Resuscitation with 100% oxygen increases injury and counteracts the neuroprotective effect of therapeutic hypothermia in the neonatal rat

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INTRODUCTION: Mild therapeutic hypothermia (HT) reduces brain injury in survivors after perinatal asphyxia. Recent guidelines suggest that resuscitation of term infants should be started with air, but supplemental oxygen is still in use. It is not known whether supplemental oxygen during resuscitation affects the protection offered by subsequent HT.

RESULTS: Wilcoxon median (95% confidence interval) hippocampal injury scores (range 0.0–4.0; 0 to ≥90% injury) were 21% O_2 normothermia (NT): 2.00 (1.25–2.50), 21% O_2 HT: 1.00 (0.50–1.50), 100% O_2 NT: 2.50 (1.50–3.25), and 100% O_2 HT: 2.00 (1.25–2.50). Although HT significantly reduced hippocampal injury ($B = -0.721$, SEM = 0.297, $P = 0.018$), reoxygenation with 100% O_2 increased injury ($B = +0.647$, SEM = 0.297, $P = 0.033$). Regression constant $B = 1.896$, SEM = 0.257 and normally distributed residuals.

DISCUSSION: We confirm an ~50% neuroprotective effect of therapeutic HT in the neonatal rat. Reoxygenation with 100% O_2 increased injury and worsened reflex performance. HT was neuroprotective whether applied after reoxygenation with air or 100% O_2. However, HT after 100% O_2 gave no net neuroprotection.

METHODS: In an established neonatal rat model, hypoxia–ischemia (HI) was followed by 30-min reoxygenation in either 21% O_2 or 100% O_2 before 5 h of NT (37 °C) or HT (32 °C). The effects of HT and 100% O_2 on histopathologic injury in the hippocampus, basal ganglia, and cortex, and on postural reflex performance 7 d after the insult, were estimated by linear regression.

Perinatal asphyxia occurs in 1 to 6 per 1,000 term human births (1) and is an important cause of severe neurologic impairment and neonatal mortality. Therapeutic hypothermia (HT) has emerged as a neuroprotective therapy in both newborn animal models (2–4) and clinical trials (5–7). The collective evidence from these studies confirms that mild therapeutic HT improves outcome in hypoxic–ischemic encephalopathy, a condition in which ~50% of cooled infants normally die or have significant neurologic disability (8) as compared with before the advent of HT treatment when ~65% had poor outcome. In the UK, the National Institute for Health and Clinical Excellence has declared that HT should be used as standard of care after perinatal asphyxia (9). HT after perinatal asphyxia is also recommended by the recent 2010 International Resuscitation Guidelines (International Liaison Committee on Resuscitation) (10). HT suppresses many of the pathways that lead to delayed energy failure and cell death after hypoxia (11,12), including generation of reactive oxygen species (13), excitotoxicity (14), and the inflammatory response (15).

Supplemental oxygen was previously recommended for resuscitation of the asphyxiated newborn infant, but after the changes in the 2010 International Liaison Committee on Resuscitation guidelines, it is now recommended that resuscitation of the term infant is best started with air, rather than pure oxygen (10). Evidence from animal studies shows that supplemental oxygen during resuscitation causes significant hyperoxia in the newborn brain (16,17), with increased generation of reactive oxygen species (18), oxidative stress (19), inflammation (20), and cerebral injury (19,21,22). Clinical studies have shown that resuscitation with 100% oxygen has no advantage over air but causes increased mortality (23) and increased time to first breath and/or cry (24–26).

It is not known whether supplemental oxygen during resuscitation affects hypothermic neuroprotection, but resuscitation with oxygen and HT have opposite effects on important contributors in the cascades after hypoxia–ischemia (HI), such as the generation of reactive oxygen species (13,18) and the inflammatory response (15,20). We used an established neonatal rat model to study whether air or 100% oxygen during 30-min reoxygenation after HI would affect the development of hypoxic–ischemic brain injury, and whether supplemental oxygen during reoxygenation would influence the neuroprotective effect of postresuscitation HT.

