Effect of eel and tempe composite flour supplementation on the nutritional status biomarkers of rats with a restricted protein diet: Data from a preclinical trial [version 1; peer review: awaiting pee

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Submission date: 18-May-2023 06:59PM (UTC+0700)
Submission ID: 2096188174
File name: 5-F1000Research_2022.pdf (734.57K)
Word count: 3462
Character count: 18464

DATA NOTE

Effect of eel and tempe composite flour supplementation on the nutritional status biomarkers of rats with a restricted protein diet: Data from a preclinical trial [version 1; peer review: awaiting peer review]

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v1 **First published:** 12 Sep 2022, **11**:1031
https://doi.org/10.12688/f1000research.122646.1
Latest published: 27 Apr 2023, **11**:1031
https://doi.org/10.12688/f1000research.122646.2

Abstract

Incorporating eels and tempe can replace and complement the content of proteins, macronutrients, and micronutrients, which may be related to curative effects for malnutrition. In addition, converting the ingredients into a form of flour can increase their shelf life and nutrient concentration. Therefore, an *in vivo* approach was undertaken to explore further the nutritional status value of biomarkers in malnourished male rats (*Rattus norvegicus*) after Eel and Tempe Composite (ETC) flour supplementation. Data was collected from blood samples (both plasma and serum) of rats in all groups, and the appropriate biomarkers were analyzed. The final data presented in this study is openly available and can be further analyzed using statistical means to determine the dose of ETC flour as the basis of clinical trials, which other researchers can reproduce. This data may also be valuable for those interested in using different analytical methods to research the same questions or even new preclinical

Open Peer Review

Approval Status *AWAITING PEER REVIEW*

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studies focusing solely on nutritional status biomarker analysis methods, including clinical trial prospects.

Keywords
Nutraceuticals, Nutritional Status, Protein Diet, Preclinical Trial, Eel, Tempe, IGF-1

This article is included in the Agriculture, Food and Nutrition gateway.

This article is included in the Data: Use and Reuse collection.

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Author roles: Mentang F: Formal Analysis, Validation, Writing – Original Draft Preparation, Writing – Review & Editing; **Taher N**: Formal Analysis, Validation, Writing – Original Draft Preparation, Writing – Review & Editing; **Nurkolis F**: Conceptualization, Funding Acquisition, Project Administration, Writing – Original Draft Preparation, Writing – Review & Editing; **Gunawan WB**: Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; **Yusuf VM**: Validation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; **Sabrina N**: Supervision, Validation, Writing – Review & Editing; **Qhabibi FR**: Formal Analysis, Visualization, Writing – Review & Editing; **Mayulu N**: Supervision, Validation, Writing – Review & Editing; **Taslim NA**: Supervision, Validation, Writing – Review & Editing; **Permatasari HK**: Supervision, Validation, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

Grant information: This work has been supported by an award grant from The International Institute of Knowledge Management (IIKM) Sri Lanka with the funding number ICOPH 2020 A 410.
The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

How to cite this article: Mentang F, Taher N, Nurkolis F *et al.* **Effect of eel and tempe composite flour supplementation on the nutritional status biomarkers of rats with a restricted protein diet: Data from a preclinical trial [version 1; peer review: awaiting peer review]** F1000Research 2022, **11**:1031 https://doi.org/10.12688/f1000research.122646.1

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Abbreviations

BW: Body Weight
CIOMS: Council for International Organizations of Medical Sciences
ETC: Eel and Tempe Composite
IGF-1: Insulin-Like Growth Factor 1
NP: Normal-Protein
RBP: Retinol-Binding Protein
RP: Restricted-Protein

Introduction

Prolonged protein undernutrition can result in malnutrition, anemia, and stunting (Wu, 2016). Malnutrition can manifest through many clinical parameters and biomarkers. The most-reported blood biomarkers of malnutrition are total protein and hemoglobin (Zhang *et al.*, 2017), while serum insulin-like growth factor 1 (IGF-1) have been underreported (Keller, 2019). There is strong evidence that functional meals can help prevent and mitigate the effects of malnutrition (Ahmed *et al.*, 2022), presumably by improving malnutrition biomarkers.

Agricultural products such as soybean (*Glycine max* L.) tempe is the potential source of protein and other nutrients which may improve malnutrition, anemia, and gut health due to their folate, iron, vitamin B12, probiotics, and isoflavone content (Ahnan-Winarno *et al.*, 2021). In addition to tempe, one of the fishery products that can be used as an alternative protein-rich functional food is eel fish (*Monopterus albus*) which has immense nutritional contents that is high in vitamin A, vitamin E, and unsaturated fatty acids (eicosapentaenoic acid and docosahexaenoic acid) (Nafsiyah *et al.*, 2018). A recent review also highlighted the role of unsaturated fatty acids on protein metabolism, synthesis, and muscle growth (López-Seoane *et al.*, 2022), which are crucial in a state of malnutrition.

