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Vitamins C and E Inhibit Apoptosis of Cultured Human Term Placenta Trophoblast

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Abstract

Preeclampsia can be lethal to both mother and baby. The prominent symptoms of this syndrome are hypertension, proteinuria and oedema, resulting from an exaggerated aseptic systemic inflammatory response, triggered by placental factors shed into the maternal circulation. Syncytiotrophoblast microparticles (STBM) are one possible factor, shed when the placenta is exposed to stressors such as hypoxia/reperfusion. These can disrupt mitochondria, triggering apoptosis and necrosis, placental pathologies which are increased in preeclampsia. We tested the effects of antioxidant vitamins C (50 μM) and E (50 μM) on trophoblast in culture, using term villous cytotrophoblast preparations. Following Percoll gradient centrifugation and MHC class I expressing cell depletion of placenta digests, syncytial fragments were removed using anti-placental alkaline phosphatase antibody. This yielded cytotrophoblasts of consistently high purity. EGF (10 ng/ml) stimulated syncytialisation and hCG and progesterone production. However, mitochondrial induced apoptosis (MIA) was evident 96 h post-isolation, as mitochondrial membrane potential loss and caspase 9 and caspase 3 activation. ROCK-1 cleavage and syncytiotrophoblast particle shedding increased concurrently with apoptosis induction. Vitamins blocked MIA and syncytiotrophoblast particle shedding and significantly increased hCG ($p < 0.005$) and progesterone ($p < 0.02$) concentrations in culture supernatants, reflecting the increased survival rates. Although more cells survived in culture, syncytialisation rate (%) was significantly reduced ($p < 0.005$). We conclude that vitamins C and E can significantly reduce mitochondrial damage generated following syncytialisation *in vitro*. However, further work is required to determine whether antioxidant vitamins interfere with normal fusion processes.

Keywords: Cytotrophoblast; Syncytiotrophoblast; Mitochondria; Vitamins C and E; Apoptosis

1. Introduction

The pregnancy specific syndrome preeclampsia, arises from maternal endothelial dysfunction, which is part of a systemic inflammatory response, present in normal pregnancy but exaggerated in preeclampsia [1,2]. These changes are thought to be due to placental factors shed into the maternal circulation [2–4]. Cellular microparticles are shed from the plasma membrane of virtually all cell types when stressed or undergoing apoptosis. Microparticles are not inert but contain cell surface proteins and cytoplasmic components of the cell of origin. They can be pro-inflammatory and procoagulant, interacting with other cells, both locally and remotely, thereby comprising a novel systemic signaling mechanism [5]. Diseases with systemic inflammatory features are characterized by increased circulating microparticles of platelet, endothelial and other origins [5]. Syncytiotrophoblast microparticles (STBM) are a pregnancy specific type of microparticle and circulating levels are significantly increased in preeclamptic women compared to women with uncomplicated pregnancies [6]. Such microparticles cause endothelial cell dysfunction *in vitro* and stimulate the release of pro-inflammatory factors and hence may contribute to the pathogenesis of preeclampsia [7,8]. Apoptosis and necrosis of syncytiotrophoblast may be a major cause of increased shedding of pro-inflammatory placental debris in preeclampsia [9–12]. Increased oxidative stress and apoptosis of syncytiotrophoblast is known to occur. If it is severe,

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mitochondrial damage depletes ATP, blocks apoptosis and switches apoptosis to necrosis [13], with release of intracellular components [14]. Normal pregnancy is itself a state of oxidative stress [15], which is exacerbated in preeclampsia, in both the placental and maternal compartments [9,12]. Poor placental perfusion in preeclampsia secondary to spiral arteries disease is considered to be a major contributory factor [16].

The importance of hypoxia re-oxygenation as a placental stressor has been demonstrated *in vitro* where it increases mitochondrial dysfunction, release of reactive oxygen species (ROS) and apoptosis of syncytiotrophoblast [17] by the mitochondrial induced pathway [18]. Key components of the pathway are cytosolic cytochrome *c*, apoptosis protease-activating factor-1 (APAF-1), caspase 9 and caspase 3. In the final stages, APAF-1 and Rho-activated serine/threonine kinase (ROCK-1) (required for formation of membrane blebs and particle shedding) are cleaved by active caspase 3 [19,20]. APAF-1, caspase 9 and caspase 3, are found mainly in the syncytiotrophoblast layer and their expression increases in the third trimester [21]. Whereas oxidative stress is potentially harmful, low levels of ROS are necessary for formation of the syncytiotrophoblast layer. Cytotrophoblasts cultured at low O_2 fail to differentiate into syncytiotrophoblasts [22,23], and over-expression of the antioxidant enzyme Cu/ZnSOD by cytotrophoblasts decreased differentiation and syncytiotrophoblast formation [24,25].

The association of placental oxidative stress with preeclampsia has prompted the investigation of antioxidant therapy to prevent its onset by the administration of antioxidant vitamins C (ascorbic acid) and E (α-tocopherol) [26]. However, in a recent randomized placebo controlled trial there was no significant effect on the incidence of preeclampsia, but a significant decrease in the birthweight of babies born to women taking the vitamins [27].

