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ORIGINAL ARTICLE

Longitudinal Evaluation of Developmental Protein Malnutrition Resembling Marasmic-Kwashiorkor Condition in Wistar Rats

Varsha AUGUSTIN[1], Murali BADANTHADKA[1]*, Madhura R.J.[1], Vinitha DSOUZA[1], B. Mohana KUMAR[2], A. Veena SHETTY[3]

[1]Nitte (Deemed to be University), Nitte Gulabi Shetty Memorial Institute of Pharmaceutical Sciences (NGSMIPS), Department of Nitte University Centre for Animal Research and Experimentation (NUCARE),Karnataka, India
[2]Nitte (Deemed to be University), K.S. Hegde Medical Academy, Nitte University Center for Stem Cell Research and Regenerative Medicine, Karnataka, India
[3]Nitte (Deemed to be University), K.S. Hegde Medical Academy, Academy, Department of Microbiology, Karnataka, India

ABSTRACT

Objectives: Protein malnutrition (PMN) is a significant public health concern that can aggravate pathological states. The impact of early malnutrition on metabolism needs extensive evaluation. Current models employ short-term diet restriction and are neither ethically correct nor clinically relevant. This study aimed to develop a PMN rat model to evaluate the effects of a low-protein diet (LPD) on physiological, hematological, biochemical, and histological changes affected by malnourishment from postweaning to the 40th week.

Materials and Methods: The PMN model was developed in Wistar rats (post-weaning) by assigning animals to patented LPD (10% protein) and a control group to a normal diet (18% protein). Developed model was confirmed by biometric, biochemcial parameters and Gomez classification of malnutrition.

Results: LPD-induced PMN showed stunted growth, altered biochemical (albumin range, 1.9 - 2.4 g/dL, total protein range, 5.1 - 6.4 g/dL), and hematological markers mean corpuscular volume (52.03 ± 1.34, 47.45 ± 0.44, p≤0.01), mean corpuscular hemoglobin (17.67 ± 0.47, 15.37 ± 0.18, p≤0.001) and mean corpuscular haemoglobin concentration (33.87 ± 0.22, 32.37 ± 0.24, p≤0.001) and significantly affected hepatic histology. A long-term study was conducted to analyze the pattern of developmental PMN and its stabilization over time.

Conclusion: The developed PMN rat model imitates clinical conditions and is confirmed as a stable, reproducible, and reliable model for short- and long-term studies. The clinical relevance of this approach opens new avenues for research in treatment, drug development, molecular interactions, and disease model development.

Keywords: Animal model, biochemical parameters, low protein diet, marasmic-kwashiorkor, protein malnutrition, rats

INTRODUCTION

Malnutrition is a condition caused by an imbalance in the intake of nutrients in terms of quantity, quality, or both at any point inlife.[1,2] A report by the Food and Agriculture Organization stated that 728 million people around the globe were malnourished in 2020.[3] World hunger statistics 2021 report a drastic increase of approximately 161 million malnourished people between 2019 and 2020. This crisis can be attributed to climate change and COVID-19 consequences.[4] Malnutrition at critical growth period results in short- or long-term metabolic impairments.[5] The metabolic activity of an individual is controlled by the nervous system, which generally develops in the early stage of life. Early undernutrition processes information to the nervous system for permanent self-programming to save energy in the form of fat and to reduce growth. This anatomical and physiological adaptation is required to secure survival under possible adverse conditions.[1,5]

*Correspondence: murali@nitte.edu.in, ORCID-ID: orcid.org/0000-0002-4313-5111
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Malnutrition is not a disease; it is considered one of the primary concerns that lead to the burden of disease in developing countries. Protein malnutrition (PMN) can be categorized into three forms based on its clinical manifestations: kwashiorkor, marasmus, and an intermediate stage termed marasmic-kwashiorkor. Clinical features depend on the severity, duration, stage of life, and degree of nutritional deficiency.[6] Kwashiorkor is typically defined as edematous malnutrition, with clinical characteristics such as skin lesions, hair loss, hypoalbuminemia[7], and hepatic abnormalities (hepatomegaly and fatty infiltrations). Whereas marasmus isa form of non-edematous malnutrition characterized by significant weight loss, lack of subcutaneous fat, muscular atrophy, and a poor weight-for- height ratio.[8] Marasmic-kwashiorkor is a clinical manifestation characterized by a combination of the clinical features of two types of malnutrition. Body composition, gastrointestinal tract, liver, kidney, tissue protein, body fluids, plasma, and hormones are targets of protein-energy malnutrition-mediated physiological and functional changes.[9]

