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Original article

Molecular and cellular effects of vitamin B12 forms on human trophoblast cells in presence of excessive folate



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ABSTRACT

Folic acid (FA) and iron are essential supplements during pregnancy. Similarly effects of vitamin B12 (B12) inadequacy and high folate and low B12 status, on pregnancy outcome are available. However there are no mandatory recommendations for B12. There are many forms of B12 viz. Cyanocobalamin (Cbl), Methylcobalamin (MeCbl), Adenosylcobalamin (AdCbl), and Hydroxycobalamin (HCbl) though there is limited consensus on which form has better efficacy. In the present study we have determined effect of various forms of B12 in the presence of two FA concentrations namely normal physiological (20 ng/mL; NPFA) and supra-physiological (2000 ng/mL; SPFA) concentration to mimic real time situation where FA is in excess due to supplementation. We assessed trophoblastic proliferation, viability, TNF α and EGFR mRNA expression, homocysteine, β -hCG and MDA levels. Trophoblastic viability was significantly suppressed at SPFA concentration and was restored by B12 treatment with Cbl, AdCbl and combination of MeCbl + AdCbl. The mRNA expressions of TNF α were up-regulated, while EGFR were down-regulated at SPFA concentrations, as validated by RT-PCR. Treatment with MeCbl + AdCbl significantly decreased homocysteine and MDA levels at SPFA concentrations. High levels of FA alone had a detrimental effect on placental health and functions as reflected by decreased viability, EGFR expression and increased TNF α expression, homocysteine and MDA levels. Combination of B12 active forms *i.e.* MeCbl + AdCbl was found to be most effective in neutralising excess folate effect *in-vitro*.

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1. Introduction

Maternal nutrient supplementation in developing countries is generally restricted to iron and folic acid [1]. As per National Institutes of Health, the recommended dosage of folate during pregnancy is 600 mcg per day but in developing countries dose prescribed is as high as 5 mg/day *i.e.* intervened at supra physiological concentration. Although early results from the fortification policy indicated beneficial outcomes in context to reduction of plasma homocysteine levels [2–4], recent reports are contradictory [5,6].

In our earlier studies we reported that there is an imbalance of vitamin B12 (B12) and folic acid (FA) due to high folate and low vitamin B12 plasma concentration in the Indian pregnant women, which was inversely associated with birth anthropometric parameters [7]. We also showed that high folate and low vitamin

B12 ratio leads to hyperhomocysteinemia during pregnancy. It also leads to hypermethylation in vegetarian population [8]. Although vitamin B12 plays an important role in pregnancy and pregnancy outcomes (like pre-eclampsia, fetal growth restriction, preterm labor *etc.*), there are no national guidelines to prescribe proper doses and forms of vitamin B12 in pregnant women [9,10].

Additionally there is confusion on use of forms of vitamin B12 viz. Cyanocobalamin (Cbl), Methylcobalamin (MeCbl), Adenosylcobalamin (AdCbl) and Hydroxycobalamin (HCbl) for the treatment of vitamin B12 deficiency. Thakar et al., [11] suggested giving a combination of the active forms of vitamin B12 *i.e.* MeCbl and AdCbl. Vitamin B12 remains vital in development and maturation of placenta [12]. Hence it is important to know the response of placental cells towards vitamin B12 in presence of folate.

Trophoblast cell lines have been employed in a variety of *in-vitro* studies of placental function including, proliferation, differentiation, hypoxia/oxidative stress responses, endocrinology, and maternal-fetal immunology [13]. Recently effect of increasing folic acid concentration was assessed on placenta health and

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function using trophoblastic cell lines viz. BeWo and HT8-8/SVneo [14]. Folic acid induced necrosis has been reported within renal tubules of mice treated with 300 mg/kg folic acid [15].

Aim of our study was to find out which form of vitamin B12 (Cbl, MeCbl, AdCbl, HCbl) and combination of active forms of vitamin B12 i.e. MeCbl + AdCbl is useful for placental growth, development and function. We used trophoblastic cell lines viz. BeWo and JEG3 as an *in-vitro* model for this study. Effects of B12 forms were studied in presence of normal physiological FA concentration (20 ng/mL) and supra-physiological FA concentration (2000 ng/mL).

We compared trophoblastic cells proliferation, cell viability, TNF α and EGF receptor (EGFr) mRNA expression levels, homocysteine levels, β -hCG expression and lipid peroxidation expression in BeWo and JEG3 cell treated with FA and combination of FA and vitamin B12 forms.

2. Materials and methods

2.1. Cell lines and cultures

BeWo and JEG3, are human trophoblast-like choriocarcinoma cell lines, derived from placenta. The BeWo and JEG3 cell line were obtained from National Centre for Cell Science (NCCS, India) and American Type Culture Collection (ATCC, USA) respectively. Both cell line were cultured in Dulbecco's modified Eagle's medium (DMEM-D2429) (Sigma Aldrich, Missouri, USA) deficient of folic acid and vitamin B12. Growth medium was supplemented with 10% FBS (Gibco, Invitrogen) and 1% Antibiotic – Antimycotic 100 $\times$ solution (ThermoFisher Scientific). The cells were maintained at 37 °C in 95% humidity and 5% CO₂. Growth rates were determined by cell count. Both BeWo and JEG3 cells were serum starved overnight in serum free media, prior to any experimental procedures described below.

