Early femoral ossification in offspring as an indicator of linear growth following maternal protein restriction: effects of protein repletion and spirulina supplementation

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Abstract. Maternal protein deficiency can impair early postnatal growth, including skeletal development in offspring. This study evaluated the effects of pre-pregnancy maternal protein restriction and postnatal nutritional recovery, with or without spirulina, on postnatal bone growth in rat offspring. The experimental animal study was conducted at IPB University (2025). Pregnant rats (n=25) were assigned to five groups: normal, protein-restricted (pre-pregnancy), protein recovery, spirulina (400 mg/kg BW/day), and protein recovery + spirulina (until postnatal day 7). Femoral ossification was assessed at D-1 and D-8 using Alizarin Red S staining. Ossification length was quantified using ImageJ and analyzed using ANOVA. At D-1, femoral ossification length did not differ significantly among groups ($p>0.05$). By D-8, significant differences were observed ($p<0.001$). Offspring from protein-restricted dams exhibited the shortest femoral ossification length. Protein recovery during lactation significantly improved postnatal femoral growth, while spirulina supplementation alone resulted in partial improvement. The combination of protein recovery and spirulina produced femoral ossification lengths comparable to the normal protein group. These findings indicate that nutritional recovery, particularly when combined with spirulina supplementation, may help mitigate the adverse effects of maternal protein restriction on early skeletal development.

1 Introduction

Maternal undernutrition during pregnancy is a major determinant of disrupted early growth trajectories, particularly under chronic protein insufficiency. Limited substrate availability during gestation constrains fetal tissue accretion and alters developmental programming of longitudinal skeletal growth. Linear growth failure, often reflected in height-for-age indices, represents the cumulative biological expression of these early constraints rather than a mere

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anthropometric deficit [1]. Such impairment originates at the level of skeletal maturation, especially through disturbances in endochondral ossification that drive bone elongation. However, mechanistic evidence directly linking maternal protein restriction to early skeletal developmental markers remains limited.

Spirulina, a nutrient-dense cyanobacterium rich in high-quality protein and micronutrients, has been proposed as a supportive strategy in malnutrition management. In Zambian children, daily supplementation significantly improved height-for-age z-scores without comparable effects on weight-related indices, suggesting a preferential influence on linear growth pathways [2]. A meta-analysis of randomized controlled trials further demonstrated modest effects on body weight and waist circumference in adults, though outside the context of maternal protein deprivation and without assessment of skeletal endpoints [3]. Experimental studies in growing rats also indicate that spirulina enhances bone modeling through increased osteocalcin and mineral concentrations, supporting its potential role in skeletal development [4].

Previous studies have primarily evaluated spirulina as a standalone intervention, with limited attention to its role under conditions of maternal protein deficiency. Moreover, few investigations have examined whether its effects may be potentiated when combined with dietary protein restoration. The present preclinical study evaluated the effects of pre-pregnancy maternal protein restriction and postnatal nutritional recovery, with or without spirulina, on postnatal bone growth in rat offspring.

2 Methods

2.1 Experimental model and dietary induction of maternal protein restriction

Female Sprague-Dawley rats were used to establish a controlled model of chronic mild protein insufficiency. The study was conducted at IPB University in 2025. The experimental protocol was approved by the Animal Ethics Committee (No. 300-2025 IPB). A total of 25 pregnant dams were randomly distributed into five experimental groups. Rats were initially assigned to either a normal-protein (17% protein) or an isocaloric low-protein diet (8% protein) at six weeks of age. The low-protein diet was administered for five weeks prior to mating to induce preconception protein restriction and was maintained throughout gestation and early lactation (postnatal day 8). Diets were modified from AIN-93G formulation [5].

After confirmation of pregnancy, protein-restricted dams were allocated into three intervention arms: continued protein-deficient diet (PD), dietary protein repletion during gestation and lactation (PR; switched from 8% to 17% protein), and protein repletion combined with spirulina supplementation (PRSP). An additional group received spirulina supplementation under sustained protein restriction (SP), while the normal control group (N) remained on the 17% protein diet throughout. Dried SP biomass was obtained from a local producer in Klaten, Central Java, Indonesia. The material was stored in airtight containers at room temperature and administered daily at 400 mg/kg body weight by oral gavage.

2.2 Offspring skeletal assessment and ossification analysis

From each litter, pups were randomly standardized to four per dam on postnatal day 1 and maintained with their respective dams until postnatal day 8 for short-term lactation assessment and standardized postnatal comparisons. The remaining pups were euthanized at birth for immediate skeletal evaluation, ensuring consistent sampling across dams and time points.