Experimental animal models serve as important sources of information for understanding the effects and consequences of various diseases and drug actions.[10] Laboratory animals are extensively used to assess the effects of variables at various degrees of malnutrition and individual pathologies related to malnutrition. The highly controlled evaluation of each nutritional parameter individually gave more consistent results in animal models than in humans. Nutritional insults like protein malnutrition during the early and developmental phases of life, induce weak hallmarks of metabolic malfunctions and can impair lifelong metabolism patterns.[11] Protein deficiency in Wistar rats induces changes in body weight and body and organ growth and leads to hepatic steatosis.[12] Malnutrition negatively affects biochemical parameters like total protein (TP) and albumin (ALB),[13,14] phosphorous[15], and triglyceride (TG).[16] Liver function markers such as alkaline phosphatase (ALP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) increase with malnutrition.[17,18]

The global prevalence of PMN requires a better understanding of the underlying pathophysiological mechanisms. The effects of undernutrition in humans are not restricted to early development but also exert lasting effects. However, studies on the consequences of long-term malnutrition and its metabolic risks are limited. It is necessary to have a stable, long-term, and clinically relevant malnutrition model to carry out this kind of research. Malnutrition models using different animals employ starvation/diet restriction, which are generally short-term models developed for context-specific experiments. This study aimed to develop a PMN rat model to evaluate the effects of a low-protein diet (LPD) on physiological, hematological, biochemical, and histological changes affected by malnourishment from postweaning to the 40th week.

MATERIALS AND METHODS

Chemicals and instruments

Semi-Auto Analyzer model: Star 21 Plus from Rapid Diagnostic Group of Companies, India; Automated hematology analyzer: Nihon Kohen, India; Biochemical reagent kits: Aspen Laboratories Pvt. Ltd., Formaldehyde (LobaChemie Pvt. Ltd., #01460). Normal diet (ND) (Amruth feeds, Pune, Maharashtra), Corn oil (Grainotch Industries Ltd., Cornlite), Sucrose (Shree Renuka Sugars Limited, Madhur sugars), Wheat bran (Liberty brand from local market), Vitamin mix (SIDDON BIOTECH, LBCE150205), Mineral mix (SIDDON BIOTECH, LBCE150203), Maize starch (SB-IMPEX, CHE150196).

Experimental animals

Healthy female Wistar rats were selected at weaning and housed in polypropylene cages with rice husk bedding at NUCARE (Nitte University Center for Animal Research and Experimentation). Standard laboratory conditions were maintained (12 hours light/dark cycle; temperature 22 ± 2 ˚C; relative humidity 60 ± 5%) were maintained with free access to food and water. All animal experiments were conducted according to the guidelines of the committee for the Purpose of Control and Supervision of Experiments on Animals (approval number: NGSMIPS/Dec-2020/2022, date: 29.12.2020).

Experimental design

The animals chosen were divided into two groups: ND and low-protein diet (LPD).[19,20] Details of the diet composition are presented in Table 1. The ND and LPD groups received anND (18% protein) and LPD (MB diet, 10% protein) *ad libitum*. Body weight and length (nose-to-anus length) were assessed weekly as biometrical parameters, and rats were classified into malnourished categories according to the Gomez classification of malnutrition.[21,3]

The body mass index (BMI) of rats was calculated using the standard formula.

Blood and tissue sampling

Blood was sampled on the first day of every alternate week by puncturing the retro-orbital plexus under isoflurane anesthesia. The collected blood samples were allowed to clot at room temperature and centrifuged at 3000 rpm for 5

Table 1. Composition of a LPD (10% protein) for the development of malnourished rat model

Serial number	Ingredients (ND)	g/kg	Ingredients (LPD)	g/kg
1	Wheat flour	56.2	ND	44.440
2	DCP (rock base)	1.8	Corn oil	2.415
3	Calcite powder	1.0	Sucrose	6.038
4	LAF premix	1.0	Wheat bran	3.019
5	Linseed	5.0	Vitamin mix	0.603
6	Maize gluten	5.0	Mineral mix	2.113
7	Roasted gram flour	25.0	Maize starch	41.360
8	Skimmed milk powder	5.0		

LPD: Low-protein diet, ND: Normal diet, DCP: Dicalcium phosphate, LAF: Laboratroy animal feed

minutes to separate the serum. At the end of the experiment, rats were euthanized using isoflurane anesthesia. Liver tissue was excised, washed, weighed, and fixed in 10% formalin for histopathological examination.

Hematological analysis

Blood samples collected in EDTA-coated tubes were analyzed immediately after collection using an automated hematology analyzer.