RESULTS

Sixty-four 7-d-old rats were randomized to the four treatment groups: 21-normothermia (NT; reoxygenation at 21%

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O_2 followed by 5 h at 37 °C), 21-HT (reoxygenation at 21% O_2 followed by 5 h at 32 °C), 100-NT (reoxygenation at 100% O_2 followed by 5 h at 37 °C), and 100-HT (reoxygenation at 100% O_2 followed by 5 h at 32 °C). No significant differences were found between groups regarding weight on the day of the insult, sex, or weight gain after the insult (**Table 1**). Median (95% confidence interval) duration of anesthesia was 5.5 (5.0–6.0) min, and the time between ligation and hypoxia was 107 (96–117) min. Regression analysis showed that sex, weight, and litter of pups did not significantly influence injury ($P > 0.2$).

Median (95% confidence interval) histopathologic injury scores in the hippocampus, cortex, and basal ganglia are shown in **Figure 1**, whereas the raw data for the hippocampus are shown in **Figure 2**. The severity of injury in the 21-NT group was similar between the regions, with Wilcoxon median pathology scores of 2.0, 2.3, and 1.9 for the hippocampus, cortex, and basal ganglia, respectively (**Figure 1**). **Table 2** shows the results from linear regression: HT significantly reduced hippocampal injury ($P = 0.018$), whereas 100% O_2 significantly increased injury ($P = 0.033$). **Table 2** further shows that also for the cortex and basal ganglia, the injury scores were higher after 100% O_2 and lower after HT; however, the effects were not significant. HT significantly reduced hippocampal injury whether the animals had been reoxygenated with 21% O_2 or 100% O_2. However, because 100% O_2 increased injury, there was no net neuroprotection of HT after 100% O_2. ANOVA revealed no statistical interaction between the effects of HT and oxygen ($P = 0.49$).

Postural reflex performance (time to rotate 180° from head down to head up in the negative geotaxis test) (**Table 3**) worsened with hyperoxia, as reoxygenation with 100% O_2 caused significantly increased time to rotate 180° ($P = 0.016$). The reduction in time to rotate observed in hypothermic animals did not reach statistical significance ($P = 0.100$). Median (95% confidence interval) time in seconds to rotate 180° were 21-NT: 4.3 (2.9–5.7), 21-HT: 3.3 (2.3–4.1), 100-NT: 5.2 (4.5–6.5), and 100-HT: 4.5 (3.5–5.9).

Table 1. Animal characteristics

	21-NT ($n = 16$)	21-HT ($n = 18$)	100-NT ($n = 15$)	100-HT ($n = 15$)
Weight at P7	10.6 (9.5–11.6)	10.6 (10.0–11.2)	10.3 (9.3–11.2)	10.3 (9.5–11.2)
Female	63%	61%	60%	53%
Weight gain at P14[a]	10.6 (9.3–12.3)	9.7 (8.4–10.7)	9.5 (8.4–10.5)	9.3 (8.3–10.9)

Sixty-four 7-d-old (postnatal day 7, P7) rats were randomized to treatment after hypoxia–ischemia; 21 = 21% O_2; 100 = 100% O_2. Wilcoxon median (95% CI) body weights in grams, and percentage female in each group.

CI, confidence interval; HT, hypothermia; NT, normothermia.

[a]Excluding five rats that died prematurely before postnatal day 14 (P14).

