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Combination of vitamin B12 active forms improved fetal growth in *Wistar* rats through up-regulation of placental miR-16 and miR-21 levels

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ABSTRACT

Aim: Epidemiological studies have indicated importance of folate and vitamin (B12) during pregnancy. Also available evidence on efficacy of B12 forms viz. Cyanocobalamin (Cbl), Methylcobalamin (MeCbl), Adenosylcobalamin (AdCbl) and Hydroxycobalamin (HCbl) in preventing or treating cobalamin deficiency is limited. The present study examines the effect of various forms of B12 in combination with folate during pregnancy and their effect on gestational outcomes.

Main method: In the present study, we examined the effect of various vitamin B12 forms in presence of recommended folate (RFol: 400 μg/day) and high folate (HFol: 5 mg/day) on gestational outcomes in female Wistar rats.

Findings: Dams dosed with excessive folate (HFol group) delivered low birth weight (LBW) offsprings ($p < 0.01$) as compared to RFol dams. Plasma homocysteine levels were found to be significantly higher ($p < 0.05$) in dams of HFol group and were reduced after vitamin B12 supplementation. Excessive folate supplementation and homocysteine levels showed inverse association with placental weight ($p < 0.01$) and placental efficiency ($p < 0.05$). B12 supplementation significantly up-regulated placental miR-16 and miR-21, associated with fetal growth which in turn reflected in improved birthweights. Supplementation with vitamin B12 forms, especially combination of active forms of cobalamins: MeCbl + AdCbl significantly increased birth weights ($p < 0.05$) and modulated gestational outcomes in RFol as well as HFol supplemented dams.

Significance: Our results indicated supplementing vitamin B12 along with folate during pregnancy had positive impact on the gestational outcomes. We have shown for the first time that combination of active forms of vitamin B12: MeCbl + AdCbl has better efficacy as compared to Cbl, MeCbl, AdCbl and HCbl alone.

1. Introduction

Fetal development and growth is a result of several factors comprising genetics, maternal nutrition, maternal metabolism and placental health and functions. In developing nations, suboptimal maternal nutrition compromises fetal growth and development. In India, vitamin B12 deficiency is commonly seen due to vegetarianism and lower consumption of milk and milk products, while the concentration of folate is adequate. A recent systematic review showed that vitamin B12 insufficiency among pregnant women across the world was common in all trimesters (20%–30%) [1]. The recommended dose of folic acid during pregnancy is 400 μg/day, however, the dosage prescribed in India is as high as 5 mg/day [2]. Although the DOHaD hypothesis began with fetal under nutrition, the role of maternal over nutrition is now recognized and a U-shaped relationship between birth weight and later health outcomes for the adult has been suggested [3,4].

In our earlier report, we demonstrated that imbalance of folic acid and vitamin B12 due to higher doses of folate was associated with global DNA methylation and poor birth outcomes in Indian pregnant women [2,5]. We also showed restoration of growth, development and function in trophoblastic cells viz. BeWo and JEG3 when supplemented with vitamin B12 in excess folate condition [6]. Vitamin B12 deficiency is also an independent risk factor leading to adverse gestational outcomes, neural tube defects and neurological complications [7–9]. Vitamin B12 deficiency in pregnancy is prevalent and has been associated with both lower birth weight (LBW), intrauterine retardation and preterm birth [10,11].

Vitamin B12 deficiency is strongly associated with

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hyperhomocysteinemia, which predisposes to adverse pregnancy outcomes [12,13]. It also increases the risk of developing preterm labor, intrauterine growth retardation (IUGR), preeclampsia and recurrent abortions [14,15].

Several studies have recommended vitamin B12 supplementation during pregnancy although there is confusion on the use of forms of vitamin B12 viz. Cyanocobalamin (Cbl), Methylcobalamin (MeCbl), Adenosylcobalamin (AdCbl) and Hydroxycobalamin (HCbl) [16]. Thakkar et al., suggested giving a combination of the active forms of vitamin B12 (MeCbl + AdCbl) for treatment of vitamin B12 insufficiency [17]. Identifying the best vitamin B12 form for supplementation remains important.

We planned to study effect of vitamin B12 forms MeCbl, Cbl, HCbl, AdCbl and combination of active forms (MeCbl + AdCbl) along with recommended dose of folate (RFol: 400 μg/day) and high dose of folate (HFol: 5 mg/day) on gestational outcomes (in terms of birth weight (BW), placental weight (PW) and placental sufficiency (BW:PW)) in female *Wistar* rats. We measured plasma homocysteine levels in dams at gestational day 19 (GD19) as a probable marker associated with an imbalance in plasma folate and vitamin B12 in pregnancy.

Previous studies have shown the role of placental miRNAs involved in the development of preeclampsia, preterm labor, IUGR etc. [18,19]. However, miRNA intermediated mechanism in placenta remains poorly explored. We examined the expression levels of two candidate miRNAs: miR-16 and miRNA-21 in placental tissue to study its association with differential maternal nutrition and fetal growth.

2. Material and methods

2.1. Animal maintenance and ethics statement

The study protocol was carried out in compliance with the CPCSEA guidelines (Committee for the Purpose of Control and Supervision of Experiments on Animals) Government of India. This study was approved by the IAEC of National Toxicology Centre, Pune (Registration No.40/CPCSEA/1999 through RP No. 39/1415). We obtained 12–14 weeks old ninety-six virgin female *Wistar* rats from a single colony to decrease genetic variability. Female *Wistar* rats were housed in polypropylene cages and maintained at 22 ± 2 °C, under standard lighting conditions (12- h light/dark cycle) with the relative humidity of 55 ± 10%.