Serum biochemical analysis

Biochemical parameters were quantified using standard commercial kits according to the manufacturer's instructions. Stored serum samples were thawed and analyzed for ALB, TP, TG, phosphorous, AST, ALT, and ALP.

Statistical analysis

Statistical analysis was performed using the mean population and standard error of the mean. The test of significance or statistical analysis was the Student's t-test. $p \leq 0.05$ was considered statistically significant. The Graph Pad Prism (version 8.4.3) (GraphPAD, San Diego, CA, USA) software was used for statistical analysis.

RESULTS

Biometric parameters

The body weight and BMI of the LPD group were significantly decreased ($p \leq 0.001$) compared with the normal age-matched rats. Body weight increased in the ND group during the first 6 weeks, after which it stabilized. The gradual rate of weight gain in LPD patients may be attributed to lower dietary protein (Figure 1A, B).The body weight and BMI of the LPD group confirm the development of a stable malnourished rat model compared with age-matched controls. The LPD group animals were categorized into various degrees of malnutrition based on the principles of Gomez classification concerning the body weight of the ND group rats. By the end of week 1, LPD shows grade II malnutrition (moderate malnutrition), progressing to grade III malnutrition (*i.e.*, severe malnutrition) from week 2 to 12. Subsequently, a slow improvement in malnutrition was observed from weeks 13 to 27. Later, malnutrition shifted to mild or grade I malnutrition (Figure 1C), which may be by survival adaptations.[22]

Table 2. Hematological parameters of ND and LPD animal groups at 10th week (n=6)

Parameter (Unit)	ND group	LPD group
WBC (103/uL)	10.53 ± 0.97	11.80 ± 1.24
RBC (106/uL)	8.36 ± 0.25	8.41 ± 0.69
HGB (g/dL)	14.75 ± 0.53	12.87 ± 0.98
HCT (%)	43.52 ± 1.56	39.82 ± 3.02
MCV (fL)	52.03 ± 1.34	47.45 ± 0.44**
MCH (pg)	17.67 ± 0.47	15.37 ± 0.18***
MCHC (g/dL)	33.87 ± 0.22	32.37 ± 0.24***
PLT (10^3/uL)	740.33 ± 60.59	681.52 ± 60.70
RDWCV (%)	12.30 ± 0.34	13.27 ± 0.15
RDWSD (fL)	25.62 ± 0.85	25.18 ± 0.32
PCT (%)	0.42 ± 0.05	0.37 ± 0.32
MPV (fL)	5.53 ± 0.03	5.48 ± 0.07
PDW (%)	15.40 ± 0.07	15.03 ± 0.12

All values are mean ± SEM. Bars represent the standard error, $p < 0.05$*, < 0.01** and $p < 0.001$** when compared to the ND group. WBC: White blood cells, RBC: Red blood cell, ND: Normal diet, LPD: Low-protein diet, HGB: Hemoglobin, HCT: Haematocrit, MCV: Mean corpuscular haemoglobin, MCH: Mean corpuscular haemoglobin, MCHC: Mean corpuscular haemoglobin concentration, PLT: Platelet, RDWCV: Red blood cell distribution width, RDWSD: Standard deviation red blood cell distribution width, PCT: Procalcitonin, MPV: Mean plateletvolume, PDW: Platelet distribution width

Hematological parameter

The hemoglobin (Hb), hematocrit (HCT), platelets (PLTs), and PLT Crit (PCT) counts in blood were lower in the LPD group than in the ND group (Table 2). Although the red blood cell (RBC) count was normal in the LPD group, RBC indices like mean corpuscular volume (MCV) (52.03 ± 1.34, 47.45 ± 0.44, $p \leq 0.01$), mean corpuscular Hb (MCH) (17.67 ± 0.47, 15.37 ± 0.18, $p \leq 0.001$) and mean corpuscular Hb concentration (MCHC) (33.87 ± 0.22, 32.37 ± 0.24, $p \leq 0.001$) were significantly low. On the other hand, the white blood cell (WBC) count increased compared to normal.

Biochemical parameters

TP and ALB, clinically relevant markers of PMN, both decreased significantly in LPD ($p \leq 0.05, 0.01, 0.001$) owing to prolonged low protein intake. The average serum ALB ranged between 1.9 and 2.4 g/dL from week 4 to week 22, and TP ranged between 5.1 to 6.4 g/dL from week 10 to week 24 in LPD (Figure 2A, B), which corresponds to clinical data and previous reports.[13,14] ALT and AST levels increased in the LPD group (Figure 2C, D) because PMN alters liver function. The significant increase in AST ($p \leq 0.01$) and ALT ($p \leq 0.05$) was positively correlated with the degree of malnutrition; grades II and III showed the highest level of injury. Serum ALP also increased substantially in the LPD group. Phosphorous levels were significantly higher in the ND group initially but stabilized later (Figure 2E, F). PMN remarkably decreased plasma TG levels (Figure 2G), possibly due to lipid accumulation in the liver, as shown in the histopathological results (Figure 3). The present study also demonstrated that the relative liver weight was higher in patients with LPD than in healthy controls (Figure 2H). Fat accumulation and hydropic changes may increase liver weight in patients with LPD.

