Dec 08, 2021

Effect of offering Phenylketonuria (PKU) mice Glycomacropeptide (GMP) before normal or low protein (LP) diet on growth, serum-phenylalanine (phe)-concentration, bone structure, performance and concentration of phe in the brain.

kirsten.ahring [1], Mads Kjolby Department Of Biomedicine[1]

[1]Kennedy Centre, PKU clinic, Copenhagen University Hospital, Denmark
kirsten.ahring : Metabolic Dietitian, Cand. Scient in Human Nutrition, Ph.D

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dx.doi.org/10.17504/protocols.io.bkg9ktz6

PKU team, Copenhagen Uni Hospital, Denmark

kirsten.ahring

Introduction: Management of phenylketonuria (PKU) is mainly achieved through dietary control with limited intake of phenylalanine (Phe) from food, supplemented with low protein (LP) food and a mixture of free synthetic (FS) amino acids (AA) (FSAA). Casein glycomacropeptide (CGMP) is a natural peptide released in whey during cheese making by the action of the enzyme chymosin. Because CGMP in its pure form does not contain Phe, it is nutritionally suitable as a supplement in the diet for PKU when enriched with specific AAs. Lacprodan® CGMP-20 (= CGMP) used in this study contained only trace amounts of Phe due to minor presence of other proteins/peptides.
Objective: The aims were to address the following questions in a classical PKU mouse model:
Study 1, off diet: Can pure CGMP or CGMP supplemented with Large Neutral Amino Acids (LNAA) as a supplement to normal diet significantly lower the content of Phe in the brain compared to a control group on normal diet, and does supplementation of selected LNAA results in significant lower brain Phe level?
Study 2, on diet: Does a combination of CGMP, essential (non-Phe) EAAs and LP diet, provide similar plasma and brain Phe levels, growth and behavioral skills as a formula which alone consist of FSAA, with a similar composition?

Material and methods: 45 female mice homozygous for the *Pah*^{enu2} mutation were treated for 12 weeks in five different groups; G1(N-CGMP), fed on Normal (N) casein diet (75%) in combination with CGMP (25%); G2 (N-CGMP-LNAA), fed on Normal (N) casein diet (75%) in combination with CGMP (19,7%)and selected LNAA (5,3% Leu, Tyr and Trp); G3 (N), fed on normal casein diet (100%); G4 (CGMP-EAA-LP), fed on CGMP (70,4%) in combination with essential AA (19,6%) and LP diet; G5 (FSAA-LP), fed on FSAA (100%) and LP diet. The following parameters were measured during the treatment period: Plasma AA profiles including Phe and Tyr, growth, food and water intake and number of teeth cut. At the end of the treatment period, a body scan (fat and lean body mass) and a behavioral test (Barnes Maze) were performed. Finally, the brains were examined for content of Phe, Tyr, Trp, dopamine (DA),3,4-dihydroxyphenylacetic acid (DOPAC), serotonin (5-HT) and5-hydroxyindole-acetic acid (5-HIAA) , and the bone density and bone mineral content were determined by dual-energy x-ray absorptiometry.
Results: Study 1: Mice off diet supplemented with CGMP (G1 (N-CGMP)) or supplemented with CGMP in combination with LNAA (G2 (N-CGMP-LNAA)) had significantly lower Phe in plasma and in the brain compared to mice fed only casein (G3 (N)). Extra LNAA (Tyr, Trp and Leu) to CGMP did not have any significant impact on Phe levels in the plasma and brain, but an increase in serotonin was measured in the brain of G2 mice compared to G1. Study 2: PKU mice fed with mixture of CGMP and EAA as supplement to LP diet (G4 (CGMP-EAA-LP)) demonstrated lower plasma-Phe levels but similar brain- Phe levels and growth as mice fed on an almost identical combination of FSAA (G5 (FSAA-LP)).
Conclusion: CGMP can be a relevant supplement for the treatment of PKU.

