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Title: The effect of resuscitation in 100% oxygen on brain injury in a newborn rat model of severe hypoxic-ischaemic encephalopathy

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Abbreviations:

HI – hypoxia-ischaemia

HIE – hypoxic-ischaemic encephalopathy

IQR – interquartile range

P7 – postnatal day 7

Abstract

Aim

Infants with birth asphyxia frequently require resuscitation. Current guidance is to start newborn resuscitation in room air. However, infants with severe hypoxia-ischaemia may require prolonged resuscitation with oxygen. To date, no study has looked at the effect of resuscitation in 100% oxygen following a severe hypoxic-ischaemic insult.

Methods

Postnatal day 7 Wistar rats underwent a *severe* hypoxic-ischaemic insult (modified Vannucci unilateral brain injury model) followed by immediate resuscitation in either 21% or 100% oxygen for 30 minutes. Seven days following the insult, negative geotaxis testing was performed in survivors, and the brains were harvested. Relative ipsilateral cortical and hippocampal area loss was assessed histologically.

Results

Total area loss in the affected hemisphere and area loss within the hippocampus did not significantly differ between the two groups. The same results were seen for short-term neurological assessment. No difference was seen in weight gain between animals resuscitated in air and 100% oxygen.

Conclusion

Resuscitation in 100% oxygen does not cause a deleterious effect on brain injury following a *severe* hypoxic-ischaemic insult in a rat model of hypoxia-ischaemia. Further work investigating the effects of resuscitation in 100% oxygen is warranted, especially for newborn infants with severe hypoxic-ischaemic encephalopathy.

(Institutional animal protocol licence number: PPL 30/2729)

1. Introduction

Infants affected by perinatal asphyxia often require cardiopulmonary resuscitation at birth, which historically included administration of 100% oxygen. A meta-analysis of 10 clinical trials, with 2133 term infants randomised to either room air or 100% oxygen during resuscitation, demonstrated a significant reduction in mortality in the infants resuscitated in room air (typical relative risk 0.32, 95% confidence interval 0.12-0.84).[1] Furthermore, a trend was seen towards a decrease in the risk of more severe hypoxic-ischaemic encephalopathy (HIE) (Sarnat stage 2 and 3) among the infants resuscitated in air. Additionally, in term infants with perinatal asphyxia undergoing therapeutic hypothermia, a higher fraction of inspired oxygen in the first six hours of life was found to be associated with adverse long-term outcome.[2] This may be because oxygen has been shown to cause oxidative injury and apoptosis in the developing brain.[3, 4] In a cohort of term infants with severe perinatal acidosis, admission hyperoxaemia was also found to be associated with a higher risk of HIE.[5] In the same study, the infants with moderate or severe HIE in association with hyperoxaemia in the first postnatal hour had a higher incidence of abnormal brain magnetic resonance imaging (MRI) findings.

The effect of hyperoxia (i.e. due to resuscitation in 100% oxygen) following a hypoxic-ischaemic (HI) insult on the immature brain has been described in animal models, and most frequently a worsening of the injury is seen.[6-9] However, one study found that there was no effect of 100% oxygen on brain histology, and a non-significant improvement in male pups resuscitated in 40% oxygen.[10] Another study found a short-term (24 hours post-insult) worsening of sensorimotor skills, but long-term (assessment at eight weeks) improvement in navigational learning, spatial and orientation skills.[11] A study by Woodworth *et al.* looked at short- and long-term behavioural effects following resuscitation

in air, 40%, or 100% oxygen in postnatal day 7 (P7) rats.[12] They found conflicting long-term results for males (improved sensory skills in both oxygen treated groups) and females (worsened motor skills in air and 40% oxygen group). We have also previously studied the effect of resuscitation in 100% oxygen in combination with therapeutic hypothermia in a P7 rat model of *moderate* HI, and found that resuscitation in 100% oxygen counteracted the effect of therapeutic hypothermia with an increase in brain damage and worsening of reflex performance.[13]

