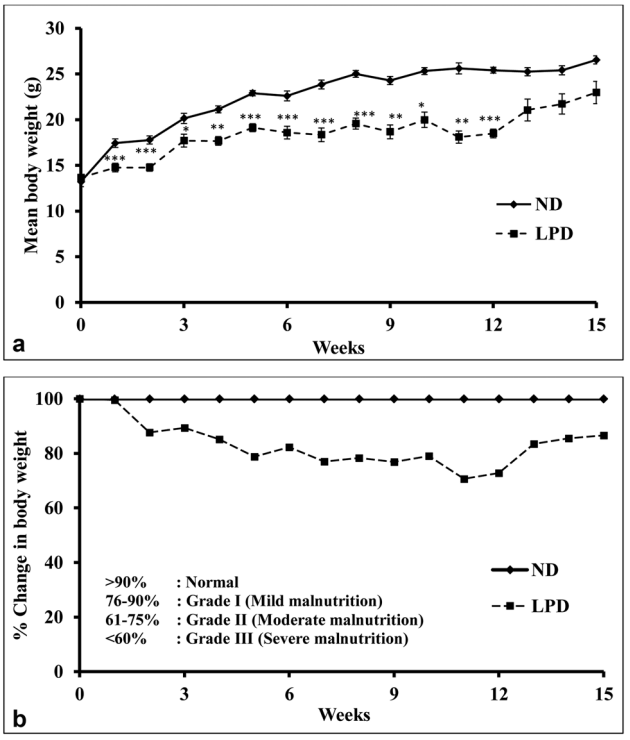

Fig. 1 (**A**) Fifteen weeks mean body weight data of ND (18%) and LPD (10%) mice and (**B**) 15 weeks percentage change in body weight data with respect to ND ($n > 6$). LPD, low protein diet; ND, normal diet.

BMI is one of the significant indicators of malnutrition. ND mice showed a gradual increase in BMI from the beginning and maintained a steady state until the completion of the study. Similarly, LPD mice also showed a slight increase in their BMI. But the increase was marginal and significantly lower than in ND mice (►**Fig. 2**). The very low BMI of the malnourished diet group compared with that of ND groups confirms the development of the stable malnourished model.

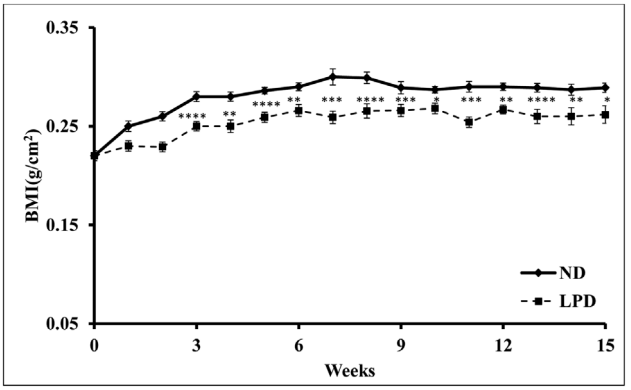

Fig. 2 Fifteen weeks BMI data of ND (18%) and LPD (10%) mice ($n > 6$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$. BMI, body mass index; LPD, low protein diet; ND, normal diet.

