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Reoxygenation with 100% Oxygen Versus Room Air: Late Neuroanatomical and Neurofunctional Outcome in Neonatal Mice with Hypoxic-Ischemic Brain Injury

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ABSTRACT: Study investigated neuroutcome in mice subjected at 7–8 d of life to hypoxic-ischemic brain injury (HI) followed by 30 min of reoxygenation with 100% O_2 (Re-O_2) or room air (Re-Air). At 24 h of recovery, mouse reflexes were tested. At 7 wks after HI spatial orientation and memory were assessed in the same mice. Mortality rate was recorded at 24 h and at 7 wks of recovery. In separate cohort of mice, changes in cerebral blood flow (CBF) during HI-insult and reoxygenation were recorded. Re-O_2 *versus* Re-Air mice exhibited significantly delayed geotaxis reflex. Adult Re-O_2 *versus* Re-Air mice exhibited significantly better spatial learning and orientation with strong tendency toward better preserved memory. Histopathology revealed significantly less hippocampal atrophy in Re-O_2 *versus* Re-Air mice. Following a hypoxia-induced hypoperfusion, Re-O_2 re-established CBF in the ipsilateral side to the pre-hypoxic level significantly faster than Re-Air. The mortality was higher among Re-O2 *versus* Re-Air mice, although, it did not reach statistical significance. Re-O_2 *versus* Re-Air restores CBF significantly faster and results in better late neuroutcome. However, greater early motor deficit and higher mortality rate among Re-O_2 *versus* Re-Air mice suggest that Re-O_2 may be deleterious at the early stage of recovery. **(*Pediatr Res* 60: 55–59, 2006)**

The resuscitation of asphyxiated neonates is aimed to re-establish tissue oxygen delivery by restoring blood flow and increasing the arterial oxygen content. Use of 100% O_2 during neonatal resuscitation is currently recommended (1). However, Re-O_2 compared with Re-Air significantly exacerbates an oxidative stress in asphyxiated neonates (2), which is one of the mechanisms of HI-brain injury (3). To avoid hyperoxia-induced oxidative stress, the efficacy of resuscitation with room air (RA) instead of 100% O_2 is being extensively studied. To date, results of these studies are conflicting. For instance, it was reported that in asphyxiated pigs the Re-O_2 restored CBF significantly faster and more complete compared with Re-Air (4). Significantly higher levels of excitatory amino acids were found in the striatum of newborn HI-piglets Re-Air compared with Re-O_2 piglets which may be indicative of less favorable neurologic prognosis for Re-Air animals (5). However, Liu. Y and co-authors, using a canine model of cardiac arrest, demonstrated that Re-O_2 *versus* Re-Air resulted in greater neurologic impairment assessed at 24 h of recovery (6). In contrast, the postischemic use of hyperbaric oxygenation, which produces substantial oxidative stress (7), significantly improved neurologic score following focal cerebral ischemia in adult rats and HI in neonatal rats (8,9). Hyperbaric oxygenation used as a mode of resuscitation from experimental heatstroke in rats was shown to be significantly neuroprotective (10). Meier and co-authors reported that hyperoxic ventilation significantly reduced short-term (6 h) mortality due to systemic ischemia following an acute hemorrhagic shock in pigs (11). The most recent meta-analysis indicates that there are not enough data to challenge current recommendation of using of pure O_2 during resuscitation (12).

The assessment of long-term neurologic handicap following HI is of paramount clinical value to define the ultimate efficacy of Re-O2 *versus* Re-Air in an asphyxiated subject. There are no reports on late neurologic outcome and reoxygenation strategy following asphyxia. This work was undertaken to provide experimental data on short- and long-term neuropathological and neurofunctional outcomes in HI-affected mice re-oxygenated with either 100% O_2 or RA immediately following the index event.

MATERIALS AND METHODS

Murine model of HI. Three day old (p3) C57/BL6J mice of both genders were purchased from Jackson Laboratories (Bar Harbor, ME) with their birth mothers. All research was conducted according to a protocol approved by the Columbia University Animal Care and Use Committee.

