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# The placental immune response is dysregulated developmentally vitamin D deficient rats: Relevance to autism

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ABSTRACT

Emerging evidence suggests that maternal or developmental vitamin D (DVD) deficiency is a risk factor for Autism Spectrum Disorders. A well-established association has also been found between gestational infection and increased incidence of autism. Placenta mediates the maternal immune response in respect to the foetus. The placenta is also a major source of vitamin D and locally produced vitamin D is an essential regulator of immune function during pregnancy. Here we investigate the effects of DVD-deficiency on baseline placental immune status and in response to the well-known viral and bacterial immune activating agents polyriboinosinic-polyribocytidylic acid (poly(I:C) and lipopolysaccharide (LPS). We show DVD-deficiency does not affect baseline inflammatory cytokines in placenta. However, when challenged with poly(I:C) but not LPS, DVD-deficient placentas from male foetuses had higher production of IL-6 and 1L-1β compared to control placentas. This suggests the developing DVD-deficient male foetus may be particularly vulnerable to maternal viral exposures. This in turn may have adverse implications for the developing male brain. In conclusion, a dysregulated placental immune response may provide a plausible mechanism for both the epidemiological links between DVD-deficiency and increased male incidence of developmental conditions such as autism.

## 1. Introduction

During pregnancy, adverse environmental factors may affect the development of the foetus. This can lead to long-term impacts on later health and disease [1]. One early environmental risk factor of increasing interest is maternal vitamin D deficiency. Vitamin D deficiency appears to be particularly common among pregnant women and has been implicated in a number of developmental conditions related to bone formation [2]. We have been studying the effects of developmental vitamin D (DVD) deficiency on brain development for more than 15 years [3]. In earlier publications, we proposed that maternal vitamin D deficiency may increase the risk of autism [4,5]. Increasingly, DVD-deficiency is being linked with several neurodevelopmental disorders [6–8]. More recently we have shown that DVD-deficiency at birth is associated with both autism-related traits [9] and clinical diagnosis of autism in children [10] in large population cohorts. Vitamin D regulates several potential mechanisms believed to be involved in the development of autism, including regulation of neurotransmitters such as glutamate, gamma-aminobutyric acid and serotonin [11,12]. Vitamin D also controls cell division and apoptosis during brain development consistent with its anti-proliferative and pro-apoptotic properties [13,14]. Therefor developmental deficiency of vitamin D may lead to brain abnormalities seen in autistic patients (for review see Ali et al. [15]).

Another widely reported prenatal environmental risk factor for neurodevelopmental disorders is maternal infection. Mild to persistent maternal infection can lead to long-term brain-related outcomes in newborns [16]. A study from Danish birth records has shown significant increased risk of autism in children born to the mothers who were infected with either viral or bacterial agents during pregnancy [17,18]. Postmortem brains from autistic individuals have increased levels of inflammatory cytokines [19]. In animal models of maternal immune activation (MIA), the offspring of maternal mice injected with lipopolysaccharide (LPS) as a bacterial mimic or polyriboinosinic-polyribocytidylic acid (poly(I:C) as a viral mimic develop brain pathology and behavioural phenotypes consistent with autism (reviewed in: -[20]). Of direct relevance to the work proposed in this study, the simultaneous acute administration of 125 dihydroxyvitamin D ($125(OH)_2D$) to pregnant mice when they receive poly(I:C) prevents autism-related phenotypes in their offspring [21]. Moreover, recent clinical trials have also shown that vitamin D supplementation improves core autism symptoms in children [22–24].

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Vitamin D is a potent anti-inflammatory agent which inhibits several key molecular pathways involved in the production of inflammatory cytokines [25]. An immunosuppressive role for vitamin D during conception and pregnancy is well-established [26,27]. However, the specific mechanisms by which prenatal infection may interact with vitamin D deficiency to affect foetal programming is still relatively unexplored.

As the placenta contains immune regulatory cells such as neutrophils and macrophages it is uniquely positioned to maintain intrauterine homeostasis against maternal immune challenges [28]. Several studies in both mothers and pregnant rodents suggest critical role of the placenta in foetal programming and transmission of maternal immune responses to the foetus [29,30]. Impairments of placental function can have deleterious impacts on foetal brain development [31]. The placenta is also a major site of vitamin D synthesis during pregnancy. Placental trophoblast cells express both the enzyme 1-α-hydroxylase (CYP27B1) which synthesises the active vitamin D hormone (1, 25$(OH)_2$D) and the vitamin D receptor (VDR). It has been shown that 1, 25$(OH)_2$D locally synthesized in the placenta promotes anti-inflammatory actions via expression of VDR [32].

In cases of maternal deficiency, the absence of available dietary vitamin D (Cholecalciferol) will lead to diminished synthesis of the active hormone and may well compromise the ability of the placenta to respond to infection. In this study, we investigated the impact of DVD-deficiency on placental immune function. We have analysed the production of inflammatory cytokines in placentas from vitamin D deficient and control dams both at baseline and in response to *ex vivo* LPS and poly(I:C) exposures. We also studied putative molecular mechanisms involved in the recognition of pathogens such as receptors of the innate immune system and other molecules underlying cytokine synthesis.