Early bone mineralization was assessed using a modified Alizarin Red whole-mount skeletal staining protocol adapted from established rodent methods [6]. After fixation and soft-tissue clearing, specimens were transferred through graded glycerol solutions for transparent imaging and morphometric analysis [7]. The length of the calcified femoral segment, representing true ossified bone, was quantified using digital morphometric analysis with ImageJ software. Measurements were performed at birth (D-1) and at postnatal day 8 (D-8) to capture early dynamic changes in skeletal maturation and longitudinal bone development. Data were expressed as mean±SD and analyzed using one-way ANOVA followed by Tukey's HSD post hoc test, with statistical significance set at $p<0.05$. Analyses were performed using SPSS for Windows.

3 Results and discussion

At postnatal day 1 (D-1), no statistically significant differences in calcified femoral length were observed among experimental groups ($p=0.098$). Ossified femoral length was broadly comparable across groups, indicating that mineralized femoral development at birth remained relatively similar despite differences in maternal dietary treatment (Table 1).

Table 1. Calcified femoral length in mm (Mean±SD) at postnatal day 1 and day 8.

Measurement	N	PD	PR	SP	PRSP	P-value
Day-1	3.36 ± 0.41^{a}	2.75 ± 0.36^{a}	3.34 ± 0.58^{a}	3.36 ± 0.43^{a}	3.44 ± 0.20^{a}	0.098
Day-8	8.11 ± 0.41^{c}	5.42 ± 0.41^{a}	7.83 ± 0.84^{c}	6.50 ± 0.58^{b}	8.26 ± 0.40^{c}	<0.001
ΔOssification	4.76 ± 0.39^{a}	2.67 ± 0.71^{b}	4.50 ± 0.78^{a}	3.14 ± 0.78^{b}	4.82 ± 0.33^{a}	<0.001

Note: N: normal control; PD: protein-deficient; PR: protein repletion; SP: spirulina supplementation; PRSP: protein repletion plus spirulina. Values are presented as mean±SD. Different superscript letters within the same row indicate significant differences among groups (Tukey's HSD test, $p<0.05$).

By postnatal day 8 (D-8), femoral ossification differed significantly among groups ($p < 0.001$), with the lowest values observed in the PD group and the greatest in the N, PR, and PRSP groups. The SP group showed partial improvement but remained below the protein-repleted groups. Delta ossification was also comparable in the N, PR, and PRSP groups, whereas the SP group showed only partial recovery with some overlap with PD (Table 1).

Insufficient protein intake during late gestation and lactation has been shown to impair bone growth and reduce mineral deposition in offspring, underscoring the essential role of adequate protein supply in healthy skeletal development. Another study on experimental animal model further demonstrates that offspring born to protein-deficient dams exhibit reduced body weight and delayed bone growth compared with controls. These alterations have been associated with lower plasma protein and albumin concentrations, decreased thyroid hormones (FT3 and FT4), reduced bone DNA, calcium, and phosphorus content, as well as disrupted bone remodeling activity-characterized by decreased alkaline phosphatase and increased acid phosphatase, suggesting a shift toward greater bone resorption [8].

Longitudinal growth of long bones, including the femur, occurs through endochondral ossification at the epiphyseal growth plate and directly reflects early linear growth. This metabolically demanding process depends on sufficient amino acid, mineral, and vitamin availability together with coordinated hormonal regulation. Previous studies suggest that protein deficiency may impair growth plate function and suppress long bone elongation even when energy intake is adequate. Protein deficiency has also been associated with impairment of the GH-IGF-1 axis, characterized by reduced IGF-1 concentrations that may improve following restoration of protein adequacy. In addition to their structural role, essential amino acids may act as signaling mediators in growth regulation; arginine and lysine, for example, have been reported to stimulate GH and IGF-1 activity [9].

Preconception protein deficiency resulted in sustained maternal metabolic disruption, reflected in altered gestational adaptation and impaired early postnatal skeletal growth in offspring. The significant reduction in femoral ossification observed under continued protein restriction underscores the sensitivity of early longitudinal bone mineralization to maternal protein adequacy. Restoration of dietary protein effectively normalized femoral length by postnatal day 8, confirming protein availability as the primary determinant of early skeletal growth recovery (Fig. 1). These findings support the importance of improving maternal protein status before and during pregnancy, particularly in populations where undernutrition contributes to early-life growth faltering and stunting risk.

Spirulina supplementation alone produced only partial improvement under sustained restriction, indicating that it could not fully compensate for inadequate total protein intake. However, when administered alongside protein repletion, spirulina was associated with femoral ossification comparable to the normal control group. This pattern suggests that spirulina may enhance metabolic recovery during nutritional restoration rather than functioning as a primary protein substitute, although the underlying mechanisms were not directly measured in the present study and are inferred from prior literature. Collectively, these findings emphasize that adequate maternal protein intake remains fundamental for skeletal growth programming, while adjunct supplementation may optimize adaptive responses during recovery from prior deficiency. This study has several limitations, including the short postnatal observation period, modest sample size, and reliance on an animal model, which may limit long-term translational interpretation.