On p7 or 8 the right common carotid artery was permanently ligated under isoflurane anesthesia. To induce anesthesia mice were placed for 20 s into plastic chamber containing isoflurane-saturated cotton tip. Anesthesia was maintained for 7–10 min. by holding the same cotton-tip near the animal's nose. Following two hours of recovery with the dam, mice were subjected to 8% O_2 balanced with N_2 for 20 min. The ambient temperature during hypoxic exposure was kept at 37 ± 0.5°C and monitored continuously at 2 cm above the bottom of the partially submerged hypoxic chamber. To achieve an even exposure to the environmental temperature, mice were placed into the plastic-net hammock inside of the hypoxic chamber. Immediately after hypoxic

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Abbreviations: CBF, cerebral blood flow; **RA,** room air; **Re-Air,** reoxygenated with room air; **Re-O_2,** reoxygenated with 100% oxygen

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exposure pups were exposed either to RA or 100% O_2 for 30 min at 25°C and then returned to their dams. Because it has been shown that carotid artery ligation without hypoxia (sham) produced neither brain damage, nor functional impairments in rodents (13,14), littermate naïve (no HI) mice were used as a control. Mice that died during hypoxia ($n = 8$) or cannibalized ($n = 11$) by their dams were excluded. The total, 89 mice were used to analyze data in the long-term survival protocol. In addition, 10 mice were used to obtain a short-term neuroanatomical outcome and 13 mice were used to study CBF. The total 112 mice were used for data-analysis. Two litters of the survived experimental mice were excluded from the study because of very high mortality rate among HI-mice and naïve littermates secondary to the death of lactating dams. Mortality rate was recorded at two time points: 24 h and 7 wks after HI-insult.

Measurement of the cerebral blood flow. On separate cohort of p8 mice under isoflurane anesthesia laser doppler probes were placed on the skull through scalp incision using fiberoptic extensions. Probes were paralleled to each other 2 mm posteriorly and 2 mm laterally to bregma. The changes in CBF were recorded before, during, and 10 min after hypoxia and expressed as a percentage of the prehypoxic level. Values of the CBF at every two minutes of hypoxia and reoxygenation were analyzed as previously described (15).

Short-term assessment. At 24 h of recovery, the neonatal mouse reflex (righting and geotaxis) performance was tested in the experimental mice and compared with naïve counterparts as described earlier (16). The same mice were housed at the animal facility until evaluation of the long-term neurofunction at 7 wks following HI-insult.

Long-term assessment. was done in adult (8 wks old) mice subjected to HI/re-oxygenation in the neonatal period. The Morris water-maze test was used according to a protocol described previously (16,17). Briefly, mice were trained (3 d/3 attempts) to locate a submerged platform in 80 cm-diameter swimming pool using central and peripheral navigational cues. On day four, the flag (central navigational cue) on the platform was removed and a mouse was given three trials to locate a platform using only peripheral navigational cues (spatial orientation skill). On day five the platform was removed and mouse was given a probe trial to locate a "nonexistant" platform. Throughout the training period the platform was located in the same quadrant of the pool, therefore the time spent by the mouse in this "platform quadrant" searching for the removed platform represented navigational memory and was expressed as a percentage of an allotted time (120 s). This test was shown to be more sensitive than rota-rod and open-field tests in detecting a neurofunctional deficit in HI-mice (17). One Re-O_2 mouse that died at the end of the water-maze test was excluded from neurofunctional data-analysis.

To ensure that performance of HI-mice in the water-maze was not altered by mouse ability to swim, the degree of motor deficit was assessed on rota-rod test. The rota-rod test was used as previously described (17) with the following modification: On the first day of testing, mice were given three training attempts. During each of these attempts, the rod-rotation speed was increased from 16 to 24 to 32 r/min by the third attempt. On the second day, the probe trial was performed during which three consecutive attempts were given to each mouse at a rotation rate of 32 r/min. The mean of all three attempts of "on-rod" running time was recorded. If a mouse was unable to run on the rod, but continued to hold on the rotating shaft for two consecutive rotations, it was recorded as a failure to run. An investigator "blinded" to the study groups, performed all neurofunctional evaluations.