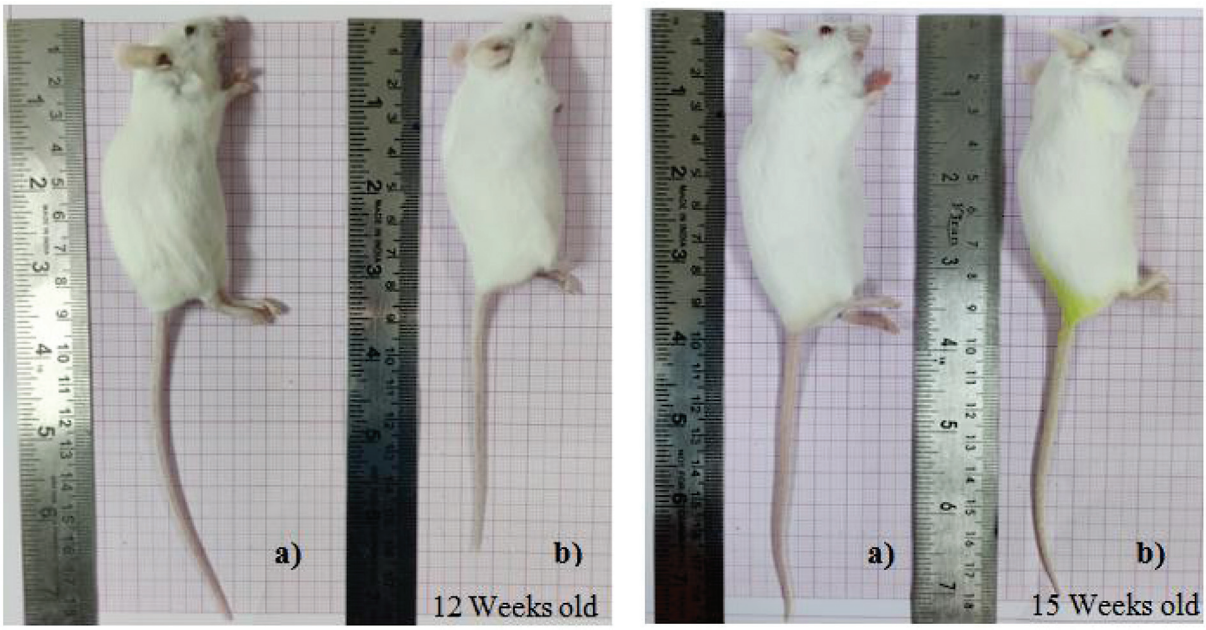

Fig. 3 Photographs showing (**a**) ND and (**b**) LPD mice at weeks 12 and 15 of the study, respectively. LPD, low protein diet; ND, normal diet.

Photographs of representative mice for each group are included (►**Fig. 3**).

Model Validation

In the present study, we did not validate the model separately by considering the 3Rs of animal ethics. Similar study was conducted earlier for the patent where male BALB/c mice were used to develop the diet composition which is used in the present study.[20] As per the patent, based on the body weight data, 6% protein diet group showed severe malnutrition from the 5th week of LPD initiation, and mortality was observed at 7th week. Considering the mortality observation, the animals receiving 6% protein diet were shifted to 10% protein diet in the 10th week. Subsequently, the body weight of these animals increased and matched to the other 10% protein diet group, indicating that the animals were stable with 10% protein diet having the characteristics of malnutrition with respect to normal as per Gomez's classification. This observation also confirms the importance of protein content in the diet, and the treatment for protein deficiency malnutrition is the higher protein content in the diet itself. Therefore, validation of the malnourished animal model in the present study is not necessary as the detailed validation using same diet combination is available in the patent.[20]

Biochemical Analysis

In contrast to ND, the LPD group showed transient liver injury as indicated group showed transient liver injury as represented by elevated AST and ALT levels on weeks 12 and

Table 2 Biochemical data of ND and LPD mice on 12th and 15th weeks

Parameters	12th wk		15th wk	
	ND	LPD	ND	LPD
Glucose	69.80 ± 9.80	98.63 ± 8.40[a]	73.31 ± 11.30	139.8 ± 13.46[b]
AST (U/L)	63.24 ± 3.81	72.27 ± 3.79	73.572 ± 9.663	168.15 ± 25.86[a]
ALT (U/L)	44.73 ± 7.20	67.28 ± 15.89[a]	77.26 ± 3.70	114.78 ± 15.58[a]
ALP (U/L)	126.5 ± 7.39	137.93 ± 7.05	144.33 ± 16.19	191.81 ± 19.18
Total protein (g/dL)	5.14 ± 0.13	5.48 ± 0.26	5.17 ± 0.48	5.57 ± 0.44
Albumin (g/dL)	2.54 ± 0.13	2.39 ± 0.04	2.60 ± 0.13	2.57 ± 0.07
Total cholesterol (mg/dL)	71.84 ± 8.42	100.65 ± 3.48[a]	61.77 ± 4.52	76.07 ± 5.53[a]
Calcium (mg/dL)	8.09 ± 0.73	8.61 ± 0.33	10.06 ± 1.27	9.29 ± 0.55
Phosphorus (mg/dL)	8.50 ± 0.23	7.54 ± 0.29	7.92 ± 0.254	7.28 ± 0.97

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LPD, low protein diet; ND, normal diet.
Note: $n = 6$.
[a]$p < 0.05$.
[b]$p < 0.01$.

