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# Placental protein tyrosine nitration and MAPK in type 1 diabetic pre-eclampsia: Impact of antioxidant vitamin supplementation ☆

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ABSTRACT

*Aim:* To examine the role of placental protein tyrosine nitration and p38-Mitogen-Activated Protein Kinase α (p38-MAPKα), Extra Cellular-Signal Regulated Kinase (ERK) and c-Jun NH2-Terminal Kinase (JNK) activity, in the pathogenesis of type 1 diabetic pre-eclampsia, and the putative modulation of these indices by maternal vitamin C and E supplementation.
*Methods:* Placental samples were obtained from a sub-cohort of the DAPIT trial: a randomised placebo-controlled trial of antioxidant supplementation to reduce pre-eclampsia in type 1 diabetic pregnancy. Placenta from placebo-treated: normotensive (NT) [n = 17], gestational hypertension (GH) [n = 7] and pre-eclampsia (PE) [n = 6] and vitamin-treated: NT (n = 20), GH (n = 4) and PE (n = 3) was analysed. Protein tyrosine nitration was assessed by immunohistochemistry in paraffin-embedded tissue. Catalytic activities of placental p38-MAPKα, ERK and JNK were measured by enzyme-linked immunosorbent assay (ELISA).
*Results:* Nitrotyrosine immunostaining was present in placebo-treated NT, GH and PE placentae, with no significant difference observed between the groups. There was a non-significant trend towards decreased p38-MAPKα activity in PE vs NT control placentae. ERK and JNK were similar among the three outcome placebo groups and vitamin supplementation did not significantly alter their activity.
*Conclusion:* Nitrotyrosine immunopositivity in normotensive diabetic placentae indicates some degree of tyrosine nitration in uncomplicated diabetic pregnancy, possibly due to inherent oxidative stress and peroxynitrite production. Our results suggest that p38-MAPKα, ERK and JNK are not directly involved in the pathogenesis of type 1 diabetic pre-eclampsia and are not modulated by vitamin-supplementation.


## 1. Introduction

Rates of pre-eclampsia are 2–4 times higher in type 1 diabetes mellitus versus non-diabetic pregnancy (Hadden, Alexander, McCance, & Traub, 2001). Increased oxidative stress is present in both type 1 diabetes and pre-eclampsia and has been hypothesized as a putative underlying mechanism (Martin-Gallan, Carrascosa, Gussinye, & Dominguez, 2003). The pro-oxidant peroxynitrite (produced from the reaction of nitric oxide with superoxide) modifies tyrosine residues in proteins resulting in nitrotyrosine residues. Although other nitrogen-related oxidants could result in the formation of nitrotyrosine, peroxynitrite is the most likely source *in vivo* (Beckman & Koppenol, 1996). Nitrotyrosine antibodies incorporated in immunohistochemical techniques were first used to demonstrate nitrotyrosine formation in human atherosclerotic plaques (Beckman et al., 1994), in lung tissue of acute respiratory distress patients (Haddad et al., 1994), Alzheimer's disease (Good, Werner, Hsu, Olanow, & Perl, 1996), and inflamed heart (Kooy, Royall, Ye, Kelly, & Beckman, 1995). Nitrotyrosine residues, localised specifically to vascular endothelium, smooth muscle and to a lesser extent syncytiotrophoblast, have been reported in non-diabetic placental tissue of pregnancies complicated by pre-eclampsia (Myatt et al., 1996). Nitrotyrosine residues have also been reported in the placentae of normotensive subjects with type 1 diabetes mellitus (Lyall et al., 1998). These findings would support the hypothesis that vascular damage could contribute to increased placental vascular resistance observed in pre-eclampsia. In addition the latter observations suggest that nitrated proteins can occur even in so called 'physiological' states, possibly due to the presence of other reactive nitrogen species which have the potential to nitrate tyrosine (Halliwell, 1997), as well as in the vasculature from women with pathophysiological conditions such as pre-eclampsia (Roggensack, Zhang, & Davidge, 1999).

p38-mitogen-activated protein kinase α (p38-MAPKα), along with other members of the MAP kinase pathways including Extra Cellular-Signal Regulated Kinase (ERK) and c-Jun NH2-Terminal Kinase (JNK), is involved in converting extracellular stimuli into

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specific cellular responses, including cell proliferation and differentiation (Bulavin et al., 1999; Daoud et al., 2005). Studies of p38-MAPKα knock out mice have demonstrated embryonic lethality at midgestation stages, in particular defective angiogenesis with loss of labyrinth, abnormal spongiotrophoblast layer and abnormal placental angiogenesis (Adams, et al., 2000; Mudgett et al., 2000; Tamura et al., 2000). Peroxynitrite treatment leads to tyrosine nitration of p38-MAPKα and loss of its catalytic activity (Webster, Macha, Brockman, & Myatt, 2006). Furthermore, pre-eclamptic placentae have demonstrated increased levels of nitrated, and decreased levels of catalytically active, p38-MAPKα (Webster, Brockman, & Myatt, 2006). To date the role of p38-MAPKα, ERK and JNK nitration in type 1 diabetic pre-eclampsia has not been determined.

The Diabetes and Pre-eclampsia Intervention Trial (DAPIT) examined the efficacy of antioxidant supplementation (vitamin C and E) in reducing the risk of pre-eclampsia in type 1 diabetic pregnancy (McCance et al., 2010). Using stored placenta from a DAPIT sub-cohort, which comprised a homogenous population of Caucasian women in Northern Ireland, the aim of the current study was to investigate the role of protein tyrosine nitration and p38-MAPKα, ERK and JNK activity in the pathogenesis of type 1 diabetic pre-eclampsia and their putative modulation by antioxidant (vitamin C and E) supplementation.