As a result of both animal data and the subsequent meta-analysis of randomised trials in newborn infants, the international resuscitation guidelines changed in 2010, and now state that resuscitation of term newborn infants should be started in air, with supplemental oxygen titrated according to oxygen saturation as measured by pulse oximetry.[14] However, early pulse oximetry in the delivery suite is not always feasible or reliable due to poor peripheral perfusion. Movement may also result in a poor trace,[15] potentially causing periods of undiagnosed hyperoxia during resuscitation. Concern has also been raised about the ability of pulse oximeters to adequately detect hyperoxaemia. This is particularly important in infants affected by severe hypoxic-ischaemic encephalopathy, who may require prolonged resuscitation with oxygen and therefore are more likely to be exposed to periods of hyperoxia than those with less severe HIE. Though periods of hyperoxia may worsen the outcome of infants with mild or moderate HIE, no sub-group analyses of the effects of resuscitation on infants with severe HIE are available, and it remains clinically relevant whether 100% oxygen during resuscitation in cases of *severe* HI worsens brain injury. Here, we describe an established newborn rat model of severe HI, and use it to investigate the effect of resuscitation in 100% oxygen on brain injury.

2. Methods

All procedures were carried out in accordance with United Kingdom Home Office regulations as approved by the University of Bristol Animal Ethical Review Panel. The well-established Rice-Vannucci model was used to create unilateral hypoxic-ischaemic brain damage.[16] Traditionally, a *moderate* insult is created leading to 40-50% hemispheric area loss. We previously established a more *severe* brain injury model by modifying the Rice-Vannucci model.[17] By increasing both the temperature during the hypoxic insult, and the duration of the hypoxic insult, 60-70% area loss is achieved. Wistar (H) rat pups of both sexes (Charles River, UK) on postnatal day 7 (P7) were used, with day of birth counted as day 1. Brain maturity in newborn P7 rats compares to human brain maturation at 32-36 weeks gestation.[18, 19] Animals were kept in an animal facility with a 12:12h light/dark cycle at an environmental temperature of 19-21°C with food and water ad libitum. The pups were weighed and examined daily for overall health status.

2.1 Procedures

Unilateral ligation of the left carotid artery was performed on P7 on eight litters, each culled to 12 pups after birth (n=96). The animals were anaesthetised for carotid ligation using 3% isoflurane in a 2:1 mix of NO_2/O_2 via a nose cone. Three animals died during the operative procedure. The animals were allowed to recover and returned to the dam for at least 30 minutes. The maximum time between ligation and hypoxic exposure was restricted to <180 minutes; this reduces variability within the model, caused by reperfusion of the brain via the well established circle of Willis in the rat.[20] The animals were randomised by litter, weight, and sex to the two treatment groups (resuscitation in air or 100% oxygen), and were placed in a custom-built chamber (Figure 1). The randomisation was kept concealed until the point of starting the resuscitation period. The animals were breathing unassisted during hypoxia, and received a constant fresh gas flow to prevent rebreathing of expired carbon dioxide.

The chamber design allows for servo-controlled temperature regulation by continuous measurement of rectal- and skin temperature using a cooling machine (Criticool Pro; MTRE, Mennen Medical, Rehovot, Israel). A water mat continuously circulates water to maintain the temperature of rats in the chamber at the set target rectal temperature. Once the rectal temperature was stable at 37℃, the hypoxic period started, using 8% oxygen for 125 minutes. Mortality during the insult was 23% (19 pups). Ten animals used for rectal- or skin temperature measurement, were excluded from further analysis, as we have previously shown that the stress of carrying a temperature probe affects brain injury.[21] At the end of the hypoxic period the animals were immediately resuscitated in either 21% or 100% oxygen for 30 minutes. Oxygen and carbon dioxide concentrations were continuously monitored throughout the insult and resuscitation period using a gas analyser (Model 4800 Charter Kontron, Andros Incorporated, Santa Clara, United States). The animals remained in the chamber for a further five hours at a constant temperature of 37℃, before returning to the dam. We have previously demonstrated that being separated from the dam for periods up to 10 hours is well tolerated, and does not result in hypoglycaemia.[17, 22] The pups were weighed daily; three animals failing to gain weight were sacrificed between P10-12 (two randomised to air, one randomised to 100% oxygen), and one pup randomised to air was eaten by the P8. Therefore, the total number of survivors on P14 was 60; 25 in the air group and 35 in the 100% oxygen group. These animals then underwent behavioural and histological assessment of neurological damage.

