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Rasbora borneensis fish and *Vigna unguiculata* legume supplementation restores reproductive hormone profiles in early-life protein-deficient female rats

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ABSTRACT

Background: Protein malnutrition during critical developmental periods disrupts hypothalamic-pituitary-ovarian axis function, impairing reproductive hormone synthesis. Evidence remains limited regarding whether locally available protein sources can reverse these endocrine disruptions.

Objective: This study evaluated the effects of seluang fish (*Rasbora borneensis*) and nagara cowpea (*Vigna unguiculata* spp. *cylindrica*) supplementation on reproductive hormone recovery in female rats subjected to early-life protein deficiency.

Methods: Thirty female Wistar rat offspring were exposed to protein deficiency via maternal low-protein diet (6% casein) during lactation, followed by post-weaning continuation for four weeks. Animals were randomized into five groups (n=6): normal control, negative control, seluang fish, nagara cowpea, and combination groups. Interventions were administered for four weeks. Serum protein, follicle-stimulating hormone (FSH), and estradiol were quantified.

Results: All interventions significantly restored serum protein and FSH levels. Estradiol recovery was remarkable, with nagara cowpea producing concentrations exceeding normal control ($p = 0.041$), followed by combination and seluang fish groups, all significantly higher than negative control ($p < 0.001$).

Conclusion: Seluang fish and nagara cowpea effectively reversed protein deficiency and restored reproductive endocrine function, representing sustainable nutritional interventions for early-life malnutrition consequences.

Keywords: estradiol, follicle-stimulating hormone, protein malnutrition, *Rasbora borneensis*, *Vigna unguiculata*

Introduction

Protein-energy malnutrition remains a critical public health challenge globally, with particularly severe consequences during critical developmental windows [1,2]. In Indonesia, particularly in South Kalimantan province, chronic protein insufficiency continues to affect low-income households whose dietary patterns rely predominantly on carbohydrate-based staples with limited access to high-quality protein sources [3,4]. This nutritional inadequacy contributes directly to growth retardation and the persistently high prevalence of stunting

among children under five years of age, affecting approximately 21.6% of this population nationwide [5]. Beyond impaired physical growth, early-life protein deficiency exerts profound and potentially irreversible effects on cognitive development, metabolic programming, and reproductive system maturation [6].

The reproductive endocrine system demonstrates particular vulnerability to nutritional deficits during perinatal and juvenile periods. Protein malnutrition disrupts the hypothalamic-pituitary-ovarian (HPO) axis through multiple mechanisms, including reduced availability of amino acids essential for gonadotropin-releasing hormone (GnRH) synthesis, diminished pituitary secretion of follicle-stimulating hormone (FSH), impaired ovarian aromatase activity, and consequently reduced estrogen production [7,8]. These endocrine disruptions may manifest without overt clinical symptoms yet carry profound implications for reproductive maturation and future fertility [9,10].

FSH and estradiol serve as sensitive biomarkers of HPO axis function and reproductive endocrine integrity, making them valuable indicators for assessing both nutritional impact and recovery potential [11–13]. Despite growing recognition of protein malnutrition's impact on reproductive development, limited research has examined whether culturally relevant, locally available food sources can support hormonal restoration following early-life nutritional deprivation. This knowledge gap is particularly significant in regions where imported or commercially processed protein supplements may be economically inaccessible or culturally inappropriate [14].

South Kalimantan possesses abundant indigenous protein sources that remain underutilized in nutritional intervention strategies, notably Seluang fish (*Rasbora borneensis*), a locally abundant freshwater cyprinid species, and Nagara cowpea (*Vigna unguiculata* spp. *cylindrica*), an indigenous legume variety traditionally cultivated in freshwater swamplands [15,16]. Seluang fish provides complete protein with balanced essential amino acid composition, including significant quantities of lysine, methionine, and leucine critical for protein synthesis and hormonal production [17,18]. Fish-derived proteins additionally supply bioavailable micronutrients including zinc, selenium, and omega-3 fatty acids that support reproductive endocrine function [19,20].

