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Ultrasound exposure of the foetal chick brain: effects on learning and memory

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ABSTRACT

Ultrasound imaging of the brain is routinely used to monitor the development and resolution of brain lesions among premature and compromised newborn human babies. However, animal studies have shown that ultrasound can cause damage to developing foetal and neonatal tissues. In this study we investigated if ultrasound of the chick brain can lead to learning and memory impairment after hatch. We exposed the brains of chicks on day 19 of a 21 day incubation period to 5 or 10 min of B-mode, or to 1, 2, 3, 4 or 5 min of pulsed Doppler ultrasound *in ovo*. Learning and memory function were assessed at day 2 post-hatch. Our results show that B-mode exposure at E19 does not affect memory function. On the other hand, 2 h after training, significant memory impairment occurred following 4 and 5 min of pulsed Doppler exposure at E19. In separate groups of chicks, short-, intermediate- and long-term memory was equally impaired suggesting an inability to learn. Further, the chicks were still unable to learn with a second training session 5 min after completion of the initial testing. These results demonstrate that extended exposure to pulsed Doppler ultrasound can adversely affect cognitive function in the chick when exposure occurs close to the time of hatch.

1. Introduction

The developing brain is particularly susceptible to injury from ischaemia, infection and a range of inflammatory and neurotoxic insults in humans. Gestational insults and problematic deliveries can lead to a variety of brain pathologies, including haemorrhages, cysts and lesions. A range of imaging modalities is available to diagnose and monitor such pathologies among newborns. These include computed tomography (CT), magnetic resonance imaging (MRI) and ultrasound.

While MRI and CT examination of neonatal brains have higher sensitivity and specificity than ultrasound (Blankenberg et al., 1996, 2000; Mirmiran et al., 2004; Rooks et al., 2008) ultrasound is considered the ideal first-line neuro-imaging tool for newborns as it provides non-invasive, cost-effective bed-side imaging without radiation or the need for sedation or anaesthesia (Maalouf et al., 2001). Because in newborns scanning can be carried out via the fontanelle without overlying bone, high quality images of brain tissue can be obtained. As ultrasound technology has advanced, so have the applications of this modality in neonatal paediatrics. Today, a large range of anatomical and haemodynamic features can be assessed and most brain pathologies detected using ultrasound (Inder et al., 2003). Because some pathologies develop only after birth, serial cranial ultrasound scans are required during the period from birth until discharge in order to accurately monitor the development or resolution of any abnormality (Perlman and Rollins, 2000; Ment et al., 2002). There is currently no consensus on the optimal timing and number of scans such neonates should receive. Protocols vary between different neonatal units (Leijser et al., 2006).

The demand for improved image resolution has seen a sharp increase of the power outputs of modern ultrasound machines (Henderson et al., 1995, 1997). The intensities of some examinations are capable of producing damage to sensitive developing foetal or neonatal tissue and this is of particular concern during pulsed or colour Doppler imaging when blood flow in the cerebral arteries is investigated using a stationary beam. The damage is believed to be due to thermal and mechanical factors causing either an increase in the tissue temperature and/or damage to cellular structures that lie within the path of the beam (Barnett, 2001). The developing brain and central nervous tissue are

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particularly susceptible to temperature increases (Edwards, 1986) and increases of only 2 °C above normal can induce damaging effects (WFUMB, 1998). Several animal studies have shown that ultrasound can lead to temperature elevations in guinea-pig and sheep foetal brain tissue of up to 5 °C at the bone/soft tissue interface (Bosward et al., 1993; Horder et al., 1998a,b,c,d) and affect neuronal migration in developing (foetal) mouse brains (Ang et al., 2006). Further, pre-natal ultrasound of mice can influence locomotor, learning and memory behaviour after birth (Suresh et al., 1996, 2002) and lead to reduced hippocampal neuron density, noradrenaline, dopamine, serotonin and 5-HIAA in adult offspring (Suresh et al., 2008).