2.2 Behavioural testing

Animals underwent negative geotaxis testing seven days after the HI insult, at P14, by an assessor blinded to the treatment allocation. This test involves placing the animal head-down midway on a 45° sloped, rough surface. The time it takes the animal to rotate 90° and

180° is noted. The animal was tested three times, and the median time was used for statistical comparison. This test reflects an innate postural response in newborn pups with fused eyelids. It appears around P11 in uninjured rats, and at P12-13 in animals following a hypoxic-ischaemic insult.[23] As a short-term outcome marker, negative geotaxis correlates well with long-term functional outcome.[24] Animals were tested at P14 to ensure the reflex had developed, and that the eyelids remained closed.

2.3 Area loss assessment

After behavioural testing on P14, animals were anaesthetised and underwent transcardiac perfusion with 10% neutral-buffered formalin. Brains were harvested and post-fixed in formalin for seven days.

Brains were cut in 3-mm coronal blocks using a standard matrix (ASI Instruments, Warren, United States of America), and embedded in paraffin. Blocks were sectioned at 5 μm and stained with haematoxylin and eosin. Two sections from the 3rd and 4th slide were scanned (Perfection V30, Epson) at a high resolution (1200dpi) and saved as a TIFF image. These two sections represent areas from the cortex, hippocampus, basal ganglia and thalami. The scanned image was viewed using Image J software (Image J version 1.43; National Institutes of Health, Bethesda, United States of America), and the midline manually identified. The brain was divided into the injured left and uninjured right hemisphere, and the area of each section measured by an assessor blinded to the treatment allocation (Figure 2A). The percentage area loss for each brain was calculated using the formula: (1 - [area left/area right] x 100). This method has been validated against a formal histopathology score.[17] Furthermore the size of the hippocampus was measured using the same technique, and left and right compared (Figure 2B).

2.4 Statistics

SPSS version 19 was used for statistical analysis. Descriptive data following a normal distribution are presented as mean ± standard deviation, and as median with interquartile range (IQR) if not normally distributed. The effect of weight, sex, litter, anaesthesia time and interval between ligation and insult on % area loss was examined using linear regression analysis. For two-group comparison the independent samples T-test or Mann-Whitney U test was used, depending on the distribution of the data. For paired comparison of area loss within the hemisphere and the hippocampus a Wilcoxon signed rank test was used. Conventional statistical significance levels were used (p<0.05 with 2-sided testing).

3. Results

Baseline animal characteristics are summarised in Table 1 and did not significantly differ between the two treatment groups. Sex, weight at P7, litter, anaesthesia time, and interval between ligation and insult, did not significantly influence the overall percentage area loss in a regression analysis.

A scatter plot of overall area loss for the hemisphere, and area loss within the hippocampus, is shown in Figure 3. Median (IQR) area loss for the left hemisphere was 73.2% (66.7-79.3) in the animals resuscitated in 21% oxygen, and 68.8% (57.4-77.2) for those resuscitated in 100% oxygen. Median (IQR) % area loss within the left hippocampus for animals resuscitated in 21% and 100% oxygen was 68.7 (64-83.5) and 66.4 (54.9-87.6) respectively. The actual area measurements on the right (uninjured) hemisphere did not differ between the 2 treatment groups, and were 43.3 ± 5.1 (mean ± SD in units as measured by Image J) for the pups in the 21% oxygen group and 42.7 ± 3.5 for the pups in the 100% oxygen group (p-value 0.33). The same was true for the left hemisphere (p-value 0.12). The distributions of % area loss for the hemisphere and the hippocampus did not significantly differ (p=0.18 and 0.631, respectively) between the animals resuscitated in air and 100% oxygen. Paired

comparison of percentage area loss within the hemisphere and hippocampus for the treatment groups did not show a significant difference, neither for the animals resuscitated in 21% oxygen (p=0.84), nor for those resuscitated in 100% oxygen (p=0.196).