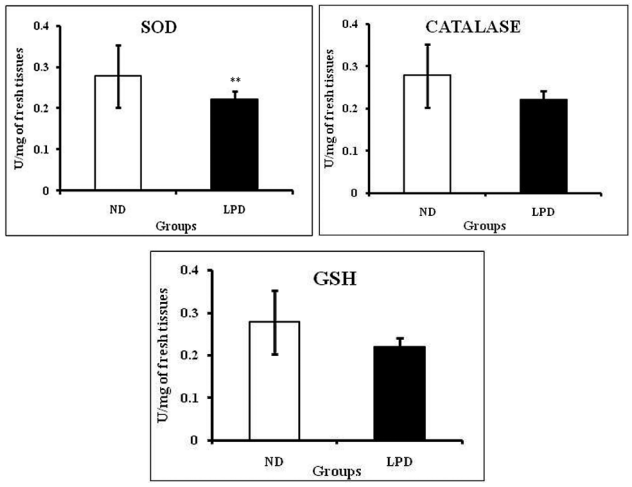

Fig. 4 Fifteenth week data of SOD, catalase, and GSH activity in ND (18%) and LPD (10%) mice ($n = 6$). $^{**}p < 0.01$. GSH, glutathione; LPD, low protein diet; ND, normal diet; SOD, superoxide dismutase.

15 serum analysis. The increase was twofold higher on week 15 than on week 12, indicating the progression of liver injury over the period. Additionally, increased TC in the LPD group supports the altered serum biochemistry in malnourished conditions. Considering liver dysfunction (►**Table 2**) as the model development and stability marker, 15 weeks are required to develop a stable malnourished mice model. This observation is further strengthened by BMI data (►**Fig. 2**) and oxidative stress parameters (►**Fig. 4**). Circulating TP and albumin levels are similar in ND and LPD. The calcium and phosphorous value obtained from the LPD group compared with the ND group was not statistically significant (►**Table 2**).

Antioxidant Assay

LPD showed a decrease in the antioxidant enzyme activity of catalase, SOD and GSH (►**Fig. 4**). However, only SOD levels showed a significant decline compared to ND.

Histopathology Observations

Histopathology observations of liver tissue from the LPD group revealed moderate alterations in the normal architecture of the tissue. Hepatocytes were enlarged, moderate hydropic degeneration, presence of Kupffer cells, and dilated sinusoids were observed (►**Fig. 5A**). These findings strengthen the observations of serum enzyme alterations. Further, LPD also shows decreased body weight/spleen ratio (►**Fig. 5C**). These observations confirm the development of a stable malnourished mice model.

Discussion

Animal models are essential to understand the pathophysiology of various diseases/conditions and drug development. Animal models resembling all clinical features of malnutrition are either scanty or unavailable. Therefore, the present study aimed to develop a long-term stable malnourished mice model that resembles a clinical scenario.

Body weight and BMI are the primary tools used worldwide to screen an individual's nutrition status. Among biochemical parameters, a lower serum albumin level has been considered as one of the main parameters for decades. However, serum albumin is intended to be a proinflammatory marker for nutritional risk assessment rather than a sole marker for malnutrition.[26,27] Other reports suggest that serum albumin is not considered a malnutrition marker in small animals.[28] Our study is to develop a malnutrition model using small animals. Hence, the similar level of albumin in ND and LPD is justified.

Alterations in TC levels are indicators of malnutrition. Deficient TC levels lead to mortality as observed in the older population.[26] Similarly, higher levels in malnourished serum indicate the chances of coronary heart disease (CHD) in later stages, as increased TC acts as a predepositing factor for CHD.[14] The increased level of TC in the LPD group is also an indicator of malnutrition. Similarly, plasma electrolyte (phosphorus, calcium) maintains homeostasis and metabolic functions of the