To date, only very limited data is available on ultrasound-induced neurological effects in human subjects. When the transducer was placed on the abdomen of the mother, reported effects included an increased incidence of left-handedness (Kieler et al., 2001, 2002) and a reported delay in speech development (Campbell et al., 1993). Since the power output of modern ultrasound machines has increased exponentially in the last decade and since the reported frequency of examinations of the newborn brain appears to increase, it is important to assess the possible damaging effects of ultrasound on cognitive function. This is of particular importance when scanning in pulsed Doppler-mode for the interrogation of blood vessel function as this mode is associated with power outputs that can be several fold higher than B-mode (Henderson et al., 1995, 1997).

The chick is an ideal model to study cognitive impairment after gestational insults because the embryos develop in the egg without maternal and placental influences. We can investigate specific effects of external insults at precise time-points during the development of the brain. The chick is particularly well suited to studies of memory ability due to well defined memory stages (short-, intermediate- and long-term memory) and the ability for training and testing soon after hatching (Gibbs and Summers, 2002). The aim of this study was to investigate if ultrasound exposure in either B- or pulsed Doppler-mode, near the time of hatch can result in memory impairment in newly hatched chicks.

2. Experimental procedures

The experimental procedures were approved by the Monash University Animal Ethics Committee. All eggs (hybrid egg laying strain—Rhode Island × New Hampshire × White Leghorn × New Hampshire) were obtained from Wagner's Poultry Enterprises (Coldstream, Victoria). Upon arrival, eggs were weighed and control and treatment groups matched to distribute egg weights equally across all groups. The eggs were incubated in a domestic, self-turning incubator (Bellsouth Multi-Equip Incubator) with exposure to 12 h light/dark cycles, at 21% oxygen with temperatures of 37.5 °C and humidity of 60% until day 19. On E19, eggs were candled to remove any unfertilised eggs and the remaining eggs were randomly assigned to one of the following groups: Group 1: controls, groups 2 and 3: sham exposure (5 and 10 min), Groups 4 and 5: B-mode exposure (5 and 10 min), Groups 6–10: pulsed Doppler exposure (1, 2, 3, 4 and 5 min). In the clinical setting, B-mode and pulsed Doppler-mode durations for neonatal cranial scans are on average 8 min and 30 s, respectively (Schneider-Kolsky, personal communication). In this study, we extended the pulsed Doppler-mode durations for up to 5 min in order to reflect scenarios in the clinic where a suspected pathology is examined in greater detail. Eggs were removed from the incubator on day E19 and the ultrasound treatments were carried out as described below. Immediately after, the eggs from each group were placed in separate Brinsea incubators and remained there until hatch. After hatch, chicks were allowed to dry inside the incubators for about 12 h. Individual chicks were leg-tagged for future identification. The chicks were weighed and placed in brooders according to treatment groups at 29 °C and provided with food and water *ad libitum*. Memory testing was performed on post-hatch day 2 using the bead discrimination task (Gibbs and Summers, 2002; Gibbs et al., 2008a,b). Within 2 h of testing, chicks were decapitated and the brains removed and weighed.