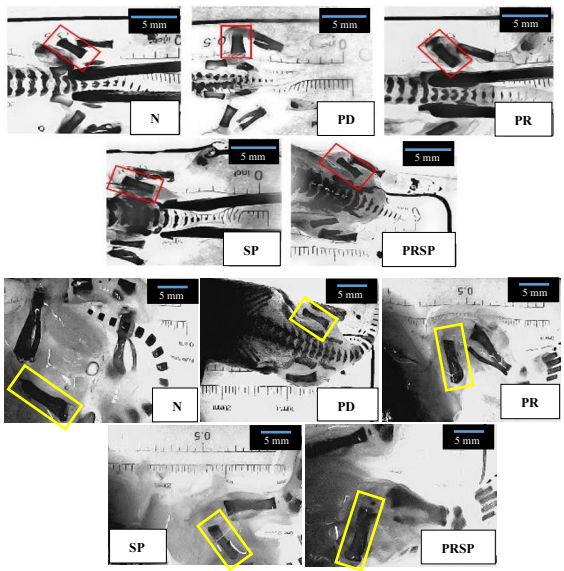

Fig. 1. Representative images of calcified femora in offspring across experimental groups (N, PD, PR, SP, and PRSP) at postnatal day 1 (red, upper panel) and postnatal day 8 (yellow, lower panel).

4 Conclusion

Sustained maternal protein restriction from the preconception period through lactation resulted in significantly reduced postnatal femoral ossification, with differences becoming evident by postnatal day 8. Restoration of dietary protein effectively reversed this impairment, confirming protein adequacy as the primary determinant of early longitudinal bone mineralization. Spirulina supplementation alone produced partial improvement but did

not fully normalize femoral length under continued protein restriction. In contrast, the combination of protein repletion and spirulina supplementation yielded ossification outcomes comparable to the normal control group. These findings suggest that in settings with prevalent maternal undernutrition, restoring baseline protein intake and supplementing with spirulina may help reduce early-life linear growth faltering and stunting risk. However, translation to humans should be cautious given species differences in skeletal development and nutritional responses. Future studies should assess whether these early skeletal benefits persist into adulthood and are matched by improvements in bone microarchitecture and strength.

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References

1. S.E. McCormack, D.L. Cousminer, A. Chesi, et al., Association between linear growth and bone accrual in a diverse cohort of children and adolescents. JAMA Pediatr. **171**, e172471 (2017). https://doi.org/10.1001/jamapediatrics.2017.2471
2. K. Masuda, Y. Inoue, R. Inoue, A. Nakamura, M. Chitundu, J. Murakami, Y. Ota, Spirulina effectiveness study on child malnutrition in Zambia, (Institute of Development Studies, 2014)
3. M. Zarezadeh, A.H. Faghfouri, N. Radkhah, E. Foroumandi, M. Khorshidi, A. Rasouli, M. Zarei, N. Mohammadzadeh Honarvar, N. Hazhir Karzar, M. Ebrahimi Mamaghani, Spirulina supplementation and anthropometric indices: a systematic review and meta-analysis of controlled clinical trials. Phytother. Res. **35**, 577-586 (2021). https://doi.org/10.1002/ptr.6859
4. J.A. Cho, S.Y. Baek, S.H. Cheong, M.R. Kim, Spirulina enhances bone modeling in growing male rats by regulating growth-related hormones. Nutrients **12**, 1186 (2020). https://doi.org/10.3390/nu12041186
5. P.G. Reeves, F.H. Nielsen, G.C. Fahey Jr., AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J. Nutr. **123**, 1939-1951 (1993). https://doi.org/10.1093/jn/123.11.1939
6. D. Rigueur, K.M. Lyons, Whole-mount skeletal staining. In: Skeletal Development and Repair: Methods and Protocols, Methods Mol. Biol. **1130**, 113-121 (2014). https://doi.org/10.1007/978-1-62703-989-5_9
7. E. Alfiah, R. Damanik, K. Roosita, M. Fahrudin, Developmental toxicity of torbangun leaves extract: Effects on mouse fetal death and late ossification. Acta Vet. Indones. **8**, 8−14 (2020). https://doi.org/10.29244/avi.8.1.8-14
8. H. Fetoui, A. Mahjoubi-Samet, F. Guermazi, N. Zeghal, Maternal low-protein diet affects bone mass and mineral metabolism in suckling rats. J. Anim. Physiol. Anim. Nutr. **92**, 448-455 (2008). https://doi.org/10.1111/j.1439-0396.2007.00732.x
9. S. Hasan, S. Naseer, M. Zamzam, H. Mohilldean, C. Van Wagoner, A. Hasan, E.S. Saleh, V. Uhley, S. Kamel-Elsayed, Nutrient and hormonal effects on long bone

growth in healthy and obese children: A literature review. Children. **11**, 1-16 (2024). https://doi.org/10.3390/children11091125