Combining eel and tempe may substitute and complement the protein, macronutrients, and micronutrient content, which may be linked with their malnutrition ameliorating effects. Moreover, turning an ingredient into the form of flour may increase its shelf life and nutrient concentration (Tomasi *et al.*, 2015). Therefore, an *in vivo* approach was performed to further explore the value of nutritional status biomarkers on malnourished rats after a supplementation of Eel and Tempe Composite (ETC) flour. A review study by Ahmed *et al.* (2022) stated that only a few of the studies reported a strong link between functional foods and malnutrition.

In this study, the data was collected from the blood samples (both plasma and serum) of rats in all treatment groups, and the appropriate biomarkers were analyzed, accordingly. The final data presented in this study can be further analyzed using statistical means to define an ETC flour dose as a basis for the clinical trial, which other researchers could reproduce. This data may also be valuable for those interested in using other analytical methods to research the same research questions or even new preclinical studies focusing solely on biomarker nutrition status analysis methods, including clinical trial prospects.

Methods

Ethics statement

The protocol for this preclinical study refers to the Animal Research: Reporting in vivo Experiments (ARRIVE) Guidelines by the Institutional Animal Care and Use Committee. See the *Reporting guidelines* section (Nurkolis and Gunawan, 2022a) for the completed checklist. The study also adheres to the Declaration of Helsinki by The Council for International Organizations of Medical Sciences (CIOMS). This study protocol has been registered at Preclinical Trials Europe preclinicaltrials.eu (PCTE0000271). Ethical approval was obtained from the Ethics Committee at Educational General Hospital, Sam Ratulangi University (approval number 039/EC/KEPK-KANDOU/III/2022). Every effort was taken to keep the experimental animals as pain-free as possible throughout the trial and by maintaining laboratory conditions at room temperature and noise-free.

Production of ETC flour

A total of 1,000 g of fresh Asian swamp eel (*Monopterus albus*) were purchased live from a local market in Jakarta. The live eels were placed in a bucket measuring 21 cm in diameter with a volume capacity of 5 L (pH 5.5) and acclimatized for 24 hours in a normal light-dark cycle before being euthanized. The Asian swamp eel were euthanised using a slaughter process such as fish processing and cleaned (including removal of the bone) and steamed for 10 minutes. After drying for 12 hours at 60 °C, the meat was grounded using a meat mincer. Then, 1,000 g of soy-based tempe was chopped and steamed for 20 minutes. Before being ground, the tempe sample was steamed and then baked for 12 hours at 60 °C. Both eel and tempe were filtered using a 60-mesh filter. According to the specifications of a previously published preliminary study (Ngadiarti *et al.*, 2022), a formulation with the highest level of folic acid (vitamin B9; 1258.53 ± 1.39 ug/100 g) and

unsaturated fatty acids (20.83 ± 0.12%) was prepared by combining eel and tempe flour samples in 1: 3.5 ratio. A complete formulation of ETC flour and the results of proximate amino acids, unsaturated fatty acids, and folate contents were presented by Ngadiarti *et al.* (2022). The ETC formulations were conducted by Ngadiarti *et al.* (2022) at the Laboratory of the Ministry of Health Polytechnic Jakarta II (Poltekkes Kemenkes Jakarta II), Jakarta 12540, Indonesia (Ngadiarti *et al.*, 2022).

Source of animals

Thirty male Albino Wistar rats (*Rattus norvegicus*) weighing 40.5 – 5.4 g, aged 3 – 4 weeks, were put in cages under typical laboratory settings (27 ± 2.2 °C) with normal light and dark cycles (12/12 hours). Before the experiment, all rats were acclimatized for a 10-day period. The minimum sample size was determined using the Frederer formula, with the equation = (t-1) (r-1)≥15. t = treatment, r = replication. t in this study is 3, then = (3-1) (r-1) ≥ 15; 2(r-1) ≥ 15; 2r-2 ≥ 15; r ≥ 8.5. So the minimum sample for each group is 8.5. The research team chose 10 (just in case there were rats that needed to be excluded, such as those experiencing moody eating and drinking) (Federer, 1966).