## 2. Methods

### 2.1. Study participants

Pre-eclampsia was defined as gestational hypertension with proteinuria using the International Society for the Study of Hypertension in Pregnancy guidelines, as previously described (Davey & Mac, 1988). Each case of hypertensive pregnancy was reviewed by the staff of the Trial Co-ordinating Centre, and diagnosis was confirmed by three senior clinicians, working independently and unaware of treatment allocation. Women received placebo or vitamin supplementation (vitamin C [1000 mg] and vitamin E [400 IU], administered on a daily basis from between 8 and 22 weeks gestation until delivery. The DAPIT inclusion and exclusion criteria have been described previously (McCance et al., 2010; Holmes et al., 2004).

### 2.2. DAPIT sub-cohort placental collection and preparation

After approval from the West Midlands Multi-Centre Research Committee (MREC/02/7/16), DAPIT placentae were collected by trained study personnel from a sub-cohort of 57 women with type 1 diabetes in Northern Ireland (between January 2004 to December 2008). One placental block (2 $cm^3$ section) was obtained from both the center and periphery (to potentially reduce any inherent structural disparity in placental composition), immediately after delivery, snap frozen in liquid nitrogen and stored at −20 °C, for MAPK activities and vitamin content analyses. In addition, formalin-fixed (4% formaldehyde), paraffin embedded placental blocks were prepared immediately for immunohistochemical analysis for the presence of nitrotyrosine residues (representative of peroxynitrite activity).

### 2.3. Immunohistochemical analysis of global protein tyrosine nitration

Sections 5 μm thick were cut from formalin-fixed, paraffin embedded blocks, deparaffinized in xylene and rehydrated through a series of graded alcohols. Sections underwent microwave antigen retrieval in 0.01 M citric acid buffer, pH 6.0, over sequential (5, 10 and 10 min) exposures.

Protein tyrosine nitration was detected by means of a commercially available kit (Labelled Streptavidin-Biotin-2: Dakocytomation, Glostrup, Denmark) according to the manufacturer's instructions. The specificity of the anti-nitrotyrosine antibody for placental tissue was confirmed with serial dilutions of the anti-nitrotyrosine antibody to determine the optimum antibody dilution for immunohistochemical detection of nitrotyrosine in placental tissue (data not shown). In brief, following blockade of endogenous peroxidase, avidin and biotin, sections were incubated at room temperature with a well validated monoclonal anti-nitrotyrosine (Myatt et al., 1996) antibody (Upstate Biotechnology, USA), 1:100 in phosphate-buffered saline (PBS), 1% bovine serum albumin (BSA). Sections were then treated (10 min at room temperature) with biotinylated anti-mouse/anti-rabbit immunoglobulin followed by a streptavidin–horseradish peroxidase conjugate, as per the manufacturer's instructions. Peroxidase activity was developed in 3,3′diaminobenzidine (DAB) for 10 min. Slides were counterstained with Gill's haematoxylin for 1 min, then dehydrated through a series of graded alcohols (70%–100%), and cleared in ammonia water and xylene before being mounted for microscopy. Sections from mouse liver tissue which were confirmed to express high levels of nitrotyrosine (MacMillan-Crowe, Crow, Kerby, Beckman, & Thompson, 1996), served as positive control tissue for immunohistochemical detection of the nitrotyrosine antibody. For each immunohistochemical run, control tissue (placenta/mouse liver) was incubated with PBS, 1% (w/v) BSA containing mouse immunoglobulin G2b (Dakocytomation) solution of an equivalent total protein concentration.

Following global protein tyrosine nitration immunohistochemistry, sections were examined by light microscopy for positivity (indicated by brown staining) and scored in a semi-quantitative fashion by two independent observers blinded to their identity (PJ and KP). Where there was disagreement between these two observers, the specimen was referred to a third observer (AMG). The intensity of nitrotyrosine immunopositivity was scored on a four-mark scale: negative (0), weakly positive (1), moderately positive (2) or intensely positive (3). The proportion of tissue demonstrating positivity was also graded semi-quantitatively according to the following scale, negative (1), ≤25% positive (2), 25%–50% positive (3) and ≥50% positive (4). A histoscore representing the product of the intensity and proportion scores was calculated for each section. The resultant histoscores (0–12) were grouped as negative (histoscore 0), low (histoscore 1–4), moderate (histoscore 5–8) or high (histoscore 9–12) (Cadden et al., 2007; Helin, Isola, Helin, Helle, & Krohn, 1989; Mizuno et al., 2006).

### 2.4. Vitamin C and E analysis

Placental lysates were prepared for vitamin C and vitamin E analysis with buffer containing 1:100 butylated hydroxytoluene (200 μM; BHT/PBS) per 0.1 g of placental tissue. Vitamin C was measured in placental lysates stabilised in a 1:10 solution of 5% metaphosphoric acid (MPA)(w/v), using a fluorometric assay (Vuilleumier & Keck, 1989) on a Cobas FARA centrifugal analyzer Roche Diagnostics, Switzerland. Vitamin E was measured by high performance liquid chromatography (HPLC) with UV detection at 292 nm (Craft, 1992), in placental lysates. Assays were standardized against appropriate National Institute of Standards and Technology reference materials. Maternal plasma and cord ascorbate (vitamin C) and serum α-tocopherol (vitamin E) [expressed per mmol of serum cholesterol] were analyzed as described previously (McCance et al., 2010).

### 2.5. P38-MAPKα, ERK and JNK activities

Placental lysates were prepared by homogenising placental tissue using a hand held homogeniser, for 3 min in 1× lysis buffer (containing protease and phosphatase inhibitors;catalogue no: 9803