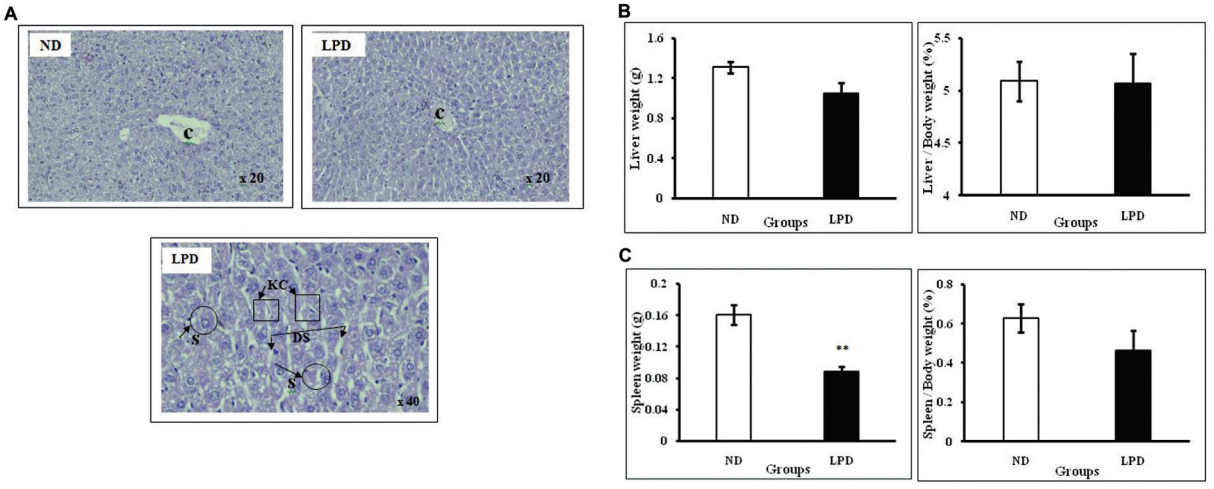

Fig. 5 (**A**) Photomicrographs of histopathological changes in liver tissue in ND and LPD mice at week 15 in magnification ×20 and ×40, (**B**) liver weight, and liver and body weight ratio, and (**C**) spleen weight, and spleen and body weight ratio ($^{*}p < 0.05$, $^{**}p < 0.01$). C, central vein; DS, dilated sinusoids; KC, Kupffer cells; LPD, low protein diet; ND, normal diet; S, swollen hepatocytes.

body, and their depletion is meant to cause metabolic disorder associated with protein malnutrition (►**Table 2**).[29]

Liver health and nutritional status are closely related as the liver plays a vital role in regulating nutritional status and energy balance.[29] In the malnourished condition, liver tends to exhibit altered functioning. Protein malnutrition is said to reduce hepatic expression of antioxidant enzyme GSH S-transferase; this may increase the concentration of free radicals resulting in oxidative stress.[30] Increased AST, ALT, and ALP as a result of protein malnutrition is a primary suspect of liver injury, indicating tissue damage due to the condition, and the increased level is considered as the biomarker of malnutrition.[14,29]

Oxidative stress is the imbalance between the production and accumulation of related oxygen species in cells and tissues. Antioxidant enzymes such as SOD, GSH, and catalase act in defense to inactivate free radicals generated due to oxidative stress.[31] Literature reports that protein-malnourished animals tend to show increased lipid peroxidation, which causes changes in the activities of antioxidant enzymes.[32,33] SOD acts as a first-line defense against oxygen toxicity and lipid peroxidation, followed by catalase and GSH. SOD undergoes the dismutation of reactive oxygen free radicals through various processes. A decrease in SOD indicates the initiation of defense against tissue damage[34] by disposing of reactive superoxide radicals formed by oxidative stress. This decrease is further observed in catalase due to the removal of toxic peroxides. Decreased level of catalase and SOD and GSH indicates free radical-mediated tissue damage (►**Figs. 4** and **5**). This decrease is due to inadequate defense mechanisms in protein-deficient animals, causing liver injury. The decreased antioxidant condition also causes decreased immune function and increased inflammation.[35] Therefore, maintenance of antioxidant status is essential in biology. The changes in antioxidant enzyme level and altered liver tissue pathology of the LPD group correlate to these findings and confirm malnutrition development in mice (►**Figs. 4** and **5**).

Alteration in spleen weight/body weight ratio and changes in histopathology architecture in the 15th week is reflected in the biochemical parameters (►**Fig. 5** and ►**Table 2**). In the clinical scenario, impaired liver function leads to altered biochemical parameters in marasmus protein malnutrition.[19] Literature also indicates that the increase in Kupffer cells results from malnutrition.[36] Considering this evidence, our study confirms that the developed mice model is stable and clinically relevant.

Conclusion

The present study explains the development of a stable malnourished model in mice receiving a LPD (10% protein). Our model mimics the clinical scenario of long-term malnutrition with lowered BMI, alteration in biochemical parameters, and clinically resembling changes in histopathology of long-term malnutrition. The developed model is cost-effective, simple, and stable for short-term and long-term studies to know the role of malnutrition in the pathophysiology of various diseases, drug action, and its kinetics in the future.

Conflict of Interest
None declared.

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