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The effect of oxidative stress induced by tert-butylhydroperoxide under distinct folic acid conditions: An *in vitro* study using cultured human trophoblast-derived cells

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ABSTRACT

Preeclampsia is a pregnancy disorder characterized by high maternal blood pressure, fetal growth restriction and intrauterine hypoxia. Folic acid is a vitamin required during pregnancy. In this work, we investigated the relationship between preeclampsia and the intake of distinct doses of folic acid during pregnancy. Considering that preeclampsia is associated with increased placental oxidative stress levels, we investigated the effect of oxidative stress induced by *tert*-butylhydroperoxide (TBH) in human trophoblast-derived cells cultured upon deficient/low, physiological and supra-physiological folic acid levels. The negative effect of TBH upon thiobarbituric acid reactive substances (TBARS), total, reduced and oxidized glutathione, cell viability, cell proliferation, culture growth and cell migration was more marked under folic acid excess. This study suggests more attention on the dose administered, and ultimately, on the overall folic acid levels during pregnancy, in the context of preeclampsia risk.

1. Introduction

Preeclampsia is a pregnancy disorder characterized by high maternal blood pressure and proteinuria at ≥20 weeks of gestation [1,2]. Preeclampsia remains an important cause of maternal and fetal morbidity and mortality, affecting 1–8% of all pregnancies [3–5]. In severe cases, preeclampsia complications may include maternal seizures, cerebrovascular accident, thrombocytopenia, renal failure, fetal growth restriction, intrauterine hypoxia, prematurity and death [2,6–9].

The placenta, a materno-fetal complex organ formed during pregnancy, is responsible for nutrient transport, gas exchange, hormone synthesis and embryo protection [10,11]. An inadequate placentation process, including inadequate trophoblast invasion and insufficient remodeling of the uterine spiral artery, results in persistent placental hypoxia/ischemia. This in turn results in an imbalance in the production of pro-angiogenic/anti-angiogenic factors into the maternal circulation [2,3,6]. Although the pathophysiology of preeclampsia remains unclear, this imbalance in favor of anti-angiogenic factors appears to be associated with the onset of maternal endothelial dysfunction, affecting all maternal organ systems [2,12]. Also, an increased oxidative stress imbalance has been described during the pathogenesis and progression of preeclampsia, leading to fetal programming of deleterious effects such as cardiovascular complications and metabolic syndrome in adulthood [13–16].

Folic acid is the synthetic form of folate, a member of B_9 vitamin family, which is essential for the biosynthesis of nucleotides, amino acids and S-adenosyl-L-methionine [17,18]. It is well established that an adequate intake of folic acid is required for the correct development of neural tube and central nervous system and the growth of placenta and fetus [19–21]. Accordingly, supplementation with folic acid (0.4 mg/day) has been recommended to all women within childbearing age from the beginning of pregnancy and until the end of the first trimester of pregnancy, while 4–5 mg/day is advised for women at high risk of neural tube disease [22,23].

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In the last decades, motivated by public health policies, many countries introduced folic acid-fortified foods, which may result in upper intake levels in women at childbearing age and in pregnant women [24–26]. Surprisingly, recent results from *in vivo* and *in vitro* models have shown that high folic acid supplementation is associated with adverse effects and metabolic imbalance in both mothers and their offspring, including predisposition to an insulin-resistant state, memory failure, development of ventricular septal defects and thinner left and right ventricular walls, neuronal hyperexcitability, diminished seizure threshold and synapse formation [27–34]. On the other hand, researchers have studied the effects of folic acid supplementation over the risk of developing preeclampsia [35–41], and a possible beneficial effect seems to exist, associated with an adequate placental development and growth, lower homocysteine levels and endothelial function improvement, resulting in a reduction in preeclampsia incidence [37,42–45].

In this work, we decided to investigate the hypothesis that a relationship exists between preeclampsia and the intake of distinct doses of folic acid during pregnancy (resulting in deficient, physiological and supra-physiological levels). Considering that preeclampsia is associated with increased placental oxidative stress, we investigated the cellular consequences of oxidative stress induction with *tert*-butylhydroperoxide (TBH) in trophoblast-derived cells cultured under different folic acid concentrations: deficient/low, physiological and supra-physiological. The concentrations of folic acid were chosen based on the human levels found in human plasma/serum under low/deficient, physiological and high concentrations. For this, the effect of TBH upon oxidative stress-related parameters, namely malondialdehyde and protein carbonyl levels and the activities of enzymatic (glutathione peroxidase) and non-enzymatic antioxidant defenses (glutathione levels) and upon cell viability, proliferation, migration and apoptosis index was assessed in cells cultured at distinct folic acid levels.