Histopathology

Histopathological examination of the liver at weeks 10, 18, 20, and 26 captures the histological changes in the liver during PMN development in rats. Liver sections of the LPD group showed ballooning degeneration due to hydropic changes. Both Kupffer cells and dilated sinusoids around the central vein are evident. Nuclear displacement toward the periphery from fat

deposition in hepatocytes with vacuoles. These remarkable changes in liver histology confirmed the stable malnourished rat model. We used only week 10 slides for comparison.

Model validation

The model was validated by examining the biometric parameters of malnourished rats that were refed with ND (18% protein). Our results showed that LPD can induce PMN in rats, resulting in body weight loss, which can be reversed by refeeding. The BMI and body weight (Figure 4) of these animals increased significantly compared with the ND group ($p \leq 0.001$). During the 15 weeks of diet rehabilitation, we observed that the refed rats exhibited catch-up growth of (60.4%) and recovered from grade II (65%) to grade I (89.36%) malnutrition according to the

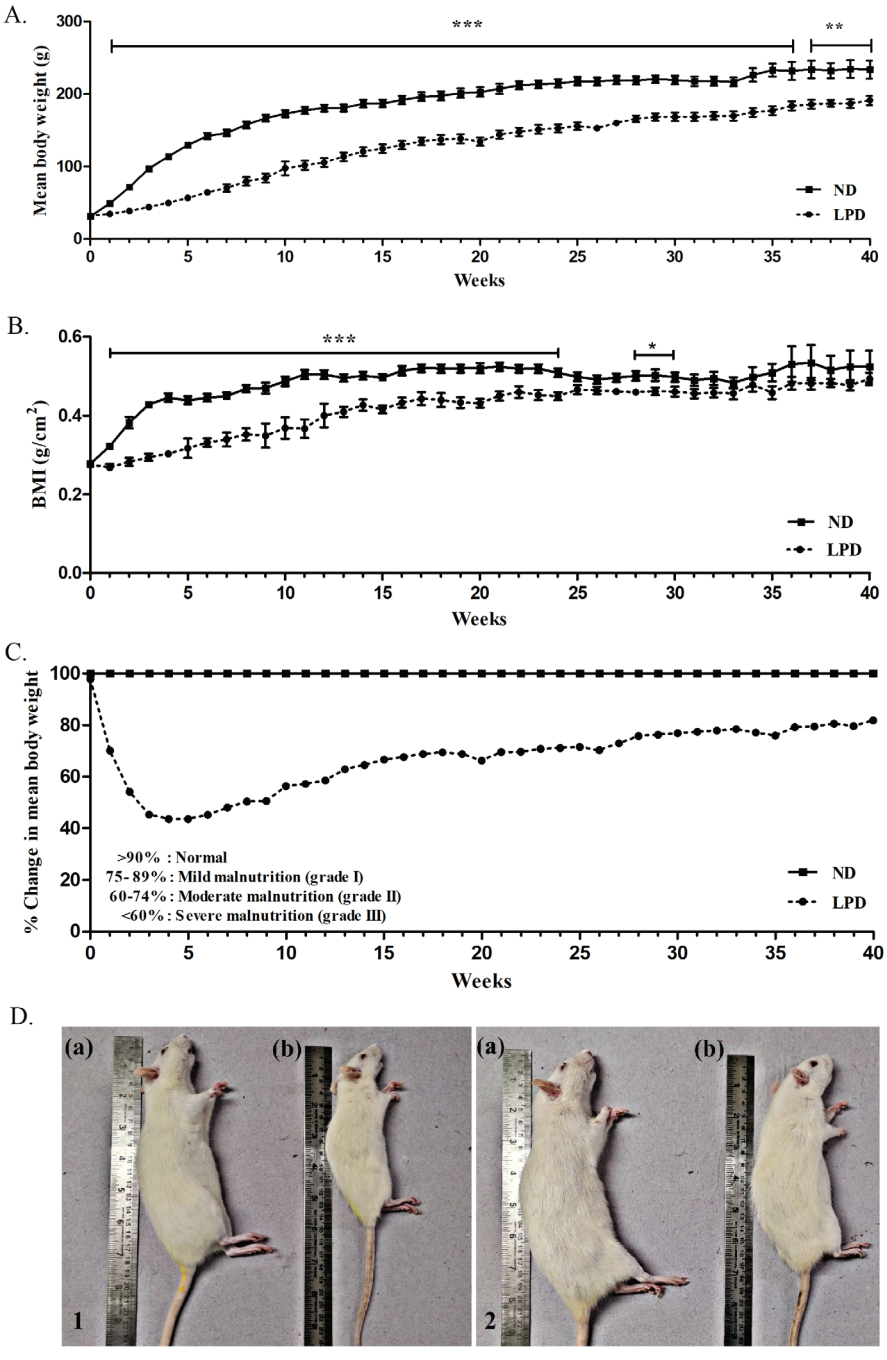

Figure 1. Graphical representation of (A) rat body weight (g) from weaning to 40 weeks in the ND and LPD groups (B) BMI of the ND and LPD groups (C). Percentage of change in body weight of LPD with respect to the ND group, and (D) photographic representation of ND (a) and LPD (b) at week 10 (1) and week 34 (2) of the study (n= 6). Significant differences are indicated by *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$

ND: Normal diet, LPD: Low-protein diet, BMI: Body mass index

Gomez classification. This finding supports the importance of protein content in the diet and demonstrates that treating protein-deficient malnutrition requires increased dietary protein intake.

DISCUSSION

As stated in the hypothesis, we developed a stable, reproducible, and clinically relevant PMN rat model that could be used in short- and long-term studies. Morphology showing stunted growth and skeletal structure in the LPD group. Skeletal muscles are the main protein reservoirs in the body and are sensitive to protein deficiency. Therefore, the depletion of differentiating muscle fibers weakens skeletal muscles,[23] leading to a decrease in weight gain. Opaque fur coating, voracious feeding, and a stooped posture were also noted.

The levels of hematology markers such as MCV, MCH, MCHC, Hb, HCT, PLT, and PCT in the PMN group were significantly reduced. Despite a normal RBC count, the altered RBC indices (MCV, MCH, and MCHC) indicate reduced Hb in the RBCs, resulting in compromised cell size, which resembles iron deficiency-induced hypochromic microcytic anemia.

