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Mild maternal iron deficiency anemia induces DPOAE suppression and cochlear hair cell apoptosis by caspase activation in young guinea pigs

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ABSTRACT

Iron deficiency (ID) anemia (IDA) alters auditory neural normal development in the mammalian cochlea. Previous results suggest that mild maternal IDA during pregnancy and lactation altered the hearing and nervous system development of the young offspring, but the mechanisms underlying the association are incompletely understood. The objective of this study was to evaluate the role of apoptosis in the development of sensory hair cells following mild maternal IDA during pregnancy and lactation. We established a maternal anemia model in female guinea pigs by using a mild iron deficient diet. The offspring were weaned on postnatal day (PND) 9 and then was given the iron sufficient diet. Maternal blood samples were collected on gestational day (GD) 21, GD 42, GD 63 and PND 9, serum level of iron (SI) or hemoglobin (Hb) was measured. Blood samples of pups were collected on PND 9 for SI measurement. On PND 24, pups were examined the distortion product otoacoustic emission (DPOAE) task, and then the cochleae were harvested for assessment of apoptosis by immunohistochemistry of cysteine–aspartic acid proteases 3/9 (caspase-3/9) and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay, and by double immunofluorescence for the colocalization of TUNEL and caspase-3. Blood samples of pups were collected on PND 24 for SI and Hb measurements. Here we show that mild maternal IDA during pregnancy and lactation resulted in hearing impairment, decreased

Abbreviations: Caspase-3 (Caspase-9), cysteine–aspartic acid proteases-3 (cysteine–aspartic acid proteases-9); DAB, diaminobenzidine; DPOAE, distortion product otoacoustic emission; GD, gestational day; Hb, hemoglobin; ID, iron deficiency; IDA, iron deficiency anemia; IHCs, inner hair cells; OHCs, outer hair cells; PBS, phosphate-buffered saline; PND, postnatal day; SI, serum level of iron; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling.

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hair cell number, caspase-3/9 activation and increased apoptotic cell number of young guinea pigs. These results indicate a key role for apoptosis in inhibition of hair cell development, caused by mild maternal IDA during pregnancy and lactation.

1. Introduction

Iron is crucial in multiple aspects of neurodevelopment (Georgieff and Innis, 2005; Lozoff, 2000). Iron deficiency (ID) is the most prevalent forms of malnutrition and the leading cause of anemia in the world (Stoltzfus, 2003). Iron deficiency anemia (IDA) is a common health problem among populations, particularly among infants, young children and childbearing age women (McLean et al., 2009). An estimated 25% of pregnancies worldwide are thought to involve IDA (Stoltzfus, 2003), because their nutritional demand increases in response to fetal and neonatal development (Jougleux et al., 2011). It is increasingly recognized that in utero and lactational IDA is associated with long-lasting detrimental effects on general nerve conductivity (Amin et al., 2010), behavior (Lee et al., 2012), and auditory function (Jougleux et al., 2011, 2013). Many of the impairments are progressive and usually irreversible, despite subsequent iron gain, suggesting a critical window of vulnerability exists during early development (Kwik-Uribe et al., 2000; Lozoff et al., 2000).

In contrast to the many studies on IDA, far less is known about the impact on cochlear hair cell development of mild IDA during the critical period. Development of the guinea pig cochleae during the perinatal and early postnatal periods is critical for establishment of normal function of the adult auditory system (Ohmura and Yamamoto, 1990). During these periods, the loss of hair cells will result in a significant decrease in the number of adult hair cells (Cotanche, 2008), even lead to permanent hearing impairment (Hori et al., 2009). In addition, progressive development is seen in the fetal auditory nervous system during the last trimester of pregnancy (Henley and Rybak, 1995). Distortion product otoacoustic emission (DPOAE) is sounds recorded in the ear canal, has been widely accepted to be generated in the cochlea as a byproduct of the actions of the cochlear amplifier (Kemp, 1979). This measure is also an ideal assessment of the effects of personal music system use (Torre et al., 2013). The outer hair cells (OHCs) are particularly vulnerable to acoustic overstimulation, and are the dominant source of DPOAEs in the mammalian cochlea (Cho et al., 2013). An emerging study suggests that mild IDA during pregnancy and lactation in guinea pigs causes auditory dysfunction in the offspring (Jougleux et al., 2013). But this emerging research did not ascertain whether inhibition of hair cell development in the offspring resulted from mild IDA during pregnancy and lactation. Thus, there is an urgent need to gain insight into the impact cochlear hair cell structure and function of mild IDA.

An in vitro test has explored that iron chelators induce cell death through its activation of a mitochondrial caspase pathway (Greene et al., 2002). Apoptosis is a crucial physiological determinant of embryonic and neonatal development (Wang and Han, 2009), and is known to play an essential role in the development of the inner ear structures (Schmutzhard et al., 2009). Caspase-9 is the key initiator caspase for the intrinsic pathway to apoptosis. Upon cleavage and activation from its proform, caspase-9 cleaves and activates caspase-3, which has been recognized as a reliable marker for the identification of apoptosis (Yan et al., 2009). Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining is a method for the detection of DNA damage and identifying apoptotic cells (Sträter et al., 1995). Nevertheless, until now little is known regarding the involvement of hair cell apoptosis in the offspring following mild IDA during pregnancy and lactation. Consequently, the present study was designed to test the effects of mild maternal IDA during pregnancy and lactation on cochlear hair cell function in young guinea pigs by DPOAE to determine the potential hearing impairment, and on cochlear hair cell morphology by immunodetection to detect apoptosis marker and DNA fragmentation marker.

2. Materials and methods

2.1. *Animals and diets*

Procedures involving laboratory animals were performed in accordance with the general principles of the Institutional Animal Care and Use Committee. All protocols were approved by China Medical University Institutional Animal Care and Use Committee. Because unlike smaller rodents, guinea pigs and human have a critical period of cerebral development during pregnancy (Jougleux et al., 2011), moreover albino guinea pigs are more susceptible to ototoxicity and less susceptible to possibly confounding systemic toxicity than pigmented ones (Schweitzer, 1993), we used albino guinea pigs (female, $n = 12$; male, $n = 6$) aged 12 week (the Center for Experimental Animals at China Medical University) with a normal Preyer's reflex. This reflex is the elicitation of startle response to auditory stimuli, which has been used for the evaluation of hearing in rodents and other animals (Jero et al., 2001). Animals were housed with a controlled temperature of $22 \pm 2\,^{\circ}C$, a relative humidity of $55 \pm 15\%$, and a 12-h light/dark cycle. The guinea pigs were ranked according to body weights, and those from the extremes of the distribution were removed from the population until only the number of animals required for the study remained. These female guinea pigs were assigned to one of two dietary groups: the first group was fed iron sufficient diet (iron content: 112.16 ± 34.90 mg/kg diet) and given tap water (control, $n = 6$). The other group was fed iron deficient diet (IDA, $n = 6$). Iron content was detected at 6.15 ± 2.32 mg/kg feed. These ingredients were based on prior studies (Leblanc et al., 2009; Jougleux et al., 2011). After three weeks, two female guinea pigs with one proven male guinea pig were placed for mating at the same time. The day when the vaginal plug was discovered was considered as gestational day (GD) 0. A total of 36 pups were born (control: $n = 19$, IDA: