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Maternal low-protein diet alters the expression of real-time quantitative polymerase chain reaction reference genes in an age-, sex-, and organ-dependent manner in rat offspring

Barent DuBois[a,1], *Jacob Pearson*[a,1], *Bonnie Hastings*[a], *Tahir Mahmood*[a], *Tammy Chan*[a], *Ali Alnakhli*[a], *Ganesh Cherala*[a,b,*]

[a] *College of Pharmacy, Oregon State University/Oregon Health and Science University, Portland, OR 97239, USA*
[b] *Department of Obstetrics and Gynecology, Oregon Health and Science University, Portland, OR 97239, USA*

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ABSTRACT

Altered perinatal environment, often manifested as low birth weight, is thought to contribute to greater susceptibility for hypertension, hyperlipidemia, and diabetes as a result of epigenetic modifications and alteration of transcriptional activity for key genes. Real-time polymerase chain reaction is a useful technique for the quantitative determination of differences in transcriptional activity. Real-time quantitative polymerase chain reaction data analyses require normalization of transcriptional activity of target genes to an endogenous control, usually a reference gene. In response to reports of altered expression of reference genes in various experimental models, we hypothesized that adverse perinatal environment alters reference gene expression. We examined the expression of the following reference genes in the offspring of a rodent maternal low-protein diet model: *β-actin*, *hypoxanthine phosphoribosyltransferase* 1, *TATA-box-binding protein*, *glyceraldehyde-3-phosphate dehydrogenase*, and *glucuronidase-β* in brain, heart, kidneys, and intestines. We found altered expression in brain, heart, and kidneys for each of the reference genes measured; these effects were age, organ, and sex dependent. *Glyceraldehyde-3-phosphate dehydrogenase* and *glucuronidase-β* were found to be the least affected by these variables, whereas *hypoxanthine phosphoribosyltransferase* 1 was the most inconsistent. Our findings underscore the importance of empirical determination of a reliable reference gene for real-time polymerase chain reaction studies in the low-protein diet model.

1. Introduction

Altered perinatal environment, often manifested as low birth weight, is linked to greater susceptibility for hypertension, hyperlipidemia, and diabetes [1]. An altered perinatal environment is thought to contribute to metabolic alteration of adult phenotype as a result of epigenetic modifications and subsequent alterations in transcriptional activity of key genes [2,3]. Real-time

Abbreviations: Actb, β-Actin; ANOVA, Analysis of variance; cDNA, Complementary DNA; Ct, Cycle threshold; Gapdh, Glyceraldehyde-3-phosphate dehydrogenase; Gusb, Glucuronidase-β; Hprt1, Hypoxanthine phosphoribosyltransferase 1; LPD, Low-protein diet; mRNA, Messenger RNA; RT-PCR, Real-time polymerase chain reaction; RT-qPCR, Real-time quantitative polymerase chain reaction; Tbp, TATA-box-binding protein.

* *Corresponding author*. Portland, OR 97239-4501, USA. Tel.: +1 503 418 0447; fax: +1 503 494 8797.
E-mail address: cheralag@ohsu.edu (G. Cherala).

[1] Barent DuBois and Jacob Pearson are recognized as co-first authors on this manuscript.

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quantitative polymerase chain reaction (RT-qPCR) is a valuable tool for the quantitation of differences in gene transcription.

In RT-qPCR, changes in the level of gene transcription are measured by normalizing target gene expression to that of a reference gene. Although there are many reference genes to choose from, an ideal pick is one with reliable and ubiquitous expression in all cell types and tissues and similar expression between experimental groups [4–7]. It is important to identify reference genes, which demonstrate little variation between experimental groups before differences in the gene(s) of interest can be quantified [8]. For example, reference gene expression is subject to change between sexes [9], organs [10], and developmental age [11]. In addition, some studies have indicated that gene expression is altered as a result of an altered perinatal environment. Denisenko et al [12] observed decreased expression of *glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*), a commonly used reference gene, in the kidneys of microswine offspring from mothers fed a low-protein diet (LPD) during pregnancy. Maternal LPD also altered the expression of 18 s and 28 s RNA in a rat model [13]. Rodent maternal LPD is a commonly used animal model to understand developmental origins of health and disease [14], making the evaluation of adequate reference genes important. However, limited data are available on the expression of commonly used reference genes in rodent maternal LPD model [15,16].