Conversely, Nagara cowpea offers plant-based protein rich in arginine and glutamic acid, dietary fiber, folate, and notably, bioactive phytoestrogens including genistein and daidzein that may exert estrogenic effects through binding to estrogen receptors [21–23]. These phytoestrogens have demonstrated capacity to modulate aromatase activity and support estrogen synthesis in experimental models [24,25]. Furthermore, cowpea varieties contain substantial concentrations of antioxidant compounds including polyphenols and flavonoids that may protect ovarian cells from oxidative damage, potentially supporting steroidogenic capacity [26–29].

Therefore, this study investigated the effects of Seluang fish, Nagara cowpea, and their combination on reproductive hormone profiles in female rat offspring subjected to early-life protein deficiency, aiming to provide experimental evidence for sustainable, locally-sourced nutritional interventions to mitigate reproductive consequences of early-life malnutrition.

Methods

Study design and ethical approval

This experimental study employed a randomized post-test only control group design to evaluate the effects of locally sourced protein supplementation on reproductive hormone recovery following early-life protein deficiency. All experimental procedures were conducted in accordance with international guidelines for laboratory animal care and use, and approved by the Health Research Ethics Committee of the Faculty of Medicine and Health Sciences, Lambung Mangkurat University (No. 038/KEPK-FKIK ULM/EC/VI/2025). Appropriate measures were implemented throughout the study to minimize animal pain and distress.

Animals and housing conditions

Ten time-mated pregnant female Wistar rats (*Rattus norvegicus*, aged 3–4 months, body weight 180–220 g) were obtained from a certified breeding facility. Animals were housed in polycarbonate cages under controlled environmental conditions (temperature 22 ± 2°C, humidity 50–60%, 12-hour light/dark cycle). Standard wood-chip bedding material was changed twice weekly to maintain hygiene. All animals had *ad libitum* access to clean drinking water throughout the study period.

Sample size determination

Sample size was calculated using Federer's formula: $(n - 1)(r - 1) \geq 15$, where *n* represents the number of animals per group and *r* represents the number of treatment groups. This formula has been validated in previous nutritional intervention studies using similar experimental designs [30]. To account for an anticipated 20% mortality rate during the experimental period, six animals were allocated to each experimental group, yielding a total of 30 female offspring for the study.

Experimental timeline and group allocation

Phase 1: Maternal protein deficiency (postnatal days 0–28). Immediately following parturition, lactating dams were transitioned to a modified low-protein AIN-76A diet (Dyets, Bethlehem, PA, USA) containing 6.0% casein as the sole protein source. This level of protein restriction is consistent with severe malnutrition models established in previous developmental nutrition studies and represents a 70% reduction from the standard 20% casein formulation [7,30]. This dietary intervention was maintained for four consecutive weeks to induce early-life protein deficiency in nursing pups through altered maternal milk composition, modeling maternal malnutrition conditions commonly observed in low-resource settings.

Phase 2: Post-weaning protein deficiency (postnatal days 28–56). At four weeks of age (postnatal day 28), female pups were weaned and maintained on the same low-protein AIN-76A diet for an additional four weeks. This post-weaning continuation ensured sustained protein deficiency during the critical developmental period encompassing sexual maturation, thereby establishing a robust model of chronic early-life protein restriction.

Phase 3: Confirmation of protein deficiency status (day 56). Following the eight-week protein restriction protocol, blood samples (approximately 200 µL) were collected from five randomly selected animals via tail vein puncture under brief isoflurane anesthesia (3% induction, 2% maintenance). Serum was separated by centrifugation at 3,000 rpm for 10 minutes at 4°C, and total protein concentration was quantified using the Biuret method [30]. Animals demonstrating serum total protein concentrations below 4.7 g/dL were confirmed as protein-deficient and included in subsequent experimental phases, ensuring uniform baseline nutritional status across all groups.