Neuropathological assessment. At the conclusion of neurobehavioral testing, the mouse brains were harvested for histopathologic assessment and volumetric analysis as described earlier (16). Briefly, serial (20 μm) coronal sections were obtained in relation to bregma and stained by Nissl (crezyl-violet). Residual tissue volume in the damaged, ipsilateral cortex and hippocampus was quantified and expressed as a percentage of the contralateral cortex and hippocampus. Brains of four mice from Re-Air group were not processed for histologic evaluation due to technical error. In separate cohort of mice at 24 h after HI the extent of cerebral injury was assessed by volumetric analysis of the triphenyltetrazolium chloride (TTC)-identifiable infarct volume as described (17).

Statistical analysis. Data are expressed as means ± SEM. One-way ANOVA test was used for comparative analysis of the extent of cerebral injury and neurofunctional performance. The mortality rate between groups was compared by χ^2 analysis. Data on changes in CBF were analyzed using ANOVA for repeated measures with Bonferroni's posthoc analysis. Data were considered statistically significant if $p \leq 0.05$ between the groups.

RESULTS

Cerebral blood flow. By the end of hypoxic exposure there was a significant decrease in CBF in both hemispheres compared with the basal prehypoxic level (Fig. 1*A, B*). In the ipsilateral hemisphere CBF was significantly ($p = 0.001$, mean ± SD = 86.6 ± 10.5%) reduced already at 2 min of hypoxia and reached the mean value of 55.2 ± 17.7% of the prehypoxic level at the end of hypoxic exposure. In the contralateral hemisphere CBF decreased to a lesser degree and reached the mean value of 89.8 ± 47% of the prehypoxic level at 20 min of hypoxia. The Re-O_2 sharply increased CBF in the ipsilateral hemisphere from 55.2 ± 17.7% at 20 min of hypoxia to 88.4 ± 30% at 2 min of O_2-reoxygenation ($p = 0.004$). In contrast, Re-Air resulted in significantly ($p = 0.006$) slower restoration of CBF compared with the Re-O_2 and reached a 90% of the prehypoxic level only by 6 min of reoxygenation (Fig. 1*A, B*). Of note, Re-O_2 resulted in significant cerebral hyperperfusion and reached mean value of 150% of the prehypoxic level in the ipsilateral, ischemic hemisphere. Re-Air did not result in hyperperfusion of the ipsilateral hemisphere (Fig. 1*A*).