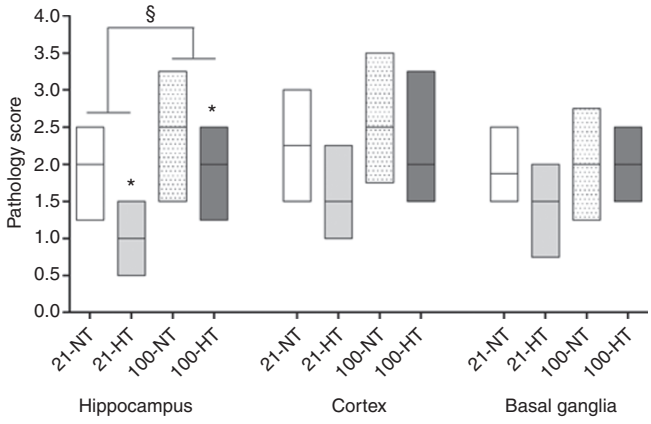

Figure 1. Pathology scores in the hippocampus, cortex, and basal ganglia. Wilcoxon median (95% confidence interval) pathology score in the hippocampus, cortex, and basal ganglia 7 d after the insult. Hippocampal injury was significantly reduced by hypothermia (*$P = 0.018$) and significantly increased by reoxygenation with 100% O_2 (§$P = 0.033$). Hypothermia significantly reduced injury whether the animals had been reoxygenated with 21% O_2 or 100% O_2, but there was no net protection after 100% O_2. The same trends were found in the cortex and basal ganglia but were not significant. The median values are almost identical to the values estimated by regression (**Table 2**); 21 = 21% O_2; 100 = 100% O_2; HT, hypothermia; NT, normothermia. Histopathology scores (range 0.0–4.0, in 0.5 intervals) corresponding to degree of brain injury: 0.0–1.0 = 0–10%; 1.0–2.0 = 10–30%; 2.0–3.0 = 30–60%; 3.0–4.0 = 60–90%; 4 ≥90%. Unshaded bars, 21-NT; gray bars, 21-HT; dotted bars, 100-NT; dark bars, 100-HT.

DISCUSSION

The main finding of this study is that the neuroprotective effect of therapeutic HT is nearly fully negated by the increase in injury seen after breathing 100% oxygen during 30-min reoxygenation before the start of HT. The effects of HT and of hyperoxic resuscitation have previously been studied separately. This is the first study to present data in a newborn survival model on the clinically relevant combination of supplemental oxygen during resuscitation and subsequent

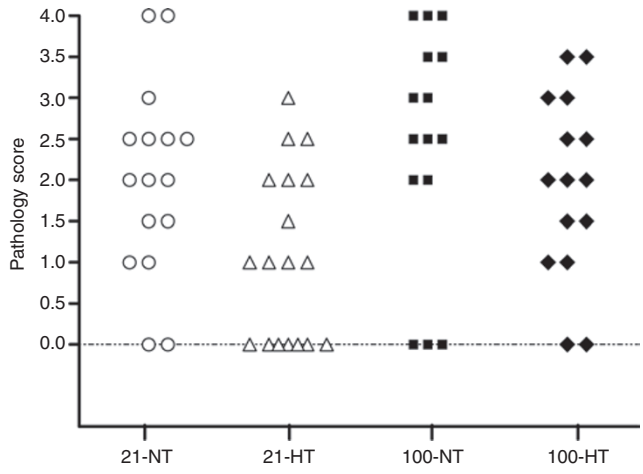

Figure 2. Distribution of individual pathology scores in the hippocampus. Individual pathology scores in the hippocampus of animals in the four groups. Histopathology scores (range 0.0–4.0, in 0.5 intervals) corresponding to degree of brain injury: 0.0–1.0 = 0–10%; 1.0–2.0 = 10–30%; 2.0–3.0 = 30–60%; 3.0–4.0 = 60–90%; 4 ≥90%. Circles, 21-NT; triangles, 21-HT; squares, 100-NT; diamonds, 100-HT; 21 = 21% O_2; 100 = 100% O_2; HT, hypothermia; NT, normothermia.

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Table 2. Effects of hyperoxia and hypothermia on pathology score

Model	Coefficient	95% CI	SEM	*P*
Hippocampus				
Constant	1.896	(1.383, 2.410)	0.257	<0.001
Hypothermia	−0.721	(−1.314, −0.128)	0.297	**0.018**[a]
100% O_2	+0.647	(0.054, 1.241)	0.297	**0.033**[a]
Cortex				
Constant	2.101	(1.481, 2.721)	0.310	<0.001
Hypothermia	−0.385	(−1.101, 0.331)	0.358	0.286
100% O_2	+0.392	(−0.325, 1.108)	0.358	0.279
Basal ganglia				
Constant	1.779	(1.316, 2.242)	0.232	<0.001
Hypothermia	−0.305	(−0.840, 0.230)	0.268	0.259
100% O_2	+0.357	(−0.179, 0.893)	0.268	0.188

Linear regression showing the independent effects of hyperoxia and hypothermia on pathology score. Dependent variable: neuropathology score in the hippocampus, cortex, and basal ganglia 1 wk after hypoxia–ischemia. Independent variables: temperature; hypothermia or normothermia; and oxygen; 21% O_2 or 100% O_2. The residuals were normally distributed.