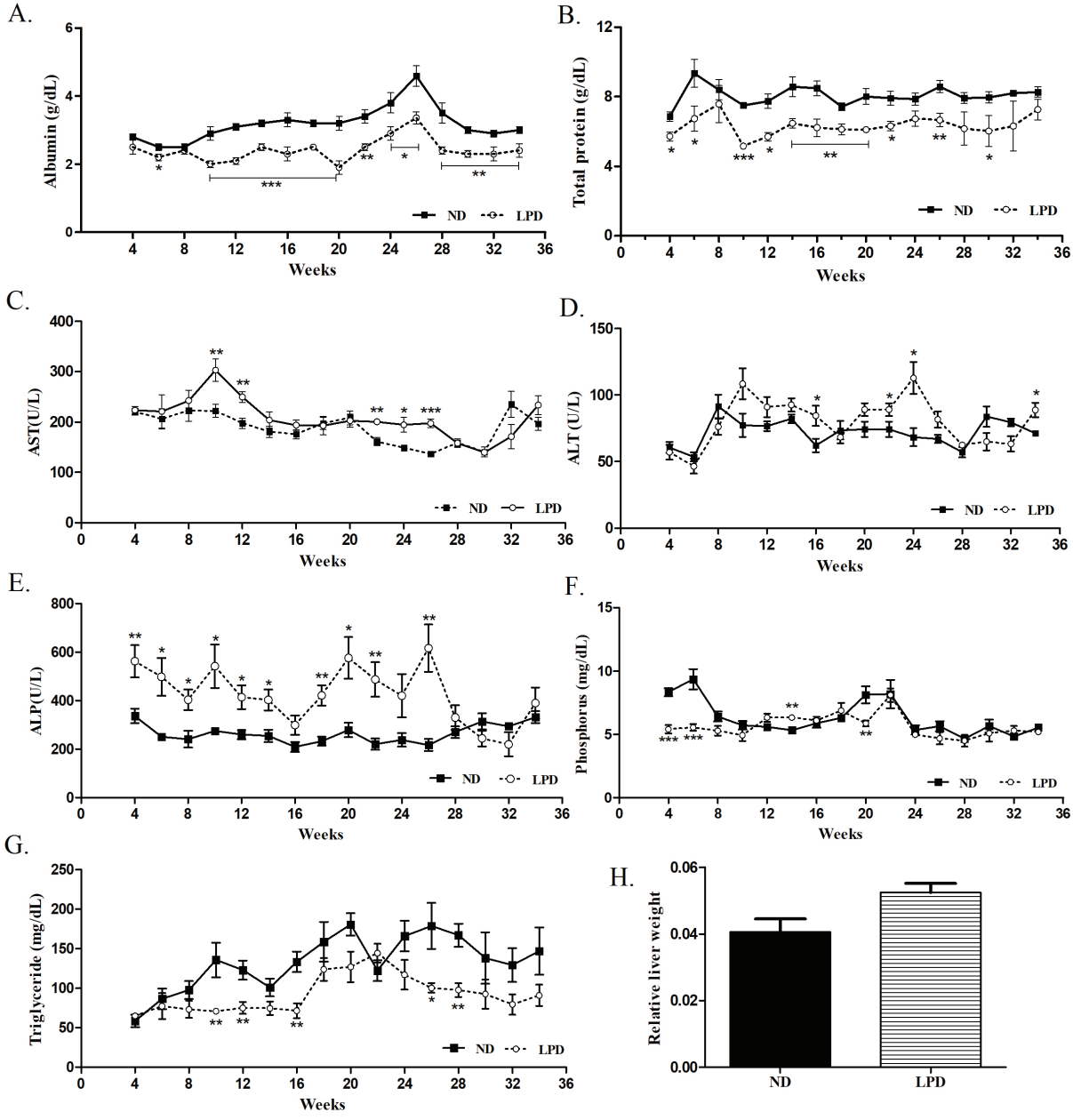

Figure 2. Serum biochemical parameters in patients with ND and LPD (n=6): (A) ALB, (B) total protein, (C) AST, (D) alanine aminotransferase, (E) ALB, (F) phosphorus, and (G) TG and (H) relative liver weight at week 10 (n= 6). Significant differences are indicated by *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$

ND: Normal diet, LPD: Low-protein diet, ALB: Albumin, AST: Aspartate aminotransferase, TG: Triglyceride