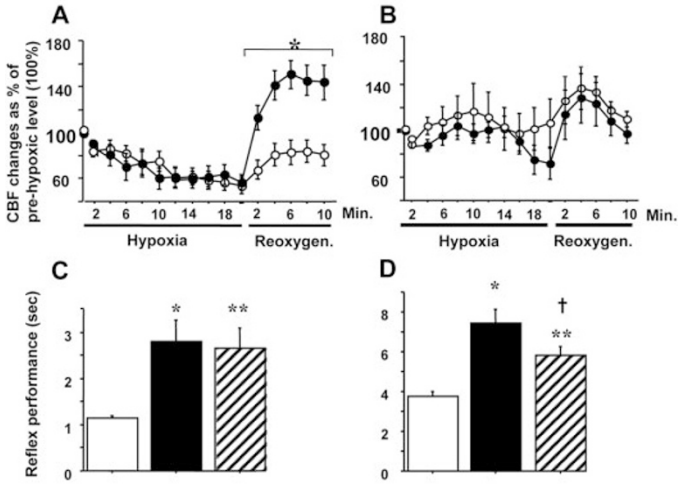

Figure 1. The CBF during hypoxia and reoxygenation in the ipsilateral (*A*) and contralateral (*B*) hemispheres in Re-O_2 (● $n = 6$) and Re-Air mice (○ $n = 7$). The CBF expressed as a percentage of prehypoxic value. * $p = 0.0013$ compared with Re-Air mice. (*C*) – Righting reflex and (*D*) geotaxis reflex performance in naïve (□ $n = 17$), Re-O_2 (■; $n = 32$) and Re-Air (▨; $n = 37$) mice. In fig. 1C * $p = 0.02$ and ** $p = 0.03$ compared with naïve mice. In Fig. 1 *D*, * $p = 0.0001$ and ** $p = 0.02$ compared with naïve mice and † $p = 0.03$ compared with Re-O_2 mice.

Short-term outcome. Analysis of the righting reflex performance revealed no difference between Re-O_2 and Re-Air-mice. However, performance of the geotaxis reflex was significantly delayed in Re-O_2 mice compared with Re-Air counterparts. HI-mice demonstrated significantly more sluggish response of both reflexes compared with naïve littermates (Fig. 1*C, D*). Cerebral infarct volume assessed at 24 h following HI in the separate cohort of mice, revealed a slightly larger cerebral infarct volume in Re-O2 mice (54.6 ± 14, $n = 5$) compared with Re-Air mice (44 ± 9.5, $n = 5$), although, it did not reach a statistical significance ($p = 0.55$).

Long-term outcome. Sensorimotor function defined as "on-rod" running time was similar in HI-affected mice (63 ± 43 s in Re-O_2 and 72 ± 54 s in Re-Air group) and naïve (92 ± 58 s) counterparts. There was no significant difference in sensorimotor function between HI-mice re-oxygenated either with Air or O_2. In contrast, navigational learning and spatial

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orientation skills were significantly better preserved in Re-O_2 mice compared with Re-Air counterparts (Fig. 2*A, B*). Re-O_2 mice were able to locate the unflagged submerged platform using only peripheral navigational cues as quickly as naïve animals. Re-Air mice, however, spent significantly more time performing the same navigational task (Fig. 2*B*). When navigational memory was tested there was a strong tendency toward better memory performance in Re-O_2 mice compared with Re-Air animals (Fig. 2*C*).

Volumetric analysis of cerebral coronal sections revealed that all HI-mice had significant tissue loss in the hemisphere ipsilateral to the carotid artery ligation side (Fig. 2*D,E*). The loss of the hippocampal volume following HI was significantly greater in Re-Air-mice compared with Re-O_2-mice (Fig. 2*D*). Hippocampal atrophy in Re-Air mice was associated with greater degree of degeneration in granular zones CA1 – CA4, compared with Re-O2 mice (Fig. 2*F,G*). Volumetric assessment demonstrated that Re-O_2 mice had a slightly better preserved cortex compared with Re-Air mice, although it did not reach statistical significance (Fig. 2*E*).

Analysis of the mortality rate revealed no statistically significant difference between Re-O_2 and Re-Air mice (Fig. 3*A*).