2.2. Diet composition, breeding and tissue collection

The composition of the diet was as per AIN 93 purified diets for laboratory rodents [20]. Folate and vitamin B12 were completely removed from the vitamin mixture of diet composition (Table 1; VRK Nutritional Solutions, Pune, India). Treatment diet and distilled water were provided ad libitum. Initially, all female rats were fed with vitamin B12 deficient diet and orally dosed with recommended folic acid [RFol; 400 mcg] for a period of 1 month. The females were then allowed to breed (sex ratio 1M:3F) and pregnancy was confirmed by the presence of a vaginal plug and/or sperm positive vaginal smear observed under the microscope. This was considered as gestational day 1 (GD1) and dams were randomly divided into different B12 forms supplemented groups as described in Table 2. Folic acid was obtained from Sigma Aldrich (Missouri, USA) while vitamin B12 forms were procured from Avanscure Life Sciences Pvt. Ltd. (New Delhi, India). As per groupings mentioned in Table 2. folic acid and/or vitamin B12 were administered to dams daily by oral intubation. The dose administered was calculated on the basis of body weight of dams (microgram/kg body weight of rat/day).

Fig. 1 shows the schematic study design of the animal experimentation. On GD19, dams were anesthetized with intramuscular administration of Ketamine (24 mg/kg) and Xylazine (10 mg/kg) to collect all placentas and liver tissues for later analysis. Litter size was recorded in each group on GD19 (Supplementary file: S1). New-borns were placed with foster mothers for further studies. Briefly, after the midline laparotomy, uterus was excised and washed in Dulbecco's phosphate buffered saline (PBS). Uterine horns were dissected under the dissecting microscope, and placentas were isolated. Placentas were weighed and immediately stored in lysis buffer and RNA later at –80 °C for later analysis. The absolute weight of placenta and neonates were recorded to analyse BW:PW ratio, as a proxy for placental efficiency. Dam's blood was collected by cardiac puncture and samples were processed for plasma separation.

Table 1
Composition of experimental diet.

Component (g/kg)	
Cornstarch	398.0
Casein (> 85% protein)	200.0
Dextrinised starch	132.0
Sucrose	100.0
Fat[a]	70.0
Fish oil	0.0
Fiber (cellulose)	50.0
Mineral mix[b]	35.0
Vitamin mix[c]	10
Folic acid	0.0
Vitamin B12	0.0
L-Cysteine	3.0
Choline bitartarate	2.5
Tert-butylhydroquinone	0.014

[a] Fat in the diet was derived from soybean oil.
[b] Mineral mixture (g/kg mixture): Calcium carbonate: 357; Potassium Phosphate: 196; Potassium Citrate: 70.78; Sodium Chloride: 78; Potassium Sulphate: 46.6; Magnesium Oxide: 24; Ferric Citrate: 6.06; Zinc Carbonate: 1.65; Manganous Carbonate: 0.63; Cupric Carbonate: 0.3; Potassium Iodate: 0.01; Sodium Selenate: 0.01; Ammonium Paramolybdate: 0.007; Sodium Metasilicate: 1.45; Chromium Potassium Sulphate: 0.275; Lithium Chloride: 0.01; Boric Acid: 0.08; Sodium Fluoride: 0.06; Nickel Carbonate: 0.03; Ammonium Vanadate: 0.006; Sucrose, 221.02.
[c] Vitamin mixture (g/kg mixture): Nicotinic Acid: 3; Calcium Pantothenate: 1.6; Pyridoxine-HCl: 0.7; Thiamin –HCl: 0.6; Riboflavin: 0.6; D-Biotin: 0.02; Vitamin E: 15;Vitamin A: 0.8; Vitamin D3: 0.25;Vitamin K: 0.075; Sucrose 977.355, was added to make total weight of the vitamin mix to 1 kg.

2.3. Estimation of plasma vitamin B12, folate and homocysteine

Plasma vitamin B12, folate, and homocysteine levels were determined on GD19. Plasma vitamin B12, folate and homocysteine concentration were measured by competitive chemiluminescent enzyme immunoassay (CLEIA) method (Siemens Centaur CP, Erlangen, Germany).

2.4. miRNA isolation and profiling

Total RNA was isolated from six placental tissues in each group using Trizol reagent (Sigma Aldrich) and was stored in RNA later at –80 °C. RNA samples of high quality with 260/280 ratios (1.8–2.0) were used in further steps. mirVana™ miRNA isolation kit was used to isolate miRNA (Ambion Inc). cDNA was synthesized using the iScript kit (BioRad, Copenhagen, Denmark) following manufacturer's protocol. Quantitative PCR (qPCR) was performed using the KAPA SYBR® Fast Universal Master Mix (Kapa Biosystems Inc., Massachusetts, USA) on ABI StepOne Plus system (Applied Biosystems, Foster City, CA, USA). Oligonucleotide sequences and qPCR conditions were used as previously described [21,22]. All samples were run in duplicates and U6 was used for normalisation. Relative expression was calculated using $2^{-\Delta\Delta Ct}$.