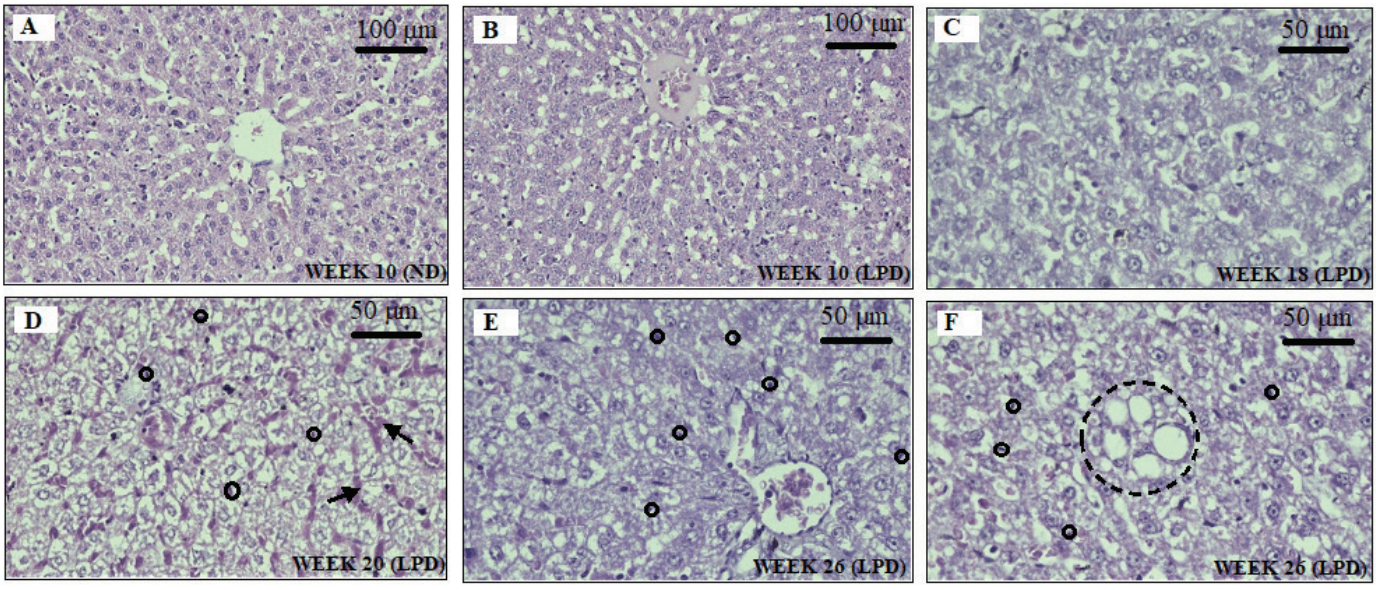

Figure 3. Photomicrographs of H&E sections of the liver (A) ND group showing normal cellular architecture with hepatocytes radiating from the central vein and sinusoidal space at week 10 of study (20X) (B) week 10 LPD group showing mild hydropic changes in the hepatocytes (20X) at week 10 (C) LPD group showing ballooning degeneration with irregular cytoplasm (40X) at week 18 (D) LPD group showing dilation of sinusoidal space (black arrows) with marked hydropic change (40X) at week 20 (E) LPD group showing central vein with marked hydropic change and presence of kupffer cells (black circles) (40X) and (F) macro-vesicular fat droplets (black discontinued circle) occupying the cytoplasm and displacing the nucleus to the periphery (40X) at week 26

LPD: Low-protein diet, ND: Normal diet

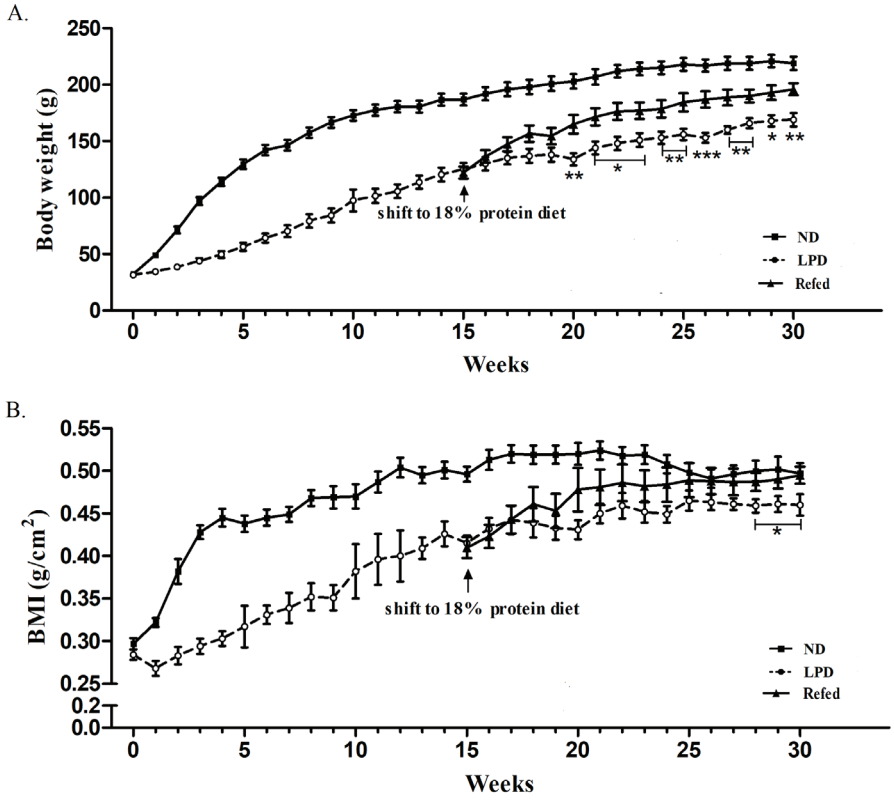

Figure 4. Graphical representations of (A) body weight (B) BMI of the ND, LPD, and refed groups during the rehabilitation period.Significant differences are indicated by *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$

ND: Normal diet, LPD: Low-protein diet, BMI: Body mass index

Red Cell Distribution Width, which increases in iron deficiency anemia[24] is possiblebecause total iron binding capacity is decreased in PMN.[25] Moderate anemia is prevalent in Kwashorkor[26] and marasmic conditions.[27] High WBC count in the LPD group, indicating susceptibility to infection. A reduction in PLT count may be attributed to compromised bone marrow activity.[28]