Phase 4: Dietary intervention (days 56–84). Protein-deficient female offspring (n=30) were randomly assigned to five experimental groups using computer-generated randomization (Random Allocation Software 2.0): (i) normal control: standard commercial rat diet (Comfeed, Jakarta, Indonesia; 1,028 kcal, 49 g protein/100 g), representing optimal nutritional conditions; (ii) negative control: continued low-protein AIN-76A diet (381.93 kcal, 6 g protein/100 g), representing sustained protein deficiency without intervention; (iii) Seluang fish group (T1): standard diet supplemented with 30% *Rasbora borneensis* powder (984.4 kcal, 56.8 g protein/100 g); (iv) Nagara cowpea group (T2): standard diet supplemented with 30% *Vigna unguiculata* spp. *cylindrica* flour (1,048.2 kcal, 54.3 g protein/100 g); (v) combination group (T3): standard diet supplemented with 15% seluang fish and 15% nagara cowpea (902.41 kcal, 54.1 g protein/100 g). All dietary interventions were administered for four consecutive weeks with *ad libitum* access. Body weight was recorded weekly using a precision balance (±0.1 g; Ohaus Scout Pro SP402, Parsippany, NJ, USA), and daily food intake was monitored to ensure consistent nutritional delivery and detect potential palatability issues.

Diet preparation

Seluang fish diet. Fresh *Rasbora borneensis* specimens were sourced from local fishermen in South Kalimantan, Indonesia. Fish were cleaned, eviscerated, and steamed at 100°C for 15 minutes using atmospheric pressure steaming to ensure pathogen elimination while preserving nutritional integrity. Steamed fish were homogenized using a mechanical grinder (National MX-T3GN, Osaka, Japan), spread uniformly on stainless steel trays (thickness ≤5 mm), and oven-dried at 60°C for 8 hours until complete moisture removal (final moisture content <5%). The dried material was pulverized to fine powder (particle size <250 μm) using a laboratory mill (Retsch ZM 200, Haan, Germany) and mixed with standard commercial feed at 30% w/w ratio. The mixture was pelleted using a laboratory pellet mill (California Pellet Mill, Crawfordsville, IN, USA; 3 mm diameter) with addition of 2% water as binder, then air-dried at room temperature for 24 hours. Pellets were stored at −20°C in airtight containers with silica gel desiccant to prevent oxidation and maintain nutritional quality.

Nagara cowpea diet. Mature *Vigna unguiculata* spp. *cylindrica* seeds were obtained from local farmers cultivating freshwater swamplands in South Kalimantan. Seeds were cleaned, sorted to remove damaged specimens, and soaked in distilled water (1:3 w/v ratio) for 12 hours at room temperature to reduce antinutritional factors including phytic acid and trypsin inhibitors. Following soaking, seeds were rinsed thoroughly three times with distilled water, then steamed at 100°C for 45 minutes using atmospheric pressure steaming to achieve complete softening and further inactivate antinutritional compounds. Steamed cowpeas were oven-dried at 60°C for 12 hours to constant weight. Dried cowpeas were ground to fine flour (particle size <250 μm) using a laboratory mill (Retsch ZM 200, Haan, Germany), mixed with standard feed at 30% w/w ratio, pelleted using identical conditions as fish diet, and stored at −20°C in airtight containers.

Combination diet. The combination diet was prepared by mixing seluang fish powder and nagara cowpea flour at equal proportions (15% each) with standard commercial feed (70%), followed by pelleting and storage under identical conditions.

Biochemical analyses

At the end of the intervention period (day 84), animals were fasted overnight (12 hours) to minimize dietary interference. Blood samples (2–3 mL) were collected via cardiac puncture under deep ketamine-xylazine anesthesia (ketamine 90 mg/kg, xylazine 10 mg/kg, intraperitoneally). Animals were euthanized immediately following blood collection by cervical dislocation under continued anesthesia. Blood samples were allowed to clot at room temperature for 30 minutes, then centrifuged at 3,000 rpm for 15 minutes at 4°C. Serum was separated, aliquoted into cryovials, and stored at −80°C until analysis.

Serum total protein. Total protein concentration was quantified using the Biuret colorimetric method [30]. Briefly, 20 μL of serum was mixed with 1 mL of Biuret reagent (containing copper sulfate in alkaline solution; Randox Laboratories, Crumlin, UK) and incubated at room temperature for 10 minutes. The resulting copper-peptide complex was measured spectrophotometrically at 540 nm wavelength using a UV-Vis spectrophotometer (Genesys 10S, Thermo Fisher Scientific, Waltham, MA, USA). Protein concentration was calculated using a calibration curve generated with bovine serum albumin standards (0–10 g/dL).