CI, confidence interval.

[a]Statistical significance.

Table 3. Effects of hyperoxia and hypothermia on postural reflex performance

Model	Coefficient	95% CI	SEM	*P* value
Constant	4.216	(3.303, 5.129)	0.457	<0.001
Hypothermia	−0.881	(−1.936, 0.174)	0.527	0.100
100% O_2	+1.304	(0.248, 2.360)	0.528	**0.016**[a]

Linear regression showing the independent effects of hyperoxia and hypothermia on postural reflex performance (negative geotaxis test) 1 wk after hypoxia–ischemia. Dependent variable: best time in seconds to rotate 180° from head-down to head-up position on a 45° slope. Independent variables: temperature; hypothermia or normothermia; and oxygen; 21% O_2 or 100% O_2. The residuals were normally distributed.

CI, confidence interval.

[a]Statistical significance.

therapeutic HT. We confirm an ~50% neuroprotective effect of therapeutic HT after reoxygenation with 21% O_2 in the neonatal rat hippocampus 1 wk after HI. We also confirm an ~25% detrimental effect of hyperoxic resuscitation. Of note, breathing 100% O_2 for 30 min after hypoxia, but before HT, increased injury nearly as much as HT protected from injury, resulting in no net neuroprotection when HT followed hyperoxia. The best outcome was seen when HT followed reoxygenation with air, and the worst scores were seen when NT followed reoxygenation with 100% O_2.

Although the effects of HT and hyperoxia were most pronounced in the hippocampus, the effects of reduced injury due to HT and increased injury due to hyperoxic reoxygenation were seen also in the cortex and basal ganglia, but did not reach statistical significance. The hippocampus is one of the most sensitive regions for hypoxic injury and is relatively more injured in the neonatal rodent models (27,28). To mimic the clinical setting in which resuscitation precedes therapeutic HT, we introduced a 30-min delay for administration of 100% O_2 or 21% O_2 before HT in this experiment. We speculate that this delay, together with an increased in core temperature from 36 °C to 37 °C during reoxygenation, may have reduced the magnitude of hypothermic protection in this experiment. Thus, the sample size might have been too small to detect statistically significant neuroprotection in the cortex and basal ganglia.

We report increased brain injury after 30-min reoxygenation with 100% O_2 after a HI insult. This is in line with results in a newborn mouse survival model, in which 30 min of 100% O_2 after hypoxia caused increased brain injury, disrupted myelination, and accumulation of nitrotyrosine (19). We chose 30 min of reoxygenation because this is a typical resuscitation time used clinically. In contrast to our results, other investigators found no difference in brain injury in rats after reoxygenation with 100% oxygen vs. air (29), or even reduced injury after 100% oxygen in mice (30). Of note, the results in the latter study may have been distorted because more animals in the 100% O_2 group died prematurely and were excluded from the analyses.

The postural reflex examined in the negative geotaxis test was significantly slower in animals reoxygenated with 100% O_2 after the insult, whereas there was a tendency toward improved performance after HT, although not statistically significant. Thus, the pathology results were supported by neurologic performance, which we have also shown previously in neuroprotective studies with both short- and long-term survival (31–33).

These results are in line with studies showing protective effects of HT (2–4,11–15) and with studies demonstrating harmful effects of hyperoxic reoxygenation (18–21). The new and important knowledge added by this study is the effect of the combination of these two important interventions recommended by the International Liaison Committee on Resuscitation guidelines (10). A recent study in a short-term-survival newborn pig model (16) suggested that the protective effect of HT was stronger after hyperoxia. However, in the present study, there was no indication that the protective effect of HT was more pronounced after hyperoxia. The results rather suggest that HT is effective both after 21% O_2 and 100% O_2, but that the starting point after hyperoxic reoxygenation is worse and HT ameliorates the injury caused by hyperoxia. Although HT and hyperoxia have opposite effects on important contributors in the cascades after HI injury, it is possible that part of hyperoxic injury is more immediate and cannot be completely reduced by subsequent HT. The lack of an interaction between oxygen concentration during reoxygenation and postreoxygenation temperature shows that there was no difference in magnitude of effect of HT between the two reoxygenation groups.

