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Prenatal hypoxia, habituation memory and oxidative stress

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ABSTRACT

Hypoxia–ischemia (HI) is characterized by a reduced supply of oxygen during pregnancy, which leads to both central nervous system and peripheral injuries in the foetus, resulting in impairment in its development. The purpose of this study was to investigate behavioural changes and systemic oxidative stress in adult animals that have been affected by HI during pregnancy. HI was induced by the occlusion of the maternal uterine artery with aneurysm clamps for a period of 45 min on the 18th gestational day. Animals from the sham group were submitted to same surgical procedure as the HI animals, without occlusion of the maternal uterine artery. The control group consisted of non-manipulated healthy animals. At postnatal day 90, the pups were submitted to behavioural tests followed by blood collection. HI adult animals presented an increase in anxiety behaviour and a lack of habituation compared to both sham and control groups. Oxidative damage, assessed by protein and lipid oxidation in serum, did not differ between HI and sham-operated animals. However, HI animals presented reduced activity of the glutathione peroxidase enzyme and increased formation of nitrite, indicating alterations in the systemic antioxidant repair system. Our results suggest an association among HI, systemic oxidative stress and behavioural alterations.

1. Introduction

Hypoxia–ischemia (HI) can be described as an event resulting in a decrease in cerebral perfusion with subsequent low oxygenation and nutrient delivery. When HI occurs during the prenatal period, it can impair neurodevelopment and lead to elevated neonatal morbidity and mortality (Letourneur et al., 2012). In humans, the incidence of low birth weight and prematurity in neonates who have undergone a systemic asphyxia event during pregnancy is about 60% (Vannucci, 2000). Between 20% and 50% of hypoxia newborn infants who present encephalopathy die within the first months of life, and more than 25% show permanent brain damage (Vannucci and Hagberg, 2004). At long-term follow-up, prenatal HI may cause deficits in learning and concentration, hyperactivity and memory impairment in children, and it may also lead to cerebral palsy and epilepsy (Marin-Padilla, 1997, 1999, 2000). The causes of HI are not always identified in humans, although it is often observed in cases of maternal smoking, anaemia, gestational hypertension, diabetes mellitus, infection, multiple births and drug abuse (Pundik et al., 2006).

Most of the current understanding of the mechanisms involved in brain damage induced by HI is derived from animal models. The key event is obviously oxygen deprivation, which affects brain metabolism. A severe HI event may deplete tissue energy reserves, leading to short-term biochemical events such as acidosis, glutamate excitotoxicity, nitric oxide (NO) production and oxidative stress in the central nervous system. This, in turn, may contribute to delayed neuronal formation, accelerated apoptosis and inflammation (Peeters-Scholte et al., 2002; Vannucci and Hagberg, 2004). Increased oxidative stress during pregnancy may result from an increase in mitochondrial activity, probably acting as a compensatory mechanism to the reduced oxygen supply during hypoxia, and also due to reperfusion and reoxygenation after this event (Reddy et al., 2011; Weis et al., 2012).

The generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) has been implicated in the pathogenesis of white matter injury in premature infants due to cell death by reactive astrocytes expressing inducible nitric oxide synthase (iNOS) (Volpe, 2003). Furthermore, a study by Li et al. (2004) shows that intermittent hypoxia in rats induces short-term increased expression and activity of iNOS in brain tissues, and that neurobehavioural deficits in the water maze were attenuated in iNOS knockout mice (Li et al., 2004).

It is well known that the NO radical reacts with superoxide anion, generating the potent antioxidant peroxynitrite. On the other hand, antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) are the first defence protecting against molecular and cellular damage caused by ROS (Valença et al., 2009). To our knowledge, there are no studies of systemic oxidative status and nitric oxide production in prenatal HI.

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Thus this study was designed to investigate oxidative stress in the bloodstream of adult animals that have been affected by HI during the intrauterine period, in addition to the habituation of memory formation, anxiety and motor behaviour that characterize these possible alterations as long-term effects of this injury.

2. Material and methods

2.1. Ethical approval

This study was reviewed and approved by the Ethics Committee of Animal Experiments of Rio de Janeiro State University (certification CEUA/028/2011).

2.2. Rat prenatal hypoxic–ischemia insult

The mating procedure was achieved by placing virgin female Wistar rats, aged 3–4 months, in a cage with males in a 3:2 proportion for 8 h. To define the first day of pregnancy, vaginal smears were used to confirm the presence of spermatozoids. Fertilized females were divided into groups of 4–5 per cage, and were kept in a temperature controlled (23 ± 1 °C) room with a 12 h normal light/dark cycle (lights on at 6 p.m.), with water and food *ad libitum*.

The surgical protocol was adapted from Robinson et al. (2005). Pregnant rats were anaesthetised intraperitoneally with Avertin® (0.5 g tribromoethanol + 0.31 mL 2-methyl-2-butanol + 39.5 mL 0.9% saline) in titrated doses up to a maximum dose of 300 mg/kg, on day 18 of pregnancy (E18). A midline laparotomy was performed, the uterine horns were exposed, and the four uterine arteries were clamped with aneurysm clips. After 45 min, the aneurysm clips were removed, the uterine horns were put back into the abdominal cavity and the wound was closed in layers with suture. In the sham-operated animals, as a surgical control group, the females had their uterine horns exposed for 45 min, but no arteries were clamped.