2.2. Vitamin B12 & folate treatment

Vitamin B12 analogues were procured from Avanscure Life Sciences Pvt. Ltd (New Delhi, India) and dissolved in distilled water to obtain 1000 ng/mL concentration and was stored light protected. Different concentration of B12 treatments (0.1, 1, 10, 100, 1000 ng/mL) were prepared by diluting the stock solution with distilled water. Stock solution and dilutions were prepared fresh prior to new experimentation procedure. Folic Acid was procured from Sigma Aldrich. The two folic acid experimentation treatments were selected to represent maternal folic acid concentration i.e. normal physiological concentration (20 ng/mL) and supra-physiological concentration (2000 ng/mL). Both cells were exposed to B12 treatments for 72 h, at which cells and/or medium were harvested for further analysis.

As maximum cell density (proliferation) was obtained at 100 ng/mL of B12 forms at both normal physiological FA concentration [NPFA] and supra-physiological FA [SPFA] concentration in both cell lines, we assessed cell viability, TNF α and EGFr mRNA expression, homocysteine levels, β -hCG expression and lipid peroxidation expression at 100 ng/mL of B12 concentration.

2.3. Trophoblastic proliferation assay

BeWo and JEG3 cells were seeded at a density of approximately 5×10^3 cells/well in a 96-well flat-bottom microplate (NEST-Biotechnology). After initial 4 h of culture, which allowed for cell attachment, medium was changed with the addition of B12 forms (0.1, 1, 10, 100, 1000 ng/mL) with NPFA and SPFA concentrations in the corresponding wells, respectively. A control

treatment of NPFA concentration (20 ng/mL) and SPFA concentration (2000 ng/mL) was included for each experimentation. The cells were cultured for another 72 h. Followed by treatment, the original medium from each well was carefully aspirated and stored in –20 °C for further analysis. The cells in well were washed twice with phosphate buffer solution, and 50 μ L of the MTT staining solution (5 mg/mL in phosphate buffer solution) was added to each well and plate was incubated at 37 °C. After 4 h, 100 μ L of di-methyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals, and absorbance was recorded with a 492 nm using enzyme linked immunosorbent assay (ELISA) plate reader. The cell densities were obtained from a standard curve constructed as a linear curve expressing the co-relation between cell densities and optical densities.

2.4. Cell viability

Trophoblastic viability was assessed using a trypan blue dye exclusion assay. Cells were seeded (5×10^3 cells/well) in a 24 well plate and incubated for 72 h with the experimental B12 form treatments at both FA concentration. Cells were harvested and assessed using haemocytometer (Rohem, India). Cellular viability was assessed as the number of viable cells (trypan blue negative) divided by the total number of cells expressed as a percentage.

2.5. Real time quantitative RT-PCR assay

The total cellular RNA was extracted using RNeasy mini kit (Qiagen) according to manufacturer's instruction. Total RNA was reverse transcribed into first-strand complementary DNA (cDNA) using High-Capacity cDNA reverse transcription (ThermoFisher Scientific) as per manufacturer's instruction. Primers for β actin and EGFr gene were designed using Gene Runner software (version 3.05). Primers were synthesised from Eurofins MWG Operons (Bangalore, India). The sequence of primers are as follows: β actin (forward: 5'- GTA GAG CCC ACC TTC CTT CC-3'; reverse: 5'- GGG AGT CAG GTG GCT TGC-3'); EGFr (forward: 5'- GGA GAA CTG CCA GAA ACT GAC C - 3'; reverse: 5'- GCC TGC AGC ACA CTG GTT G - 3') and TNF α (forward: 5'- GTG ACA AGC CTG TAG CCC A - 3'; reverse: 5' - ACT CGG CAA AGT CGA GAT AG - 3') [16]. Real-time PCR was performed on ABI Step One Plus system (Applied Biosystems) and SYBR Green chemistry (Applied Biosystem) was used for the PCR and all experiments were in triplicates using β actin as the internal control. The results were analysed by relative quantification, using $\Delta\Delta C_T$ method [17]. The reaction condition was as follows: denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 60 s.

2.6. Measurement of homocysteine level

Homocysteine levels were estimated from cell lysate using Homocysteine ELISA kit (Cell Biolabs, Inc). Briefly, cells were centrifuge at 2000 rpm for 10 min at 4 °C and were sonicated in 1 mL of cold PBS. After centrifugation at 12,000 for 15 min, supernatant was removed and was used for homocysteine analysis as per manufacturer's instruction. The readings were taken at 450 nm using ELISA plate reader.

2.7. Measurement of trophoblast β -hCG secretion

BeWo and JEG3 5×10^3 cells/well were treated with B12 forms for 72 h. The conditioned media was used to estimate β -hCG levels using hCG Human ELISA kit (Abcam®) according to the manufacturer's instructions and readings were taken at 450 nm using a ELISA plate reader.

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