The Rice–Vannucci model is the most frequently used model of neonatal brain injury. It is a selective brain injury model and does not allow for examination of the systemic and multiorgan effects of hyperoxia and HT after HI. These findings therefore need to be confirmed in a large animal model

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in which effects on the whole body can be studied. Despite its limitations, results from this neonatal rat model have been reproduced in larger models such as the newborn pig and the fetal sheep and have been confirmed in large clinical randomized trials.

We conclude that hyperoxic reoxygenation causes increased brain injury and that HT is significantly neuroprotective after both normoxic and hyperoxic reoxygenation. Because there was no net protection of HT after hyperoxia, one way to optimize outcome after HI could be to avoid hyperoxia before therapeutic HT.

METHODS

All procedures were performed under Home Office license in accordance with UK regulations as approved by the University of Bristol animal ethical review panel. The Rice–Vannucci neonatal HI rat model has been in use for more than 30 y (34). It is a unilateral hemispheric stroke model based on ischemia (ligation of the left common carotid artery) followed by hypoxia (90 min in a hypoxic chamber of 8% oxygen). The maturation of the rat brain at postnatal day 7 is "near term-equivalent" to the human. Neuroprotection from postinsult HT after both short- and long-term survival has previously been shown in the postnatal day 7 rat model (31,35). Hypothermic protection was further confirmed in newborn pigs (2–3,36) and fetal sheep (4,37) before successful clinical trials of therapeutic HT (5–7). For the postnatal day 7 rat model to show long-term neuroprotection, at least 3 h of 5 °C reduction in core temperature is needed (31–33,38). We reduced experimental variability by brief duration of anesthesia (median 5.5 min; two experienced researchers performing ligations in parallel), short duration (<180 min) between carotid ligation and the beginning of hypoxia, and tightly controlled temperature, oxygen, and CO_2 levels in our specially designed exposure chamber (32). The chamber temperature was servo-controlled to target rectal temperature, which was continuously monitored (<0.1 °C deviation) in one probe animal in each chamber (CritiCool; MTRE, Yavne, Israel).

Procedures

Ligation of the left carotid artery was performed under general anesthesia (isoflurane, 3.5% induction, 1.5% maintenance, in NO_2/O_2 (2:1)), followed by <180-min recovery with the dam. Five animals died during surgery and were excluded from the analyses. Seventy-two postnatal day 7 Wistar rats of both sexes from six litters were subjected to hypoxia (8% O_2 in N_2) for 90 min at a rectal temperature of 36.0 °C (**Figure 3**). Four temperature probe rats were excluded from all analyses because the stress of carrying a rectal probe may affect outcome (39). Four rats died during hypoxia, before randomization and treatment allocation, and were excluded from the analyses. The 64 pups that survived the hypoxic challenge were randomized, allowing all litters equal representation in each of the four groups. Pups were also randomized between groups for weight and sex. After hypoxia, groups 1–4 were randomized to one of four postinsult treatment groups receiving either 21% O_2 or 100% O_2 before NT or HT: 21-NT, 21-HT, 100-NT, and 100-HT. The 100-NT and 100-HT groups received initial 30-min reoxygenation with 100% O_2 at 37 °C, followed by 5 h of breathing 21% O_2 at either NT (37 °C) or HT (32 °C). The 21-NT and 21-HT groups received initial 30-min reoxygenation with 21% O_2 at 37 °C, followed by 5 h of either NT (37 °C) or HT (32 °C). After 5 h of NT or HT, the pups were returned to the dam and maintained in a 12:12 h dark/light cycle at an environmental temperature of 22 °C with adequate food and water. The weight of each pup was recorded daily to detect failure to thrive as an indicator of injury. Animals that continued to lose weight and died between day 4 and 7 after the insult (postnatal days 11–14; one 21-NT, no 21-HT, one 100-NT, and three 100-HT) were considered as premature deaths. Because both death and disability are main outcomes in clinical asphyxia studies, we also included animals with premature death in the analyses. These animals were given a pathology score similar to that of the worst outcome in a survivor from the same litter and treatment group.