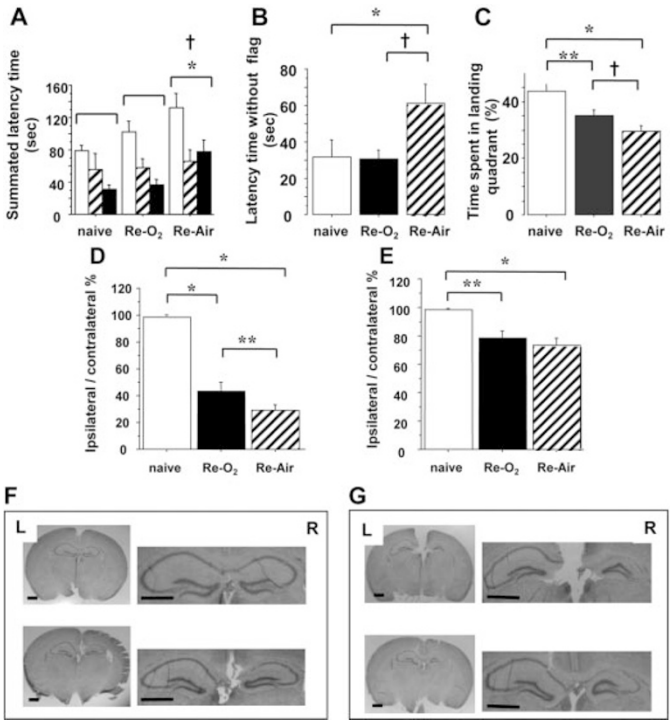

Figure 2. Spatial learning (*A*), orientation (*B*) and navigational memory (*C*) in adult naïve ($n = 14$) mice and adult Re-O_2 ($n = 18$) and Re-Air ($n = 27$) mice. *Panel A*: Day 1 (□), Day 2 (▨); Day 3 (■) of water-maze training. * $p = 0.002$ compared with naïve mice, and † - $p = 0.018$ compared with Re-O_2 mice. *Panel B*: * $p = 0.04$ and † $p = 0.02$ compared with naïve and Re-O_2 mice respectively. *Panel C*: * $p < 0.0001$ and † $p = 0.07$ compared with naïve and Re-O_2 mice respectively and ** $p = 0.02$ compared with controls. (*D,E*) – Volume of residual cerebral tissue in the ipsilateral to the carotid artery ligation side hippocampus (*D*) and cortex (*E*) in naïve ($n = 5$), Re-O_2 ($n = 17$) and Re-Air ($n = 23$) mice. *Panel D*: * $p < 0.0001$ compared with naïve mice and ** $p = 0.018$ compared with Re-O2. *Panel E* * $p = 0.002$ and ** $p = 0.003$ compared with naïve mice. Fig 2 *F,G*; Nissl-stained coronal sections of whole brains and corresponding hippocampuses obtained from Re-O_2 (*F*) and Re-Air (*G*) mice. Scale bar = 1000 μm.

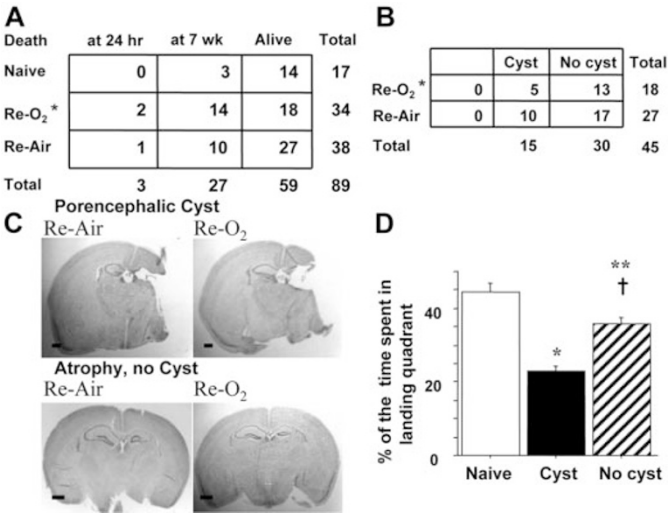

Figure 3. (*A*) Mortality rate among naïve and HI-mice at 24 h and 7 wks following HI-insult. * χ^2 $p = 0.27$ compared with Re-Air mice. (*B*) Incidence of porencephaly in Re-O_2 *vs* Re-Air mice. * - χ^2 $p = 0.19$ compared with Re-Air mice. (*C*) Nissl-stained coronal cerebral sections obtained from adult HI-mice with porencephaly and HI-mice with subtle hemispheric/hippocampal atrophy. Scale bar = 1000 μm. (D) Navigational memory in adult naïve (□; $n = 14$) mice, and HI-mice with (■; $n = 14$) and without (▨ $n = 23$) porencephaly. * $p < 0.0001$ and ** $p = 0.025$ compared with naïve mice, † $p < 0.0001$ compared with cyst-mice.