Experimental design

According to de Oliveira *et al.* (2011), all rats were supplemented with restricted-protein diet treatment (RP; 4% w/w protein) for four weeks and separated into three groups, with 10 rats per group. Shuffled blinded envelopes simple randomization (Taken at random from the population). We consider no confounders in this study since they were controlled by choosing the gender of the rats (male, not mixed) and the trials in the laboratory were carried out by a professional veterinary in animal studies. The allocation process and experiment were conducted by NM with the assistance of a professional veterinary. The assessment of the outcomes and data analysis were done by FN and FM. Rats that experienced moody eating and drinking and exhibited stress were excluded. Group C (control) was given an *ad libitum* diet of normal protein (NP; 23% w/w protein), but did not supplement with ETC. Rats in groups A and B got the same RP diet for 4 weeks but were orally given ETC dosages of 100 and 200 mg/kgBW. These dosages were based on the lower and higher limits for stomach safety in rats (Eichenbaum *et al.*, 2011). Blood samples were taken and analyzed for their protein, hemoglobin, and IGF-1 serum levels. The flowchart of this study is presented in detail in Figure 1.

Primary outcomes for this preclinical trial are defined as:

1. Protein (mg/dL)
2. Hemoglobin (mg/dL)
3. IGF-1 (pg/mL)

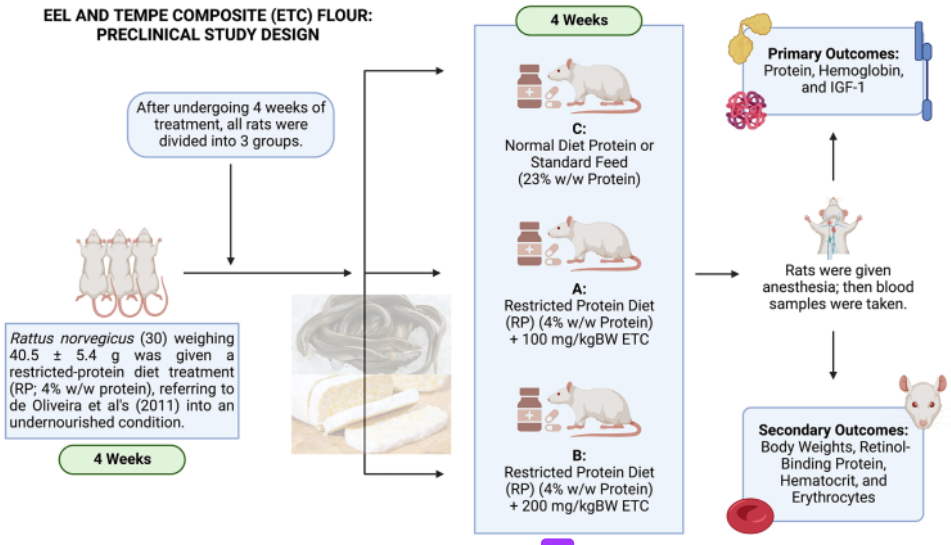

Figure 1. Study design (malnourished/undernourished rats). IGF-1, insulin-like growth factor 1.

Table 1. Composition of normal and restricted-protein diets.

Components	Control/Normal protein diet (NP; 23% Protein)	Restricted-protein diet (RP; 4% protein)
Sucrose	12.72	20.00
Cornstarch	52.75	64.25
Casein (88% protein)	23.33	4.55
Mixture of mineral salts*	3.20	3.20
Vitamin mixture*	1.60	1.60
Soybean oil	4.80	4.80
Fish oil	1.60	1.60
Total energy (Cal/100 g)	382	382

Data are presented as % per 100 g diet.
*The combination of salts and vitamins used in diet formulation is referred to AIN 93 recommendation (de Oliveira *et al.*, 2011).

Secondary outcomes for this preclinical trial define as:

1. Body Weight or BW (g) = Initial Body Weight (g); Final Body Weight (g) and Weight Gain (g/day)
2. Erythrocytes ($\times 10^6$/uL)
3. Haematocrit (%)
4. Retinol-Binding Protein (RBP, ng/mL)

The details of feed composition are shown in Table 1.

Sample collection and preparation

At the end of the intervention, the rats were fasted overnight and given ketamine anesthesia. To allow clotting at room temperature, a 2.5 mL blood sample is obtained from liver tissue and kept in a dry, clean tube without anticoagulants. Hemoglobin, hematocrit, and erythrocyte levels were determined from the blood plasma samples based on the method introduced by Arnaud *et al.* (2017) (see also the *Biomedical analysis* section for a brief description). The sample was then centrifuged for 20 minutes at 3,000 rpm to extract the protein, IGF-1, and Retinol-Binding Protein (RBP) from the blood serum for further analysis.