Follicle-stimulating hormone (FSH). Serum FSH concentrations were determined using a commercially available rat-specific enzyme-linked immunosorbent assay (ELISA) kit (Catalog No. E0369Ra, BT Lab, Shanghai, China) following the manufacturer's protocol. Samples and standards were analyzed in duplicate. The assay sensitivity was 0.05 ng/mL with an inter-assay coefficient of variation (CV) <10% and intra-assay CV <8%. Optical density was measured at 450 nm using a microplate reader (BioTek ELx800, Winooski, VT, USA). FSH concentrations were calculated from a standard curve using four-parameter logistic curve fitting (Gen5 software, BioTek).

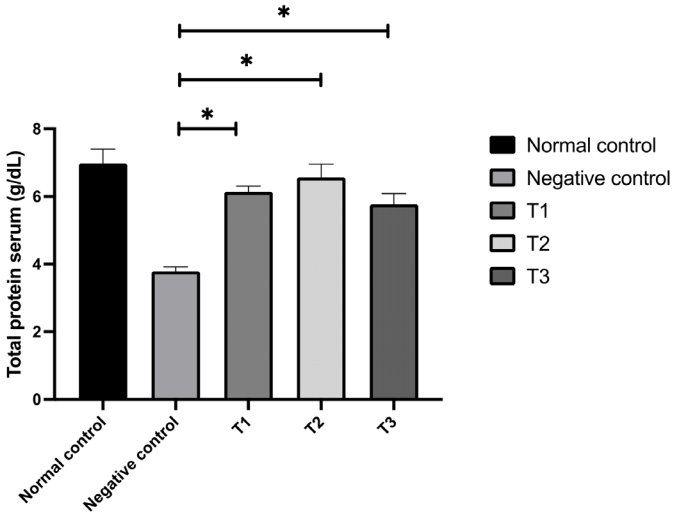

Figure 1. Serum total protein levels across experimental groups. Data presented as mean ± SD (n=6 per group). T1: seluang fish supplementation; T2: nagara cowpea supplementation; T3: combination supplementation.*p<0.001

Estradiol (E2). Serum estradiol levels were quantified using a rat-specific ELISA kit (Catalog No. E0148Ra, BT Lab, Shanghai, China) according to the manufacturer's instructions. Samples and standards were processed in duplicate. The assay sensitivity was 5.0 ng/L with an inter-assay CV <12% and intra-assay CV <9%. Absorbance was measured at 450 nm using the same microplate reader. Estradiol concentrations were determined from a standard curve using polynomial regression analysis. All biochemical assays were performed by trained personnel blinded to group assignments to minimize potential bias.

Data analysis

Data are presented as mean ± standard deviation (SD) for normally distributed variables. Normality was assessed using the Shapiro-Wilk test, and homogeneity of variance was evaluated using Levene's test. Comparisons among the five experimental groups were performed using one-way analysis of variance (ANOVA), followed by Tukey's honestly significant difference (HSD) *post hoc* test for multiple pairwise comparisons when ANOVA revealed significant differences. Statistical significance was set at $p < 0.05$. All statistical analyses were conducted using SPSS version 26.0 software (IBM Corporation, Armonk, NY, USA).

Results

Effects of dietary interventions on serum total protein

Protein deficiency induction successfully reduced serum protein concentrations to levels consistent with systemic malnutrition. Following four weeks of dietary intervention, all treatment groups demonstrated significant improvements in serum protein status compared to the negative control (Figure 1). The normal control exhibited serum protein concentration of 6.98 ± 0.421 g/dL, reflecting optimal nutritional status. In contrast, the negative control showed severely depressed serum protein levels of 3.79 ± 0.127 g/dL, confirming sustained protein deficiency below the normal threshold of 4.7 g/dL.

All groups receiving dietary interventions demonstrated significant restoration of protein levels. The nagara cowpea group achieved the highest recovery at 6.57 ± 0.389 g/dL, followed by the seluang fish group at 6.14 ± 0.166 g/dL, and the combination group at 5.77 ± 0.320 g/dL. One-way ANOVA revealed significant differences among groups ($p < 0.001$). Post hoc analysis showed that all three intervention groups differed significantly from the negative control ($p < 0.001$), while the nagara cowpea and seluang fish groups achieved protein levels comparable to the normal control ($p > 0.05$).

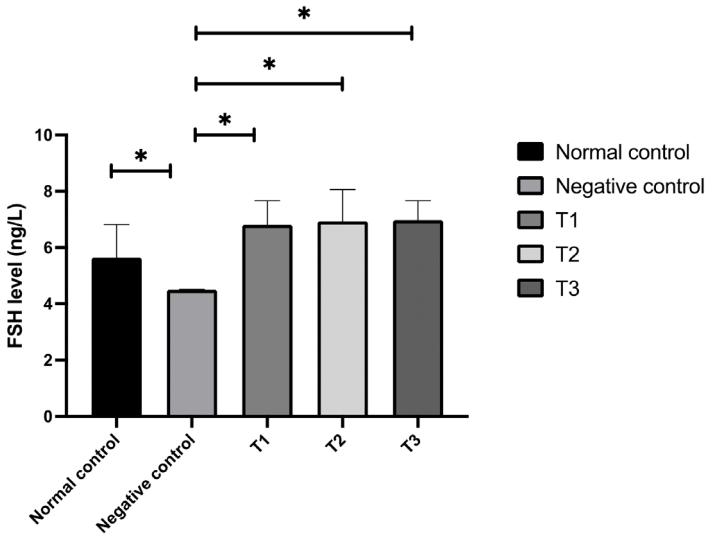

Figure 2. Serum follicle-stimulating hormone (FSH) levels across experimental groups. Bar graph represents mean ± standard deviation (n=6 per group). T1: seluang fish supplementation; T2: nagara cowpea supplementation; T3: combination supplementation.*p<0.001

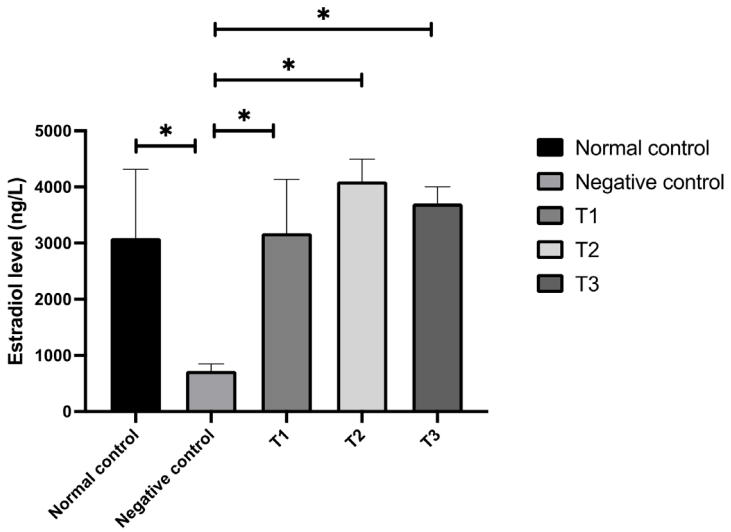

Figure 3. Serum estradiol (E2) levels across experimental groups. Bar graph represents mean ± standard deviation (n=6 per group). T1: seluang fish supplementation; T2: nagara cowpea supplementation; T3: combination supplementation.*p<0.001

Effects of dietary interventions on follicle-stimulating hormone concentrations

Serum FSH concentrations demonstrated significant suppression in protein-deficient animals and significant recovery following dietary interventions (Figure 2). The negative control exhibited significantly reduced FSH levels of 4.50 ± 0.022 ng/L, reflecting impaired pituitary gonadotropin secretion secondary to chronic protein restriction. The normal control maintained FSH concentration of 5.64 ± 1.177 ng/L, representing physiological baseline levels.