DISCUSSION

A 30 min postHI reoxygenation with 100% O_2 compared with RA resulted in: (1) Significantly faster restoration of hypoxia-depressed CBF in the ipsilateral, ischemic hemisphere, (2) Significantly delayed sensorimotor geotaxis response assessed at 24 h after index event and (3) Significantly better long-term neurologic outcome in surviving adult HI-mice. This is the first report to describe both early and late neuroanatomical and neurofunctional outcomes in the same animals subjected to HI and reoxygenated with either 100% O_2 or RA. Significantly delayed geotaxis reflex performance at 24 h following HI in Re-O_2 mice compared with Re-Air counterparts indicates that at the early stage of recovery Re-O_2 mice had a greater degree of neurofunctional deficit. P. Temesvari and coworkers using a pneumothorax-induced asphyxia model in piglets reported significantly greater degree of early (4 h of recovery) neurodeficit in Re-O_2 piglets *versus* Re-Air counterparts, although, histopathologically-defined extent of neurodamage was not different (18). Cerebral infarct volume assessed at 24 h following HI in our study showed no difference between Re-O_2 and Re-Air mice. Thus, it seems that Re-O_2 compared with Re-Air following HI exacerbates short-term neurofunctional deficit without a robust morphologic evidence of more extensive cerebral damage. It is possible that the method (triphenyltetrazolium chloride-staining) used to assess anatomical extent of neurodamage in Re-O_2 *versus* Re-Air mice was not sensitive enough to detect differences. It is also possible that cerebral hyperoxia-induced biochemical derangements result in delayed histopathological changes, yet caused a greater early sensorimotor deficit in Re-O_2 mice. It has been shown that extracellular striatal dopamine level which can exert neurotoxicity, was two folds

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higher in Re-O_2 piglets compared with Re-Air animals following systemic hypoxic injury (19). Although, early assessment of the neurofunction is an important outcome measure, the late assessment of neurohandicap ultimately defines the efficacy of therapeutic interventions in asphyxiated neonates.

The mortality rate is an important variable to consider in assessment of long-term neurologic deficit. The mortality rate in both groups was not statistically different (χ^2 $p = 0.27$). However, there was a higher death-rate among Re-O_2 mice (46.7%) compared with Re-Air animals (29%). Therefore, significantly better neurologic outcome in surviving adult Re-O_2 mice compared with Re-Air counterparts could have been biased, because the Re-O_2 mice, that survived had a milder degree of HI-injury. To support this statement, all surviving HI-mice were stratified according to the presence or absence of porencephalic cyst in their brains (Fig. 3*B,C*). Among Re-O_2 mice, 5 of 18 (27%) animals had porencephaly. In contrast, among Re-Air group 37% of mice developed porencephaly (Fig. 3*B*). Porencephaly indicates more severe HI-injury compared with those animals which developed only atrophy in the ipsilateral hemisphere (17). Regardless of the reoxygenation strategy, HI-mice with a porencephalic cyst exhibited the most profound neurofunctional deficit compared with those HI-mice that only developed ipsilateral hemispheric/hippocampal atrophy (Fig. 3*D*). If Re-O_2 mice exhibited a mortality rate and rate of porencephalic cyst formation more comparable with Re-Air mice, the long-term neuroutcome may have been similar in both groups. Alternatively, the decreased rate of porencephaly formation in Re-O_2 mice may indicate a beneficial effect of reoxygenation with pure oxygen on natural evolution of HI-brain injury.