Depletion of amino acid precursors possibly led to a significant decrease in ALB levels in the LPD group.[29] ALB is synthesized solely in the liver on polysomes bound to the endoplasmic reticulum. After synthesis, ALB is transported from the rough endoplasmic reticulum to the Golgibodies and released directly into the systemic circulation. In PMN, loss of hepatic RNA and disaggregation of polysomes together upsets ALB synthesis.[30] Clinically, hypoalbuminemia is more severe in children with kwashiorkor than with marasmus.[31] Serum ALB levelsof less than 2.3 g/dL are considered undernourished. Lower ALB levels in LPD correlate with earlier studies and are clinically relevant.[14]

Both AST and ALT levels are elevated in PMN, possibly because of hepatic tissue damage. Malnutrition affects hepatocytes and releases these enzymes into the bloodstream under clinical conditions.[32] Our PMN model mirrors these clinical data, thereby validating the model.[33] Impaired bone development and liver function raiseALP. Elevated ALP in PMN[34] has already been reported. Early rise (initial weeks) in ALP might be the result of compromised bone development rather than liver dysfunction.[35] This observation is strengthened by low phosphorous levels during early development. PMN depletes phosphorous levels, impairing bone development, and causing defects in bone maturation. Compensatory osteoblast activity is increased as a positive feedback mechanism to overcome this impairment, thereby elevating ALP.[36] In later stages, liver dysfunction is accompanied by elevated ALP levels, which correlates with AST and ALT levels.