Biomedical analysis

A fresh blood plasma sample was used to evaluate the hemoglobin levels using the Rat Hemoglobin ELISA Kit (#AB157733). The sample was rinsed in a 1% phosphate buffer salt (PBS, pH 7.4) solution until the liquid cleared. The sample was then concentrated for 20 minutes at 3,000 rpm to produce the supernatant component. Protein levels and IGF-1 were measured with The Rat Protein Assay ELISA Kit (#MBS3808613) and The Rat IGF-1 ELISA Kit (#MBS268050) at a wavelength of 450 nm. Erythrocyte and hematocrit levels are measured using the Rat Erythrocyte Protoporphyrin ELISA Kit (#RTES01121).

Dataset

This *in vivo* research was successfully carried out, and the raw data from the research can be accessed via the *Underlying data* section (Nurkolis and Gunawan, 2022b).

Table 2 proved that undernourished rats supplemented with a higher dose of ETC flour (group B) would have a higher protein, hemoglobin, and IGF-1 serum than groups A and C. This data suggests that ETC flour may improve nutritional status biomarkers via a dose-dependent manner.

The data presented in Table 3 revealed a higher increase in weight gain in group B than in group A and C. All secondary outcomes and characteristics, except for food intake, were best in group B. These findings reinforce the previous statement that ETC flour works in a dose-dependent manner.

Table 2. Primary outcomes data.

Groups	A	B	C
Protein (mg/dL)	5.04 ± 0.66	5.85 ± 0.55	3.68 ± 0.54
Hemoglobin (mg/dL)	19.22 ± 0.39	21.31 ± 0.80	15.99 ± 0.79
IGF-1 (pg/mL)	281.87 ± 3.81	321.04 ± 2.07	36.53 ± 4.35

A = RP Diet + 100 mg/kgBW; B = RP Diet + 200 mg/kgBW; C = NP (Normal Protein/Standard dry pellet). RP, restricted protein diet. All data are presented in the form of Average ± SEM.

Table 3. Secondary outcomes and characteristics of rats.

Groups	A	B	C (Normal/NP)
Initial Body Weight (g)	44.72 ± 4.51	43.70 ± 3.44	42.37 ± 1.14
Final Body Weight (g)	161.26 ± 7.14	162.51 ±5.81	155.77 ± 4.49
Weight Gain (g/day)	4.16 ± 0.21	4.24 ± 0.21	4.05 ± 0.18
Food Intake (g)	6.30 ± 0.54	5.81 ± 0.64	5.65 ± 0.58
Water Intake (mL)	5.53 ± 0.49	5.92 ± 0.37	5.54 ± 0.56
[1]FER (%)	66.44 ± 5.61	73.90 ± 9.28	72.52 ± 9.20
Erythrocytes ($\times 10^6$/uL)	6.38 ± 0.63	7.55 ± 0.95	5.47 ± 0.66
Haematocrit (%)	56.04 ± 4.57	61.19 ± 4.51	48.09 ± 2.46
[2]RBP (ng/mL)	6420.02 ± 168.96	7597.92 ± 211.52	5315.94 ± 122.00

A = RP Diet + 100 mg/kgBW; B = RP Diet + 200 mg/kgBW; C = NP (Normal Protein/Standard dry pellet). RP, restricted protein diet. All data are presented in the form of Average ± SEM.
[1] Food Efficiency Ratio (FER, %) = (Body Weight Gain (g/day) /Food Intake (g/day)) × 100.
[2] Retinol-Binding Protein (RBP, ng/mL).

Data availability

Underlying data

Figshare: RAW Data for An Eel and Tempe Composite Flour Supplementation on the Nutritional Status Biomarkers of Rats with A Restricted Protein Diet.xlsx, https://doi.org/10.6084/m9.figshare.19782784.v1 (Nurkolis and Gunawan, 2022b).

This project contains the following underlying data:

- RAW Data for An Eel and Tempe Composite Flour Supplementation on the Nutritional Status Biomarkers of Rats with A Restricted Protein Diet.xlsx (The measured serum protein, hemoglobin, and IGF-1 levels from each group of rats observed in this study).

Reporting guidelines

Figshare: ARRIVE Checklist for 'Effect of eel and tempe composite flour supplementation on the nutritional status biomarkers of rats with a restricted protein diet: Data from a preclinical trial', https://doi.org/10.6084/m9.figshare.19799731.v2 (Nurkolis and Gunawan, 2022a).

Acknowledgements

Special thanks to Professor Dr. Nurpudji Astuti Taslim, MD., MPH, Sp.GK (K) (Chairman of the Indonesian Association of Clinical Nutrition Physicians), for the motivation and supervision in conducting this research during the pandemic.

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