All dietary intervention groups demonstrated significant elevation in FSH concentrations. The combination group attained the highest FSH levels at 6.97 ± 0.0695 ng/L, followed by the nagara cowpea group at 6.93 ± 1.130 ng/L, and the seluang fish group at 6.81 ± 0.856 ng/L. Statistical analysis revealed significant differences among groups ($p = 0.012$), with all intervention groups differing significantly from the negative control ($p < 0.01$). Notably, all three intervention groups achieved FSH concentrations comparable to or exceeding the normal control ($p > 0.05$).

Effects of dietary interventions on estradiol concentrations

Estradiol concentrations exhibited profound suppression in protein-deficient animals and dramatic recovery following dietary interventions (Figure 3). The negative control demonstrated significantly reduced estradiol levels of 724 ± 124.68 ng/L, representing a 77% reduction compared to normal controls and indicating substantial impairment of ovarian steroidogenic function. The normal control maintained estradiol concentration of 3,091.83 ± 1,221.96 ng/L, reflecting physiological estrogen production capacity.

All dietary intervention groups produced significant restoration of estradiol concentrations ($p < 0.001$). Most Notably, the nagara cowpea group achieved the highest estradiol concentration of 4,103.50 ± 393.09 ng/L, significantly exceeding even the normal control ($p = 0.041$). This superior estrogenic response suggests potential contributions from bioactive phytoestrogens present in cowpea. The combination group attained estradiol levels of 3,704.33 ± 296.64 ng/L, while the seluang fish group reached 3,179.33 ± 957.96 ng/L. All three intervention groups differed significantly from the negative control ($p < 0.001$) and achieved concentrations comparable to or exceeding the normal control.

Discussion

This study demonstrates that locally sourced seluang fish and nagara cowpea effectively reverse systemic protein deficiency and restore reproductive endocrine function in female rats subjected to early-life protein malnutrition. All dietary interventions produced significant improvements in serum protein status, FSH concentrations, and estradiol levels, indicating recovery of both general nutritional status and hypothalamic-pituitary-ovarian axis function. These findings provide experimental evidence supporting the therapeutic potential of indigenous, culturally relevant food sources in mitigating reproductive consequences of early-life nutritional deprivation.

The successful restoration of serum protein concentrations across all intervention groups confirms the bioavailability and metabolic utilization of proteins from both sources. The negative control's severely depressed serum protein levels (3.79 g/dL) are consistent with clinical protein-energy malnutrition [30]. The superior performance of nagara cowpea (6.57 g/dL) may reflect its high digestibility and favorable amino acid profile, particularly enriched in arginine and glutamic acid, which support hepatic protein synthesis [21,22]. Fish protein demonstrated comparable efficacy (6.14 g/dL), consistent with the established high biological value of animal-derived proteins containing complete essential amino acid profiles [18,19].

The significant elevation in FSH concentrations across all intervention groups indicates successful restoration of pituitary gonadotropin secretion. Protein malnutrition impairs FSH synthesis through reduced amino acid availability for GnRH and gonadotropin production, diminished leptin signaling, and dysregulation of hypothalamic Kiss1/kisspeptin neurons that govern GnRH pulsatility [31,32]. High-quality protein provision likely restored amino acid pools necessary for neuropeptide synthesis, normalized leptin concentrations, and supported metabolic recovery of neuroendocrine regulatory centers [7]. That all intervention groups achieved FSH levels comparable to or exceeding normal control suggests complete functional recovery of the hypothalamic-pituitary component, which is particularly significant given that early-life protein restriction can program persistent alterations in neuroendocrine function [10,33].

The profound suppression of estradiol in protein-deficient animals (77% reduction) and its remarkable recovery represent the study's most striking findings. Protein malnutrition impairs ovarian steroidogenesis through reduced cholesterol availability, decreased aromatase enzyme expression and activity, diminished FSH-mediated stimulation of granulosa cells, and oxidative stress-induced follicular damage [34–36]. Nutritional rehabilitation likely addressed these deficits by restoring substrate availability, supporting enzyme biosynthesis, and alleviating oxidative stress [37,38].