Dipirone was injected intraperitoneally for post-operative pain relief (100 mg/kg). The females were monitored until they recovered from the anaesthesia, and then returned to the cage in a standard condition with water and food *ad libitum*. Gestation continued normally until the 23rd day.

Foetal loss was 35.2% for the HI group, and 27.2% for the sham group, which was not significantly different. After birth, the number of pups was randomly restricted to 8 per brood, to facilitate equal breastfeeding. After weaning, at postnatal day 30 (P30), male rats were separated and housed with 6 in each cage for further experiments. Both behavioural assessment and oxidative stress analysis were performed on postnatal day 90 (P90).

2.3. Behavioural assessment

A third additional group was included in the behavioural tests as controls. The animals in this control group were male Wistar rats that were not subjected to any surgical intervention.

2.3.1. Open field test

This test was performed to evaluate locomotor activity, anxiety level and habituation response of the animals when placed in a new environment. The testing apparatus consisted of a square polypropylene box divided into 16 squares of equal size, of which 12 were peripheral (P) and 4 central (C). Each animal was placed in one of the corners and its behaviour was recorded for 3 min with a digital video camera for posterior analyses. The animal was removed from the box, which was then cleaned and dried for the next test session. Each animal performed the test twice, 24 h apart, under the same conditions of temperature, luminosity, cleaning and visual cues.

The ambulation in C and P squares was measured, and one square was counted only when the animal placed all four paws on it. Locomotor activity was calculated as the number of total squares crossed by the animals (C + P). Anxiety behaviour was calculated as the percentage of activity in the central area, with C and P squares crossed by the animals divided by 4 (C/4) and 12 (P/12), respectively, to avoid misinterpretations of the locomotor activity of the animals (Filgueiras et al., 2009). Therefore, anxiety behaviour was calculated as [(C/4) / (C/4 +P/12) ∗ 100]. To verify habituation memory, locomotor activity (C + P) was compared between tests 1 and 2 (Vianna et al., 2000).

2.3.2. Elevated plus maze test

This is a well-established and widely used method to test anxiety levels in animals exposed to a conflict situation between exploring the open elevated arms and a natural tendency to hide in the enclosed arms (Bhattacharya and Satyan, 1997; Korte and De Boer, 2003). The test consists of an acrylic apparatus in the shape of a cross formed by four arms: 2 open (OA) and 2 closed (CA), with the high ground at a standard height. The animals were placed in the arms intersection of the apparatus, facing one of the CA in the beginning of each test, and they could move freely. Each test lasted three minutes. The number of entries in both OA and CA and the time spent in OA were recorded for the calculation of the following variables. Exploratory behaviour was calculated as the percentage of entries in OA [entries in OA/(entries in OA + entries in CA) × 100]. The total time spent in the OA was used as a measure of anxiety behaviour (Anand et al., 2012). The percentage of entries in OA was calculated by this formula [entries in OA / (entries in OA + entries in CA) × 100]. The percentage of entries in OA, and the total time spent in OA were classified as measures of anxiety (Anand et al., 2012). One entry was considered when the animal entered with four paws in the arm.

2.4. Oxidative stress analysis

At P90, at least 48 h after behavioural tests, the animals were anaesthetised with sodium thiopental (70 mg/kg, i.p.) and blood was collected by aortic puncture for biochemical analysis. Serum was obtained by centrifugation of whole blood at 2500 rpm for 15 min and stored at −80 °C until analysis. Total protein content was determined by the Bradford method (Bradford, 1976).

2.4.1. Determination of the activity of serum antioxidant enzymes

Total superoxide dismutase (SOD) activity was assayed by measuring the inhibition of adrenaline auto-oxidation at 480 nm (Bannister and Calabrese, 1987). Catalase (CAT) activity was measured in terms of the rate of decrease in hydrogen peroxide (H_2O_2) at 240 nm according to the Aebi method (Aebi, 1984). Glutathione peroxidase (GPx) activity was evaluated according to Flohé and Günzler (1984), by monitoring the oxidation of NADPH measured spectrophotometrically at 340 nm in the presence of H_2O_2.

2.4.2. Nitrite assay

Serum nitrite levels, an indirect measurement of nitric oxide content, were measured spectrophotometrically (540 nm) by a method based on the Griess reaction (Valença et al., 2009).

Table 1
Anxiety behaviour assessed by open field test.

	Control (n = 16)	Sham (n = 15)	HI (n = 16)
Test 1 (%)	10.0 ± 2.2	11.3 ± 3.3	9.6 ± 3.1
Test 2 (%)	1.8 ± 1.0*	2.5 ± 1.2*	3.2 ± 1.7

Anxiety behaviour analyzed by formula [(C/4) / (C/4 + P/12) × 100]. Data presented as mean ± SE. HI, hypoxic–ischemic group; C, central squares; P, peripheral squares.
*Different from test 1 ($p < 0.05$).