Trophoblast-derived cell lines such as BeWo and HTR-8/SVneo cell lines have been extensively used and proven to be effective in studying placental features [46–48], as they show morphological characteristics and hormonal secretions similar to normal human first and third-trimester trophoblast cells *in vivo*, respectively [46,49–51]. The BeWo cell line, used in the present work, shows most of the characteristics of villous trophoblast, including syncytial fusion [52,53], syncytin 1 and 2 regulation [54] as well as secretion of hormones such as hCG, hPL progesterone and estradiol [49]. The HTR-8/SVneo cell line, which we used in a few experiments, is an immortalized extravillous trophoblast cell line reported to exhibit proliferative and invasive characteristics similar to those of the parental cells [51].

2. Materials and methods

2.1. Materials

The following reactants were used: [^{3}H]-thymidine ([methyl-^{3}H]-thymidine; specific activity 79 Ci/mmol) (GE Healthcare GmbH, Freiburg, Germany), fetal calf serum (Gibco, Life Technologies Corporation, CA, USA), albumin from bovine serum, penicillin/streptomycin/amphotericin B solution, *tert*-butylhydroperoxide (TBH), decane, folic acid, folic acid-free RPMI 1640 medium, glutathione reductase from baker's yeast (*S. cerevisiae*), RPMI 1640 medium, malondialdehyde, β-NADH-Na_2 (nicotinamide adenine dinucleotide reduced disodium salt hydrate), β-NADPH (β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate), hydrochloric acid, ethyl acetate, ethanol, paraformaldehyde, sodium hydroxide, sodium pyruvate, sulforhodamine B (SRB), 2-thiobarbituric acid, trichloroacetic acid sodium salt, 2,4-dinitrophenylhydrazine, guanidine hydrochloride, trypsin-EDTA solution, ethylenediamine tetraacetic acid (EDTA) N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), 2-vinylpyridine (Sigma, St. Louis, MO, USA); L-glutamine, L-asparagine, perchloric acid and triton X-100 (Merck, Darmstadt, Germany); In Situ Cell Death Detection Kit, Fluorescein, 4′,6 Diamino-2-phenylindole (DAPI) (ROCHE Diagnostics, Germany).

2.2. Cell culture

The BeWo cell line was obtained from the Deutsche Sammulung von Mikroorganismen and Zellkulturen (Braunschweig, Germany) and HTR-8/SVneo cell line was a gift kindly donated by Prof. Charles H. Graham [51] (Department of Anatomy & Cell Biology, Queen's University at Kingston, Canada). The cell lines were cultured in RPMI 1640 medium (containing L-asparagine (0.05 g/L) and L-glutamine (0.3 g/L)) supplemented with 20 mM HEPES, 15% heat-inactivated fetal calf serum (BeWo cells) or 5% heat-inactivated fetal calf serum (HTR-8/SVneo) and 1% penicillin/streptomycin/amphotericin B (100 U/ml, 100 μg/ml and 0.25 μg/ml, respectively). BeWo and HTR-8/SVneo cells were maintained in a humidified atmosphere of 5% CO_2 at 37 °C and were used between passage numbers 17–39 and 117–130, respectively [55–57]. Cells were divided into three experimental groups, which were cultivated under three different concentrations of folic acid: a deficient/low concentration of folic acid (cells cultured in RPMI medium containing 1 nM folic acid), a physiological concentration (cells cultured in RPMI medium containing 20 nM folic acid) and a supra-physiological concentration (cells cultured in standard RPMI 1640 medium, which contains a high concentration of folic acid – 2.3 μM) [58–62]. Culture medium was changed every 2–3 days and split was realized every 7 days. For subculturing, BeWo and HTR-8SV/neo cells were removed enzymatically with trypsin 0.25% or trypsin-EDTA 0.05%, respectively. Afterwards, split 1:3 (BeWo) or 1:6 (HTR-8SV/neo) was done and cells were transferred to plastic culture dishes (21 cm^2; ø 60 mm; TPP®, Trasadingen, Switzerlan). Cell culture in physiological and deficient/low folic acid concentrations were obtained after an adaptation period of 4 weeks, as described previously by Tavares et al. [63]. For the experiments, BeWo and HTR-8SV/neo cells were seeded on 24-well plastic cell culture clusters (2 cm^2; ø 16 mm; TPP®, Trasadingen, Switzerlan) and cultured for 8 and 3 days, respectively.

2.3. Induction of oxidative stress with tert-butylhydroperoxide (TBH)

In order to induce oxidative stress, BeWo and HTR-8SV/neo cells were treated with TBH (50 μM, 100 μM or 300 μM; dissolved in decane), for 24 h, in serum-free culture medium. Similar concentrations were previously used in BeWo cells by our group [57]. Control cells were exposed to an identical concentration of decane (1% (v/v)) in serum-free culture medium.

2.4. Evaluation of oxidative stress

2.4.1. Quantification of lipid peroxidation (malondialdehyde) levels

The formation of thiobarbituric acid-reactive substances (TBARS assay) is used as a lipid peroxidation biomarker. At the end of the 24h-treatment period of BeWo cells (cultured at three different folic acid conditions) with TBH (or the respective solvent), the reaction was started by addition of 50% acetic acid to each sample, followed by a centrifugation for 2 min at 6000 rpm. Then, 1% 2-thiobarbituric acid was added to the supernatant and the reaction