DOI

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https://doi.org/10.1371/journal.pone.0261150

kirsten.ahring , Mads Kjolby Department Of Biomedicine 2021. Effect of offering Phenylketonuria (PKU) mice Glycomacropeptide (GMP) before normal or low protein (LP) diet on growth, serum-phenylalanine (phe)-concentration, bone structure, performance and concentration of phe in the brain.. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bkg9ktz6

protocol

Ahring KK, Dagnæs-Hansen F, Brüel A, Christensen M, Jensen E, Jensen TG, Johannsen M, Johansen KS, Lund AM, Madsen JG, Brøndum-Nielsen K, Pedersen M, Sørensen LK, Kjolby M, Møller LB (2022) The effect of casein glycomacropeptide versus free synthetic amino acids for early treatment of phenylketonuria in a mice model. PLoS ONE 17(1): e0261150. doi: 10.1371/journal.pone.0261150

protocol ,

Aug 29, 2020

Dec 08, 2021

41217

Protocol for mouse study

1 Project manager: Kirsten Ahring

Advisor: Frederik Dagnæs-Hansen

Project title:Effect of offering Phenylketonuria (PKU) mice Glycomacropeptide (GMP) before normal or low
protein (LP) diet on growth, serum-phenylalanine (phe)-concentration, bone structure, performance and
concentration of phe in the brain.

Sponsor:Arla Foods Ingredients Group P/S, Sønderhøj 10-12, 8260 Viby J

Project location:Bartholin Building, University of Aarhus, Wilhelm Meyers Allé 4, 8000 Aarhus C

Permission according to Experimental Animal Inspectorate:2013-2935-14-00382

Mouse strain: PAH^{enu2} homozygous breeding.
The mice receive phe free diet ad libitum with 9 ml phe in 392 ml water

Project start: November 2014

Abbreviations:
PKU: Phenylketonuri
GMP: Glycomacropeptide
AA: Amino Acids
Phe: phenylalanine

Project overview

2 The study includes the following:
- Mice: 40 PKU mice, females
- 0-4 weeks of life, the mice are breastfed by their mothers, who all live on a phe-poor diet. It is possible for the pups to eat from the mothers' food, but they are mainly fed on breast milk. Project period from 5-16 weeks of life with Frederik Dagnæs-Hansen (FDH), then on to behavioral testing
- Blood sampling and weighing every week / 3 times a week respectively
- PKU diet and other diets from Brogaarden (https://brogaarden.eu)
- Blood sample analyzes: Phe / bull analyzes are performed by David Hougaard (DH) at the Statens Serum Institute (SSI)(https://en.ssi.dk/about-us) and amino acid profiles by Mette Christensen (MC) at Copenhagen University Hospital (Rigshospitalet (RH))
- Behavioral tests and body scans (Mads Fuglsang (MF))
- Bone analyzes (Annemarie Brüel (AMB))
- Brain tests (Michael Petersen (MP))

Introduction

3 PKU is a congenital metabolic disease caused by the lack of the liver enzyme phenylalanine hydroxylase (PAH), which converts phenylalanine (Phe) to tyrosine. Deficiency of this enzyme causes accumulation of phe and results in irreversible brain damage if treatment is not started immediately after birth. Treatment consists of a low-protein diet, supplemented with amino acid (AA) supplements. The treatment is restrictive and complicated and therefore often associated with poor compliance due to the bad taste of AA supplements. Glycomacropeptide (GMP) is a natural peptide, isolated from cheese whey, with a far more pleasant taste and can therefore potentially be an alternative to AA supplements.

Developmental objective:

4 Mouse project aims to support the human project by collecting similar data where possible. CGMP is a relatively new treatment option for PKU patients, but long-term human studies have not yet been performed. In this part of the project, we will clarify whether amino acid supplements given in the form of CGMP have improved efficacy, compared to free, synthetically produced, (AA). This will be measured on bone status, phe / bull level, amino acid profiles, behavioral tests and phe content in the brain. Clarification of these questions will be of great basic scientific value and of great value in relation to the daily counseling of PKU patients, regarding the choice of amino acid supplements.