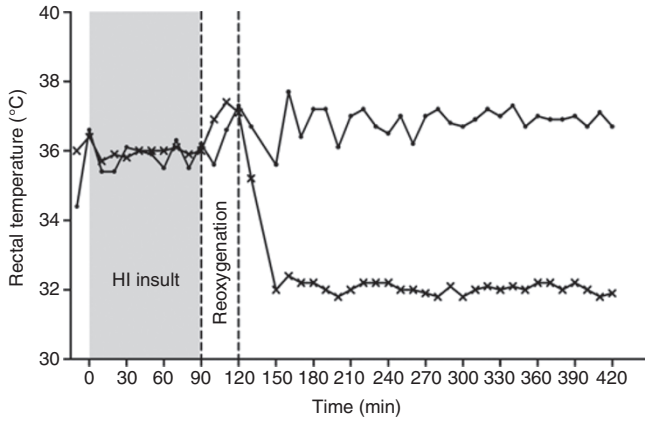

Figure 3. Experimental design and rectal temperature recordings during the experiment. Rectal temperature recordings in temperature probe rats—two typical individual traces. Experimental design: unilateral carotid ligation and 90-min hypoxic exposure at 36 °C in a chamber with 8% O_2 (HI insult), before 30-min reoxygenation at 37 °C in pure oxygen (100% O_2) or air (21% O_2), followed by 5-h recovery at 37 °C (NT) or 32 °C (HT). This resulted in four groups: 21-NT, 21-HT, 100-NT, and 100-HT. Shaded circles, NT; multiplication symbols, HT. HI, hypoxia–ischemia; HT, hypothermia; NT, normothermia.

Early Behavioral Testing (Negative Geotaxis Test)

At 2 wk of age, 1 wk after the HI insult, the animals were subjected to the negative geotaxis test, which tests an innate postural reflex that develops while the eyes are still fused in the second week of life in healthy rat pups. This test is the short-term survival test that correlates best with both short- and long-term pathology and long-term functional outcome (staircase test) in the postnatal day 7 rat model used in this study (32). The rats were placed with the head down on a 45° slope, and the time taken to rotate 180° to a head-up position was recorded (32,40). The shortest time out of three attempts was used for statistical analysis. Testing was performed by an individual unaware of treatment allocation.

Histopathology

At 2 wk of age, 1 wk after the HI insult, transcardiac perfusion with 4% phosphate-buffered (0.1 mol/l) formaldehyde was performed under deep isoflurane/N_2O anesthesia. The brains were kept in 4% formaldehyde until further processing. Coronal 3-mm blocks were cut through the brain using a standard matrix for uniformity (ASI Instruments, Warren, MI) and were embedded in paraffin. Blocks were sectioned at 3 µm and stained with hematoxylin and eosin. A pathologist blinded to treatment allocation evaluated the degree of brain injury in three regions (cortex, basal ganglia, and hippocampus) using a validated scoring system with a nine-step scale from 0.0 (no injury) to 4.0 (≥90% injury) (35). This scoring scale has been validated against cell counting previously and is also used by other investigators (35,38,41).

Statistics

Statistical analyses were performed with SPSS version 17 (SPSS, Chicago, IL) and StatExact (Cytel Studio 7; Cytel, Cambridge, MA). Because histopathologic and neurologic test data were skewed, nonparametric tests (Wilcoxon–Mann–Whitney test and Hodges–Lehman estimates of median) were used for comparisons between groups (42). Data are given as Wilcoxon median (95% confidence interval) unless otherwise specified. Effects of HT, hyperoxia, sex, weight, and litter of pups on histopathologic injury score in the brain and on neurologic testing were estimated by linear regression.

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The regression analysis was supplemented by ANOVA to assess whether there was an interaction between the effects of hyperoxia and HT. Two-sided testing with $P < 0.05$ was considered statistically significant.

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