The superior estrogenic response in the nagara cowpea group, with estradiol levels significantly exceeding even normal control (4,103.50 vs. 3,091.83 ng/L, p = 0.041), warrants particular attention. This phenomenon cannot be attributed solely to protein provision and likely reflects bioactive phytoestrogens present in cowpea. Legumes contain substantial concentrations of isoflavones including genistein, daidzein, and glycitein that exert weak estrogenic activity through binding to estrogen receptors, with preferential affinity for ERβ [24,25,39,40]. Phytoestrogens can also modulate endogenous estrogen synthesis by upregulating aromatase expression in granulosa cells [41,42]. The combination of direct estrogenic effects and enhanced endogenous production may explain the supraphysiological estradiol concentrations observed, aligning with previous research demonstrating that legume phytoestrogens can elevate circulating estrogen levels [25,43].

These findings carry significant implications for addressing protein malnutrition in resource-limited settings. Both seluang fish and nagara cowpea represent locally available, culturally familiar, and economically accessible protein sources demonstrating therapeutic efficacy comparable to or exceeding standard commercial diets. Their successful application supports integration into community-based nutritional rehabilitation programs targeting vulnerable populations, particularly during critical developmental periods when nutritional interventions yield maximal benefit [1,14]. Indigenous foods offer advantages including reduced costs, enhanced cultural acceptability promoting adherence, support for local economies, and environmental sustainability. The demonstrated efficacy of either protein source alone provides intervention flexibility based on regional availability, seasonal variation, and dietary preferences.

Several limitations warrant acknowledgment. First, this animal model may not fully replicate human protein-energy malnutrition's complexity, including concurrent micronutrient deficiencies, infectious disease burden, and psychosocial stressors. Second, the four-week intervention, though sufficient to demonstrate hormonal recovery, does not address long-term outcomes including fertility, pregnancy outcomes, or transgenerational effects. Third, we did not quantify specific bioactive compounds such as phytoestrogen content in cowpea or omega-3 fatty acids in fish, limiting mechanistic interpretation. Fourth, absence of histological examination prevents assessment of follicular development and steroidogenic cell morphology that would provide deeper mechanistic insights.

Future research should address these gaps through complementary approaches. Phytochemical analysis quantifying isoflavone profiles and omega-3 content would elucidate bioactive compound contributions. Histological examination assessing follicular populations, granulosa cell morphology, and aromatase immunoexpression would provide mechanistic insights into estradiol recovery. Fertility studies evaluating estrous cyclicity, ovulation rates, and pregnancy outcomes would determine functional reproductive capacity beyond hormonal biomarkers. Long-term follow-up investigating whether early nutritional rehabilitation prevents premature reproductive senescence would inform optimal intervention timing [10,33]. Additionally, randomized controlled trials in malnourished adolescent girls or pregnant women would provide critical evidence for clinical application, incorporating anthropometric parameters, menstrual cycle regularity, and reproductive hormones while assessing acceptability and adherence in real-world settings.

Conclusion

This study demonstrates that seluang fish (*Rasbora borneensis*) and nagara cowpea (*Vigna unguiculata* spp. *cylindrica*) effectively reverse systemic protein deficiency and restore reproductive endocrine function in female rats subjected to early-life protein malnutrition. Both protein sources significantly improved serum protein status, FSH, and estradiol levels, with nagara cowpea producing superior estrogenic response likely due to phytoestrogens. These findings support indigenous protein sources as sustainable, accessible interventions to mitigate reproductive

consequences of early-life malnutrition, warranting clinical validation in human populations.

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Author contributions

Conceptualization, DDS, and T; methodology DDS, T, SL, J, NZ, DIA; investigation and data curation, DIA MZ, MAZ; formal analysis, T, and DIA; writing – original draft, DDS, DIA; writing – review & editing, DDS, T, SL, J, NZ, DIA, MZ, MAZ; supervision, DDS, and T; project administration, DDS, and T; funding acquisition, DDS All authors have read and approved to the published version of the manuscript

Declaration of interest

The authors declare that none of them has any conflict of interest with any private, public or academic party related to the information contained in this manuscript

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