In the present study, we examined the expression of the following reference genes in the brain, heart, kidneys, and intestines of the offspring of the rodent maternal LPD model: *β-actin* (*Actb*), *hypoxanthine phosphoribosyltransferase 1* (*Hprt1*), *TATA-box-binding protein* (*Tbp*), *Gapdh*, and *glucuronidase-β* (*Gusb*). The selection of reference genes and organs was determined by their widespread use in molecular biology experiments and relevance to the field of fetal programming, respectively. We hypothesized that maternal LPD alters the expression of these genes, affecting RNA quantification. The study findings include age-, organ-, and sex-dependent alteration in the expression for each of the reference genes measured. Our findings underscore the importance of empirical determination of a reliable reference gene for RT-qPCR studies in the LPD model. This study adhered to recently published MIQE guidelines for RT-qPCR experimentations [17], strengthening the validity of our findings.

2. Methods and materials

2.1. Diets

Modified versions of the AIN76A semipurified diet (control) and the corresponding isocaloric low-protein formulation (LPD) were purchased in pellet form from Purina Test Diets (Richmond, IN). The semipurified control and LPD diets contained 19% and 8% crude protein in the form of casein, respectively. The detailed ingredient compositions of both diets are shown in Table 1.

Table 1 – Ingredient composition and proximate analysis of the diets fed to rats

	Control	LPD
Casein-vitamin free	210	88
Dextrin [a]	436	434
Sucrose	150	274
Lard	50	50
Corn oil	50	50
Powdered cellulose	30	30
RP vitamin mix#10 [b]	20	20
RP mineral mix#10 [c]	50	50
DL-methionine	1.5	0.63
Choline chloride	2	2
Proximate analysis values		
Energy (physiological fuel value), kJ/g	17.07	17.28
Protein %	18.63	7.75
Fat %	22.05	21.78
Carbohydrate %	59.32	70.47

AIN76A semipurified diet (control) and the corresponding isocaloric low-protein formulation (LPD) were purchased in pellet form from Purina Test Diets. Values are in grams per kilogram.
[a] Dextrinized corn starch.
[b] Provided per kilogram of diet: thiamin 20 mg, riboflavin 20 mg, pyridoxine 20 mg, nicotinic acid 90 mg, calcium pantothenate 60 mg, folic acid 4 mg, biotin 0.4 mg, vitamin B12 20 μg, vitamin A 22000 IU, vitamin E 50 IU, vitamin D3 2200 IU, vitamin K 20 mg.
[c] Provided per kilogram of diet: calcium 6 g, phosphorus 4 g, sodium 2.1 g, potassium 4 g, magnesium 0.69 g, manganese 65 mg, iron 60 mg, copper 15 mg, zinc 20 mg, iodine 0.6 mg, selenium 0.2 mg, chromium 3 mg, chloride 2.4 g, sulfate 1.2 g, cobalt 3.2 g, fluoride 5 mg, molybdenum 0.8 g.

2.2. Experiment with dams

The study was approved by the Institutional Animal Care and Use Committee of the Oregon Health & Science University, Portland, OR. Virgin female Sprague-Dawley rats (Charles River Laboratories, Inc, Wilmington, MA) were mated by housing 1 male rat with 2 female rats. Day 1 of pregnancy was assigned upon observation of sperm in the daily morning vaginal smears, at which time rats were randomly assigned to 2 diet groups. Each group consisted of 5 to 7 pregnant rats, and these rats received their assigned diet throughout pregnancy and lactation.

2.3. Experiments with offspring

Upon birth, pups were sexed, and litter size was noted. All litters were culled to 12 pups (6 males and 6 females) on the day of birth and further culled to 8 pups (4 males and 4 females) on day 4 after birth. Step-wise culling was practiced to ensure a uniform litter size during lactation period [18,19]. No selection criteria were used when culling pups. Offspring from both groups were weaned on day 28 after birth and were housed in isosexual groups according to perinatal diet treatment. Pups from litters in both groups were weaned onto a nonpurified diet. It is therefore important to note that different dietary treatments were administered only during gestation and lactation. One male and female offspring from each litter in both groups were randomly chosen and euthanized using CO_2 asphyxiation on days 28, 65, 90, and 150 after birth. Kidneys, intestines, heart, and brain were collected from all euthanized animals, weighed, and snap-frozen in liquid nitrogen and stored at –80°C.