## 2. Materials and methods

### 2.1. Subjects

All procedures performed in the study were approved by the University of Queensland ethics committee under the guidelines of National Health and Medical Research Council of Australia. Female Sprague-Dawley rats (Animal Resource Centre, Western Australia) were housed in opaque Makrolon cages (510 mm × 330 mm × 190 mm) with wire lids. Animals were kept at a constant temperature of 21 °C ± 2 and 60% humidity on a 12 h light/dark cycle. Gestational vitamin D deficiency was induced in female rats by feeding a vitamin D deficient diet from the age of 4 weeks (Speciality Feeds, WA) for a period of six weeks prior to mating at 10 weeks of age. This diet does not affect sera levels of calcium and phosphorus in dams. Pregnant animals remained on a vitamin D deficient diet throughout gestation until placentas are harvested. Control females were fed standard rat chow which contains cholecalciferol (AIN93G) during the entire process.

### 2.2. Tissue collection

At embryonic day (E) 18, pregnant rats were euthanized by single intraperitoneal injection of Lethobarb (Virbac Australia). Maternal blood was collected from inferior vena cava and subsequently, serum was separated. Maternal vitamin D deficiency was confirmed by measuring sera 25-hydroxyvitamin D levels. The uterus was extracted from pelvic cavity under sterile conditions and the placenta of each foetus was collected. Tails were sampled from the matching foetus to determine the sex of the foetus that each placenta was exposed to. This was done via polymerase chain reaction using sex-determining region Y (SRY) specific primers [33]. To assess the effects of locally produced steroids on placental immune response to inflammatory agents, placentas from male foetuses positioned between two neighbouring males (2M-males) or between two neighbouring females (0M-males) were selected. Similarly for female placentas, only female foetuses positioned between females on either side (2F-females) or between males (0F-females) were selected (Fig. 1). The baseline levels of cytokines in placenta were measured independent of intrauterine position.

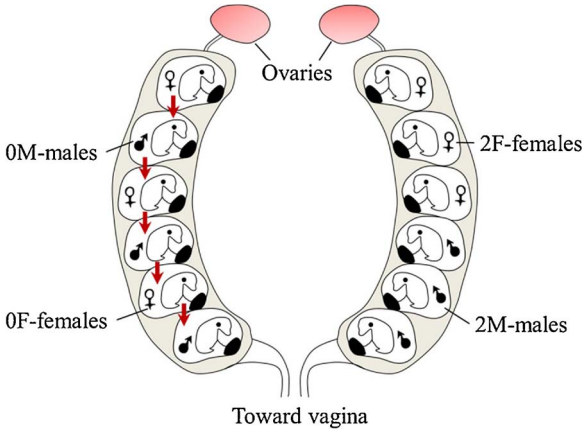


**Fig. 1.** "Schematic representation of both uterine horns in a litter bearing mammal illustrating the transport of steroids in the uterus".
Intrauterine position can affect exposure to either androgens or estradiol by transport of steroids via amniotic fluid between adjacent foetuses [34]. The direction of this transport is predominantly toward vagina as shown by red arrows. Male foetuses which were positioned between males are denoted as 2M-males and between female foetuses as 0M-males. Similarly, female foetuses surrounded by females or males are referred as 2F-females and 0F-females respectively.

### 2.3. Baseline cytokine assay in placenta and maternal sera

A customised multi-spot immunoassay system (proinflammatory rat panel 1) was purchased from Meso Scale Discovery (MSD Rockville MD USA) to measure levels of IL-1β, IL-6, IL-10 and TNF-α within one sample. The kit was supplied with lysis buffer (R60TX-2), lyophilized calibrator to develop standard curve and internal controls. The method was followed according to the instructions provided in the product insert. Lyophilised standard was reconstituted in assay buffer and serially diluted to obtain desired concentration to develop a standard curve. Samples were prepared by homogenizing the whole placenta in lysis buffer (1:10 w/v). Homogenates were centrifuged at 13,000 revolutions per min (rpm) for 20 min at 4 °C and supernatants were carefully collected and stored at −70 °C. A total of 150 µg of placental protein or 50 µl of maternal sera, standards or controls were added per well and the plate was incubated overnight on shaker at 4 °C. The wells were washed using wash buffer followed by assay diluent containing 10 SULFO-TAG™-labelled, cytokine-specific detection antibodies and re-incubated at room temperature for 2 h. The wells were washed again and the supplied buffer added and plate fluorescence detected in a SECTOR Imager 2400.

### 2.4. IL-17 assay

At the time of testing rat IL-17 could not be incorporated in the multi-spot immunoassay platform. Therefore the level of IL-17 in the cultured placental slices was measured by ELISA (Abcam-ab119536) following the manufacturer's instructions. Prepared samples and standards (100 µl) were loaded into each well followed by addition of 50 µl of biotin conjugate. Plate was covered with adhesive film and incubated for 2 h at room temperature on a shaker set at 400 rpm. In the following steps, streptavidin-HRP (100 µl) and tetramethylbenzidine (100 µl) were added and plate was incubated at room temperature for 30 min. Reaction was stopped by adding 100 µl of stop solution into each well. Plate was read using POLARstar OPTIMA reader (BMG Labtech). Level of IL-17 was determined using a standard curve.