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The purpose of the project is to test the following:

5 Off diet: Whether CGMP as a supplement to normal diet can lower phe content in the brain significantly

On diet: Whether CGMP as the primary amino acid source in combination with essential amino acids and low protein diet results in similar plasma and brain phe as well as growth as artificially produced AA supplements in PKU mice

The PKU mice are divided into 5 groups of 8 mice and fed after the end of the breastfeeding period (4 weeks) for 12 weeks with different experimental diets. An overview is shown in the table below.

Group 1	Group 2	Group 3	Group 4	Group 5
Off diet	Off diet	Off diet	On diet	On diet
CGMP-20	CGMP-20 +leu/tyr/trp (SHIELD)	Control	CGMP-20 & AA mix	AA mix (Same AA profile as CGMP-20& AA mix)

Data collection and monitoring at Aarhus University (Frederik Dagnæs-Hansen and Jani Kær):

6 Body weight - weighed 3 times a week for 12 weeks in all 5 groups (life week 5-16)

Results are sent by email to Kirsten Kiær Ahring (KKA): kirstenahring@hotmail.com
Expected start: November 2014

Blood samples are taken every week for 12 weeks in all 5 groups for analysis of phe / bull content
except weeks 10 and 16, where blood is drawn for amino acid profiles for analysis on RH.
The mice are fasted from 7 in the morning until blood sampling is performed between kl 10-12. The fasting period is noted (hours and minutes)

The samples are taken on PKU filter paper, frozen and sent in piles to (or handed over personally to Kirsten
Kiær Ahring):

Clinical Biochemistry, Immunology & Genetics
Statens Serum Institut
Artillerivej 5, 84 / 149B
2300 Copenhagen

Total amino acid (AA) profile:

7 Blood samples are taken 3 times as described in the procedure below: per week 6, 11 and 16

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The mice are fasted from the morning at 6.30 until blood sampling.
The fasting period is noted (hours and minutes). The blood samples are taken between 10-12

IMPORTANT INFO ABOUT SAMPLING
1) EDTA whole blood (preferably 100 µL min. 50 µl) is taken out in a suitable microtube NB! EDTA whole blood must NOT be frozen or cooled, as in this case plasma is hemolyzed, which could lead to contagion of AA from platelets.

2) Plasma is isolated immediately after sampling by centrifugation (we use 2800 G for 10 min at room temp). NB! When whole blood is allowed to stand, glutamine will be converted to glutamic acid, asparagine to aspartic acid, arginine to ornithine, etc. It is therefore important that plasma is isolated as soon as possible (<1 hour after sampling)

3) Plasma is frozen immediately after centrifugation at min. -18 ° C and preferably -80 ° C. NB! When the plasma is allowed to stand at room temperature, the conversion of glutamine to glutamic acid, asparagine to aspartic acid, etc. takes place rapidly. therefore, the sample must be frozen immediately

4) For prolonged transport / shipping, the plasma samples must be sent on dry ice.

Blood samples from “start = week0 corresponding to 5 weeks of life” and “end = week 12, corresponding to 16 weeks of life” sent on dry ice in groups of 24 samples from 12 mice at the same time.

The samples are sent to the following address (or handed over in person to Kirsten Kiær Ahring):

Att.: Mette Christensen (MC)
Metabolisk Laboratorium
Klinisk Genetisk Klinik RH 4062 Rigshospitalet
Blegdamsvej 9
2100 København Ø

At the end of the test period, the PKU mice are passed on to:

8 Mads Fuglsang Kjølby
Institut for Biomedicin
Aarhus Universitet
Victor Albeck Bygningen
Vennelyst Boulevard 4
8000 Aarhus C, Denmark

9 **The mice are passed on after behavioral tests at Mads Fuglsang to:**
Anne-Marie Brûel (AMB)
Institut for Biomedicin - Forskning og uddannelse, Syd
Wilhelm Meyers Allé 3, bygning 1233, lokale 222
8000 Aarhus C

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The mice are sacrificed, and bone is removed and cleaned. (Brain is taken at the same time. This is done by
Mads Fuglsang Kjølby).

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