Negative geotaxis results are summarised in Figure 4. The median (IQR) time to rotate to 90° was 3.4 (2.4, 4.2) seconds for the animals resuscitated in 21% oxygen and 3.8 (2.7, 7.8) seconds for the pups resuscitated in 100% oxygen (p=0.261). For rotation to 180° the timings were 6.6 (4.9, 10.5) seconds for those resuscitated in 21% oxygen and 8.0 (5.4, 9.8) seconds for those in 100% oxygen (p=0.697).

4. Discussion

In a newborn survival model of severe HI, resuscitation with 100% oxygen resulted neither in short-term neuro-behavioural changes, nor in an increase in brain injury. This finding differs from that seen in a P7 rat model of moderate HI, where brain injury and short-term neuro-behaviour worsened following resuscitation in 100% oxygen[13]. Newborn infants with severe HI invariably require resuscitation at birth with likely periods of hyperoxia. Based on these results, it may be that increased oxygen administration, and subsequent hyperoxia, during resuscitation of infants with severe HIE may not worsen injury, however this requires further investigation.

The postulated mechanism of increased brain damage following hyperoxic resuscitation where there is a moderate HI insult include inflammation[25] and generation of reactive oxygen species.[9] Based on the findings in this study we speculate that in case of a severe HI insult, no increase in damage is seen because mitochondrial dysfunction and oxidative stress have reached their maximum limits with overwhelmed anti-oxidant defences. In a healthy brain, a fine balance exists between reactive oxygen species and antioxidant defences.[26] Hyperoxia leads to an increase in reactive oxygen species, but this in

itself leads to an upregulation of the antioxidant defences, which could ameliorate brain damage caused by hyperoxia. In animal models of adult stroke, short periods of normobaric oxygen administration improved tissue oxygenation and perfusion[27] without an increase in oxidative stress[28]. If started early following the stroke, this resulted in a reduction in infarct size.

Furthermore, cerebral blood flow probably plays an important role in the effect of hyperoxia on brain damage. Oxygen is a potent vasoconstrictor in the renal, coronary and cerebral vasculature, potentially leading to diminished perfusion of vital organs.[29] Hyperoxaemia in preterm infants <32 weeks gestation caused an increase in cerebral blood flow velocity, whilst the opposite effect was seen in infants ≥32 weeks in a study by Basu *et al*.[30] Hyperoxia is known to cause a reduction in end-tidal carbon dioxide and an increase in cerebral vascular resistance,[31] which in turn could lead to a reduction of cerebral oxygen delivery. Hypocarbia following oxygen administration is caused by an increase in ventilation triggered by peripheral and central (brainstem) chemoreceptors.[32, 33] Hypocarbia causes cerebral vasoconstriction and periods of hypocarbia worsen outcome in both term and preterm infants.[34, 35] By adding carbon dioxide to the hyperoxic gas mixture the reduction in cerebral blood flow can be opposed.[36] The direction in which this intricate balance of hyperoxia-induced hypocarbia, an increase in the partial pressure of oxygen in arterial blood, cerebral vasoconstriction, actual oxygen delivery to the brain, and the interaction between oxidative stress and anti-oxidant defences shifts when using 100% oxygen during resuscitation, may differ depending on the insult severity. This may explain the differences in outcome seen.

In newborn rats around P7, the cortex and hippocampus are the areas preferentially damaged in models of HI.[37] There is a clear maturation effect, with mainly cortical damage

below P5, and involvement of the hippocampus at P5-P10. There is then a switch towards more hippocampal damage in comparison to cortical damage from P13 onwards. [37] In our study, we found that the distribution of area loss in the hemisphere and the hippocampus was the same, suggesting that both the cortex and hippocampus are equally vulnerable following a severe insult at P7. When looking at the effect of resuscitation in 100% oxygen on the area of the uninjured (right) hemisphere, no significant differences were seen between the two treatment groups. This indicates that 100% oxygen did not affect the uninjured hemisphere and therefore that the lack of effect of 100% oxygen on proportional area loss is not due to increased damage in the ipsilateral hemisphere.