In this study we aimed to determine the effects of vitamins C and E on human cytotrophoblast and syncytiotrophoblast *in vitro*, with particular regard to mitochondrial induced apoptosis in syncytiotrophoblast and the shedding of STBM. Cytotrophoblasts prepared from term placenta by the method of Kliman et al. [28] are significantly contaminated with syncytial fragments [29,30], which would be expected to contain activated caspase 3 [29] and confound the monitoring of apoptosis after plating. Hence, they were removed by an additional immmunodepletion step, which yielded highly pure villous cytotrophoblasts that syncytialised *in vitro* in the presence of EGF.

2. Materials and methods

2.1. Tissue

Term placentae ($n = 22$) were obtained with informed consent after delivery by elective caesarean section in the John Radcliffe Hospital, Oxford. Women with medical complications or previous perinatal deaths were excluded. Only singleton pregnancies without fetal abnormality or fetal growth restriction, i.e. <10th centile) and ≥37 weeks gestation were included. Maternal blood pressures were consistently <140/90 with no proteinuria (i.e. >2+ protein on two separate occasions) during the current pregnancy. The Central Oxford Research Ethics Committee approved this study.

2.2. Isolation and culture of human term villous cytotrophoblast

Villous cytotrophoblasts were prepared using a modification of our previous method [31]. After digestion partially pure cytotrophoblasts were isolated using a 5–70% Percoll gradient (Amersham Biosciences, Buckinghamshire, UK). The fraction between densities of 1.042 g and 1.068 g was aspirated, washed with MEM-F (Minimum Essential Medium (Invitrogen Ltd, Paisley, UK; cat no. 31095-029) containing 1% antibiotic and antimycotic solution (Sigma) and 10% FCS (heat inactivated fetal calf serum)) and pooled, before cells were counted. Cells were purified further by negative selection using immunomagnetic beads (Dynabeads® pan mouse IgG, Dynal Biotech Ltd, Wirral, UK) coupled to antibodies raised against MHC class I (clone W6/32; Serotec, Oxford, UK), to remove non-trophoblast cells, and placental alkaline phosphatase (PLAP) (NDOG2) (Table 1) to remove syncytial fragments [6]. The manufacturer's protocol for a bead-to-contaminating cell ratio of 1:4, assuming 50% contamination, was used. These surface antigens were chosen because they resist trypsin digestion [30].

For culture, the purified villous cytotrophoblasts were resuspended at 1.4×10^6 cells/2 ml MEM-F (12 well plates, Nunc) or 500,000 cells/500 μl MEM-F (four well plates, Nunc). Plates were pre-coated with fibronectin (10 μg/ml; Sigma). All cultures were maintained at 10% O_2 and 5% CO_2, and treated with recombinant human EGF (10 ng/ml; Bachem, Merseyside, UK) for the first 72 h of culture to induce syncytialisation. Separate cultures were carried out to test the effects of continued EGF supplementation over the entire 144 h culture period. No difference was found in cell viability with continued EGF use compared to EGF supplementation only in the first 72 h of culture (data not shown). Vitamin treated cells received vitamin C (50 μM L-ascorbic acid; Sigma) and vitamin E (50 μM (±)-α-tocopherol, prepared from synthetic phytol; Sigma) throughout the culture period. These doses were the maximum plasma levels measured in a controlled trial of vitamins C and E supplementation for preventing preeclampsia [32]. Culture medium and additives were refreshed every 24 h.

2.3. Flow cytometric analysis of cytotrophoblast preparation purity

Cell purity was analyzed flow cytometrically as described previously ($n = 22$ preparations) [31]. Cells were fixed in 4% paraformaldehyde (PFA; TAAB Laboratories, Berkshire, UK) in PBS (endotoxin free PBS, Sigma) and permeabilised (0.1% saponin in PBS), but live cells were retained to analyze Class I MHC. Cells were resuspended in PBS containing glucose (20 mM; Sigma) and 5% NHS (heat inactivated pooled normal human serum) and incubated (1 h, 4 °C) with primary FITC conjugated or unconjugated mouse monoclonal Abs (Table 1) followed by incubation (1 h, 4 °C) with FITC-goat anti mouse IgG when unconjugated antibodies were used. Ten thousand events were analyzed (Beckman Coulter Epics Altra flowcytometer, Expo 32 software). Gates were set on size versus fluorescence scatter plots so that ≤1% of cells stained positive in the appropriate negative controls (Fig. 1).

2.4. Immunofluorescence microscopy

Freshly isolated cytotrophoblasts were plated on fibronectin-coated four well plates. Where desmosomal protein was stained to quantify syncytialisation cells were first stained before fixation with MitoTracker Red (100 nM; Invitrogen; 15 min, 37 °C) in the dark (see below). Cells for desmosomal protein and active caspase 3 staining were fixed with ice-cold methanol (Fisher Scientific UK Ltd., Leicestershire, UK) and stored at 4 °C in PBS until processing. For NDOG2 Ab staining, cells were fixed in 4% PFA in PBS, otherwise cells were permeabilised by incubation in PBS containing 0.5% Triton-X 100 (Sigma) (10 min, room temperature (R/T)). All cells were blocked (1 h, R/T) with PBS containing 10% NHS and 0.1% Tween-20, then incubated overnight (O/N, 4 °C) with primary antibodies diluted in blocking buffer (Table 1) before washing (2 × 5 min) in PBS. Negative controls comprised mouse or rabbit IgG alone. Cells were next stained with Alexa 488 conjugated secondary antibody (Table 1) in blocking buffer, the nuclei stained with Hoechst 33342 (1 μg/ml in PBS; Invitrogen) and washed (2 × 5 min) and stored in