An important finding is that Re-O_2 restores CBF significantly faster in the ischemic hemisphere. This result is consistent with the report by A. Solas and co-authors who demonstrated that 30 min of Re-O_2 was superior to Re-Air in restoration of microcirculation in cerebral cortex of piglets following HI-insult (20). This was associated with significant reduction of excitatory amino acids level in the striatum suggestive of more favorable neurologic outcome in Re-O_2 HI-piglets (5). In preterm lambs subjected to HI-insult the severity of cerebral regional hypoperfusion strongly correlated with increased apoptotic cell death-rate (21). In our study, improved late neurologic outcome in Re-O_2 mice compared with Re-Air mice may be secondary to the more efficient restoration of hypoxia-depressed CBF during Re-O_2. Significantly faster restoration of CBF in the ipsilateral hemisphere implies a significantly better O_2-delivery to the postischemic cerebral tissue if mice were Re-O_2 *versus* Re-Air. Given, that the primary goal of resuscitation is to re-establish effective O_2 -delivery to ischemic organs, the Re-O_2 can be viewed as a superior reoxygenation strategy to the use of RA. The use of a separate cohort of mice to record CBF changes in our experiments limits the strength of the association between the pattern of cerebral perfusion during reoxygenation stage and late neurologic outcome. Nevertheless, we speculate that the more rapid and effective restoration of O_2-delivery to the postischemic brain is beneficial and may account for the improved late neurologic outcome in Re-O_2 mice compared with Re-Air counterparts. It has been reported that following a prolonged asphyxia (15 min of cardiac arrest) higher cerebral perfusion during resuscitation was associated with significantly improved neurofunctional outcome in rats at 5 d of recovery (22).

The limitation of our study is that the duration of reoxygenation was arbitrarily chosen as 30 min to mimic the clinical situation during resuscitation of severely asphyxiated neonates. This 30 min of Re-O_2 not only re-established cerebral perfusion more quickly to the prehypoxia level, but resulted in a significant hyperperfusion/hyperemia in postischemic brain. Although, more rapid CBF-recovery was associated with neuroprotection following focal ischemia in rats (23), the postischemic cerebral hyperperfusion adversely affected neurologic outcome in rats subjected to focal ischemia/reperfusion injury (24). It is possible that the initial beneficial effect of Re-O_2 is ameliorated by prolonged reoxygenation which leads to hyperoxia-induced cerebral hyperperfusion and enhancement of oxidative stress. Perhaps, significantly worse geotaxis reflex performance in Re-O_2 mice is related to this pathophysiological sequence. Physiologically unjustified, prolonged (24 h) use of pure O_2 after restoration of O_2 tissue delivery has been shown to adversely affect postHI recovery in neonatal rats (25).

Our study does not link the duration of Re-O_2 with ongoing changes in CBF induced by reoxygenation. Similarly, in other studies, the duration of postischemic reoxygenation with use of pure oxygen varies from 5 min to 24 h regardless of the resuscitation-induced changes in CBF (4,5,25). This may limit the clinical value of experimental data. A. Solas and co-investigators demonstrated that the shortening of Re-O_2 from 20 min to 5 min did not change the pattern of CBF-restoration during reperfusion stage in asphyxiated piglets, although, the highest values of mean arterial blood pressure, and cortical blood flow were observed in animals Re-O_2 for 20 min (4). In this study the 100% O_2-driven restoration of CBF instantly reached the level of cortical perfusion which was observed only following 10 min of Re-Air in asphyxiated pigs. It is possible that brief (1–2 min) initial use of Re-O_2 accelerates the achievement of the primary goal of resuscitation, the restoration of O_2-delivery, which can be beneficial. Subsequent switch to the Re-Air may limit a postischemic hyperemia.

In conclusion, the Re-O_2 for 30 min compared with that with room air following HI-insult, restores cerebral blood flow significantly faster, but results in hyperemia of the postischemic brain. Long-term neurologic outcome in Re-O_2 mice is significantly better compared with Re-Air mice. However, the greater early sensorimotor deficit coupled with increased mortality rate among Re-O_2 *versus* Re-Air mice, does not allow us to define which reoxygenation strategy is more beneficial for neurologic recovery in HI-mice. Experimental studies that link the reoxygenation-induced restoration of tissue O_2-delivery with ultimate neurologic outcome are needed before any revision of an existing guideline for resuscitation of asphyxiated neonates.

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