PMN decreases fatty acid oxidation, leading to increased lipogenesis and TG storage in the liver.[2,7] Hepatocytes recognize the amino acid profile, controlled by dietary protein intake that alters hepatocyte TG secretion,[37] resulting in decreased serum levels. Further, a reduction in TG secretion can also be attributed to a reduction in the rate of very low-density lipoprotein synthesis, resulting in hypertriglyceridemia during protein deprivation.[38] TGs in the serum are frequently low in kwashiorkor, but they are normal or increased in the marasmus condition.[10]

Severe but reversible liver changes are characterized by hepatocyte ballooning due to hydropic changes in the tissue. Edema enlarges the cell, and characterized by irregular cytoplasmic accumulation of water and fat droplets in the vacuoles. Initially, the vacuoles are small and surround the nucleus. Subsequently, the vacuoles become more prominent and displace the nucleus to the periphery, forming a signet-ring structure. The fat deposited in the vacuoles is predominantly TG. The accumulation of TG in the liver leads to fatty changes that decrease hepatic TG secretion.[39]

In the present study, LPD showed the presence of hepatomegaly, which, along with fatty liver, constitutes an essential clinical feature of kwashiorkor. Likewise, children with marasmus also present with hepatic steatosis and hepatomegaly, demonstrating clinical relevance.[40] The fatty liver is more intense in kwashiorkor than in marasmus. In marasmus, the liver increases the synthesis of plasma lipoproteins in response to an excess of fatty acids, whereas in kwashiorkor, the liver, which cannot dispose of fatty acids, accumulates lipids in the liver.[10]

In the present study, LPD showed the presence of hepatomegaly, which, along with fatty liver, constitutes an essential clinical feature of kwashiorkor. Likewise, children with marasmus also present with hepatic steatosis and hepatomegaly, demonstrating clinical relevance.[40] The fatty liver is more intense in kwashiorkor than in marasmus. In marasmus, the liver increases the synthesis of plasma lipoproteins in response to an excess of fatty acids, whereas in kwashiorkor, the liver, which cannot dispose of fatty acids, accumulates lipids in the liver.[10]

The clinical features of kwashiorkor include hepatomegaly, fatty liver, hair loss, stooped posture, hypoalbuminemia, and low serum phosphate, TP, and TG levels. However, there was no edema in PMN rats, which is a key indicator of kwashiorkor. Interestingly, PMN rats also exhibited marasmic features, such as significant weight loss, low BMI, lack of subcutaneous fat, muscular atrophy, fat deposition in the liver, and a poor weight-for-height ratio. Hence, our model clinically represents the marasmic-kwashiorkor condition.

Study limitations

The study focused on biochemical, hematological, and histological changes but did not thoroughly investigate other molecular interactions involved in malnutrition.

CONCLUSION

The PMN rat model using a 10% protein diet mimicked the clinical manifestations of the marasmic-kwashiorkor condition. This model is inexpensive to develop, easy to maintain, repeatable, predictable, ethical, and clinically relevant. The model can be used in research in the fields of drug kinetics, disease model development, drug interaction studies, and drug discovery. Being a long-term model, the LPD-induced malnutrition rat model will also be appropriate for studying maternal and intragenerational malnutrition. The PMN model can be appropriately and adaptively modeled to suit different experimental situations to evaluate multiple clinical and biological attributes.

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Ethics

Ethics Committee Approval: All animal experiments were conducted according to the guidelines of the committee for the Purpose of Control and Supervision of Experiments on Animals (approval number: NGSMIPS/Dec-2020/2022, date: 29.12.2020).

Informed Consent: Informed consent is not required.

Authorship Contributions

Surgical and Medical Practices: V.A., Concept: V.A., M.B., Design: V.A., M.B., Data Collection or Processing: V.A., M.R.J., V.D., Analysis or Interpretation: V.A., Literature Search: V.A., M.R.J., V.D., Writing: V.A., M.R.J., V.D., B.M.K., A.V.S.

Conflict of Interest: The authors have no conflicts of interest to declare.

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