2.1. Ultrasound scanning procedure

Eggs were scanned one at a time. On day E19, each egg was taken out of the incubator and a small opening made into the air sac at the blunt end of the egg to expose the chorioallantoic membrane. The resulting opening was approximately three cm in diameter. The egg was then transferred to an egg holder. The egg was kept warm by a pre-warmed heating pad and an over-head heating lamp positioned to maintain a constant temperature of about 37 °C around the egg. The air temperature around the egg was monitored continuously throughout the scanning period to ensure that heat loss was kept to a minimum during experimentation. Pre-warmed ultrasound gel was applied to the opening of the egg to provide a coupling medium between the chorioallantoic membrane and the transducer. The ultrasound machine used was a Philips HDI 5000 SonoCT with a 15–7 MHz transducer (linear array, Hockey stick). This equipment is currently in use in hospitals for obstetric and neonatal imaging. A 'musculo-skeletal, superficial' application setting was used in this study, together with a focal depth of 1–1.5 cm. The derated I_{SPTA} (intensity) of this transducer is 97.2 mW for B-mode and 576 mW for pulsed Doppler-mode (Philips Medical Systems). These values are well within the recommended power outputs for foetal and neonatal use, with maximal recommended level of 720 mW (WFUMB, 1998). The brain was quickly located in B-mode by sweeping over the entire membrane and locating the orbits which are easily identified by their hypo-echoic appearance. Close to hatching, chicks are positioned inside the egg with the right orbit facing upwards towards the air sac (Rogers, 1990). Hence, the head is in close contact with the chorioallantoic membrane. Axial views of the brain were obtained and images optimized by adjusting focal depth, contrast and time gain compensation for each egg/chick. As soon as optimal images were obtained, B-mode or pulsed Doppler-mode exposures were commenced according to the experimental protocol. For pulsed Doppler-mode exposures, the transducer beam was positioned close to the midline between the hemispheres over the intermediate medial mesopallium (IMM) (Fig. 1). The foetal chicks moved during the scanning procedures which prevented localisation of one of the cerebral blood vessels for continuous, uninterrupted exposure. As a consequence, the exposure was stopped each time the chick moved and then re-commenced once the chick was motionless again. Durations of each exposure session were added up until the cumulative total duration was reached (Table 1). Sham-exposed chicks were scanned in an identical manner, except the transducer power was switched off. The mean time required to locate the brain for the experimental groups was calculated and added to the planned exposure time in the sham-exposed chicks in order to mimic the exact scanning durations and conditions encountered in exposed chicks. The mechanical (MI) and thermal indices (TI) that are automatically displayed in real-time on the ultrasound screen were recorded at least twice in each scan, during both B- and pulsed Doppler-mode. These FDA-approved output display standards are provided by equipment manufacturers in order to provide an estimation of the risk to cause thermally (TI) or mechanically (MI) mediated biological effects in interrogated tissues. After scanning, the ultrasound gel was gently washed off with warm water. Shell caps, collected from unfertilised eggs were loosely placed over the exposed air sac to prevent the chorioallantoic membrane from drying out and the eggs returned to their respective incubators until hatching on E21. Control eggs were left in the incubator and not handled at any stage. On the first day post-hatching, chicks were weighed and placed in brooder boxes according to their allocated treatment group and kept at 29 °C with food and water *ad libitum*.

2.2. Survival of chicks after scanning

Some of the eggs did not hatch and some chicks did not survive the hatching process. Table 2 shows the number of chicks that died during the days between scanning and hatching. No chicks died in the control group. Although the number of chicks that died before hatching in the other groups varied between 7% and 30%, there was no systematic relationship between sham and treated chicks. It was concluded that the loss associated with the experimental procedures was most likely due to the removal of the shell and exposure of the chorioallantoic membrane rather than the exposure to ultrasound *per se*.

2.3. Memory testing using the bead discrimination task

Memory testing was carried out on the second day post-hatching. The test used was the bead discrimination task which has been described previously in detail (Gibbs and Summers, 2002).

Briefly, chicks were transferred in pairs to individual testing pens (approximately 20 cm × 20 cm). In order to familiarise the chicks to the beads, a chrome bead was first introduced 1 h prior to the testing. This bead was 2 mm in diameter and dipped in water to encourage the chicks to peck. Thirty minutes later, and 30 min prior to training, a blue and a red glass bead (4 mm diameter), dipped in water were introduced to the chicks in two successive sessions 2.5 min apart. We found that the chicks have no preference for either colour and peck at both blue and red beads at the same rate. For the training session, the chicks were presented with an identical red bead dipped in methyl anthranilate (100%, Sigma–Aldrich Inc.), a bitter tasting chemical. Chicks quickly learn to associate the red colour with a bitter taste and if memory formation proceeds normally, they will avoid pecking at red beads in future testing trials. The memory retention test involves presenting the chicks with a clean red bead for 10 s, followed 2.5 min later by presentation of a clean blue bead. The number of pecks on each coloured bead is recorded. Chicks that do not peck on the red bead during training, or do not peck at the blue bead during testing are excluded from the analysis. The bead discrimination tests were conducted by an independent investigator. Memory retention was calculated as the ratio of the