The negative geotaxis test has been used as a short-term outcome marker in the HI immature rat model.[23, 24] In a recent study the test was used in a moderate model of HI in 7-day old rats and correlated well with long-term outcome.[38] Absolute values from individual studies are not comparable due to difference in angle and surface material used. The method has not been reviewed yet in the severe HI rat model.

Most animal resuscitation studies use a duration of 120 minutes for the duration of the reoxygenation period.[7, 8, 10, 12] We used 30 minutes of resuscitation because this more closely replicates newborn resuscitation in the delivery suite, and allowed comparison with our previous study of hyperoxia in the moderate model. The resuscitation period followed immediately after the end of the insult to allow fast reoxygenation, as may be expected during an emergency resuscitation in the delivery room.

The severe HI rodent model used in this study closely translates to neonatal stage 3 hypoxic-ischaemic encephalopathy based on the Sarnat classification system.[39] We see a much higher mortality during the insult in the severe model compared to the moderate model (23-28% versus 0-10% in our experience), and the survivors display a significantly

higher brain area loss (median 60-70% versus 40-50% in the standard moderate model as used in our laboratory). The Rice-Vannucci model is notorious for its large intra-model variability. We tried to reduce this variability by keeping the anaesthesia period short (typically 3-4 minutes), and the interval between ligation and the start of the hypoxic period <180 minutes. All surgery was carried out by the same two experienced operators. Temperature during the insult was servo-controlled, and following the period of resuscitation, the animals remained separated from the dam for a further five hours in the servo-controlled environment to allow a constant temperature in the recovery phase. This is a study using a newborn rat survival model of unilateral brain damage, which did not include investigation of systemic or other organ involvement. Larger animal models, for example the newborn pig or fetal sheep HI model, may be required to confirm these findings, as well as the systemic sequelae of 100% oxygen administration in severe HI brain injury, before they can be directly applied to human neonates. Importantly, the Rice-Vannucci rodent model used in this study has previously allowed translational research findings that were replicated in the larger animal models and confirmed in human studies.

5. Conclusion

This study shows that using 100% oxygen during resuscitation following a severe hypoxic-ischaemic insult does not worsen brain injury in a rat model of hypoxia-ischaemia. Further research in a larger animal model is required to confirm these findings, as well as investigate the systemic effects of using 100% during resuscitation following a severe hypoxic-ischaemic insult.

Conflict of Interest:

The authors declare no conflict of interest.

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Legends to Figures and Tables:

Figure 1: Chamber design for the hypoxic insult and resuscitation period. Pups were placed on a heat-conductive floor (A) in individual spaces (B). This was inserted into a gas-tight chamber (C). The floor was in thermal connection with the water mat beneath (D) connected to a Criticool machine via 2 ports (E), allowing even temperature control of the floor. Fresh gas entered at the back of the chamber and exited at the front.

Figure 2. 2A: Illustration of the % area loss calculation of the left hemisphere using a scanned haematoxylin and eosin stained slide, using Image J. 'R' indicates the right side and 'L' the left side of the brain. In this example, 60.7% area loss was seen on the affected side. 2B: shows the same technique for calculation of area loss within the hippocampus. A line is manually drawn around the left and right hippocampus and area loss calculated using the same formula as described in Figure 2A.

Figure 3: Box plot with overlying scatter plot showing percentage area loss for surviving animals resuscitated in air (n=25) and 100% oxygen (n=35) for 30 min calculated for the whole hemisphere and hippocampus only. A Mann Whitney-U test showed no significant differences between the animals resuscitated in air and 100% oxygen.

Figure 4 Median (interquartile range) for time to rotate to 90° and 180° for postnatal day 14

animals resuscitated in air or in 100% oxygen. The animals were allowed 3 attempts and the

median was used for analysis. No statistical differences were seen between the two groups.

Table 1 Animal characteristics. Continuous variables are expressed as mean ± standard

deviation.