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Hypothermia plus Erythropoietin for neonatal neuroprotection? Commentary on Fan et al. and Fang et al.

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Neonatal neuroprotection for hypoxic ischemic brain injury remains elusive. Preclinical studies of mild hypothermia in multiple animal models in several species (primarily rodents, piglets and sheep) showed significant benefit, and this therapy was therefore brought to clinical trials. Some of the preclinical trials showed little or no benefit, and these experiments allowed investigators to better define parameters in which hypothermia was effective. Important factors included: timing and duration of therapy, depth of cooling, and use of anesthetics or morphine to prevent shivering. Multiple randomized controlled clinical trials of therapeutic hypothermia have now been completed, and meta-analyses of these trials definitively show benefit for infants with moderate to severe hypoxic ischemic encephalopathy (HIE), with number needed to treat between 7 and 9 [1,2]. In general, the benefits of hypothermia were greater in animal experiments than in human trials. This discrepancy is likely because preclinical experiments are carried out in otherwise healthy animals under controlled settings with the type, degree, and timing of injury all known. Used clinically, hypothermia improves both survival and the neurologic outcomes of those who survive, but the effect is only modest. Fifteen percent of cooled neonates with moderate to severe neonatal encephalopathy due to presumed hypoxia ischemia still die, with 25% of qualifying infants suffering severe long term neurodevelopmental impairment. Hence the search continues for therapies that will further improve outcomes.

Erythropoietin (Epo) has great potential to be such an agent. In published preclinical studies, Epo has neuroprotective and neuroregenerative effects in the brain with improvement rates after neonatal brain injury ranging from 34 to 79% [3]. Mechanisms of Epo neuroprotection include receptor-mediated, cell-specific effects that occur both early and late in the healing process, and non-specific effects that also modulate the response to injury. Epo has anti-inflammatory [4–6], anti-excitotoxic [7], anti-oxidant [8], and anti-apoptotic [9], effects on neurons and oligodendrocytes [10]. It also promotes neurogenesis [11,12] and angiogenesis [13], which are essential for injury repair and normal neurodevelopment. Epo effects are dose-dependent, and multiple doses are more effective than single doses [9,14,15]. The studies by Fang et al.[16] and Fan et al.[17] published in this issue both question whether Epo plus hypothermia might be more protective than either Epo or hypothermia alone. Surprisingly, the Fang study

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showed no benefit of 8 hours of hypothermia; Epo treatment improved the histopathological outcome in males only, and combined therapy showed no benefit (or harm). In contrast, the Fan study showed neuroprotection after 3 hours of hypothermia (females greater than males), improvement in sensorimotor function (but not histopathological damage) with Epo alone, and combined therapy showed only modest benefit in sensorimotor function (males only). How can we resolve these differences, and why are these studies discordant with previously published work?

The species and strain of animals used to model injury, mechanism of injury (stroke vs. hypoxia-ischemia), experimental design, and statistical issues can all affect outcomes. Cell-specific and regional vulnerability to brain injury changes with developmental stage, and these differ by species and strain; rats and mice have slightly different rates of brain development, and even within mice, different strains respond quite differently to hypoxia. Other less obvious factors might confound results. For example, unplanned maternal or neonatal stress (did the vivarium place barking dogs next to the rat room?) or unintentional changes in environmental factors (room temperature, noise, humidity, etc.) can affect outcomes.

So what factors might have affected the outcomes in the Fang and Fan studies? The Fang study was done in a laboratory that has previously shown robust Epo neuroprotection. Important differences between their current and previous studies include mechanism of injury (MCAO occlusion- no hypoxia vs. unilateral HIE), and developmental stage with the early studies using day 10 animals rather than P7 [14,15]. There are also several potentially important differences between the Fang and Fan studies. While both studies used the Vannucci model of unilateral brain injury in P7 rats, the severity of injury differed (120 minutes vs. 90 minutes of hypoxia) as did duration and degree of hypothermia (8 hours vs. 3 hours; 30.8°C cranial temp vs. 32.5°C rectal temp), the temperature of control animals (33.8°C cranial temp vs. 36.5–37°C rectal temp), Epo preparation (R&D Systems vs. EPEX), Epo dose (1000 vs. 5000 U/kg) and dosing intervals. Both studies mentioned power calculations, but it is not clear that the extreme variability seen in the untreated brain injured group was taken into account in these calculations. It appears that despite the prolonged period of hypoxia (120 min), some animals in the Fang study remained uninjured while others were severely affected. The uninjured animals increase within-group variability and ideally would be identified in real time and excluded from all treatment groups. It is possible that in such small treatment groups these uninjured animals were randomly unevenly distributed, thus affecting the results. It is striking that neither therapy showed clinically significant neuroprotection in the Fang study, suggesting there is something important to be learned about resistance to therapy.

Rodents are the most commonly used animals to model neonatal brain injury, so it is worthwhile considering some of the difficulties in translating information learned from rodents to humans. The rodent brain is lissencephalic with a much smaller proportion of white matter than is present in humans. Foci of neurogenesis and timing of myelination are different. These factors may be important when studying the effect of an early insult on later brain development. The rate of rat or mouse maturation is very accelerated relative to humans with each day of rat development corresponding to more than a week of human

development. However, the time course of response to injury appears to be similar in both species. Thus as brain injury unfolds over hours to days, the developmental context changes differentially in rodents compared to humans (as injury evolves from P7 to P10 in a rat, this timeframe would roughly span 32 weeks to term in a human infant). Since the cellular and regional vulnerability of brain varies by developmental stage, the effect of brain injury and its repair may be quite different in rodents than humans. We do not know how these different time frames affect dosing duration, dosing interval, or how response to therapeutics interact with evolution of injury. For example, in humans and larger animal models it is known that 72 hours of hypothermia is more beneficial than 12 or 24 hours. How does this translate to rat pups? Are 3 or 8 hours sufficient? Or is 24 or 72 hours required for neuroprotection? Therapy should optimally target the timing of response to injury, the pattern of cell death and inflammatory response, followed by repair. Differences in drug metabolism may also be important when translating preclinical trials to humans. We have seen that in extremely low birth weight infants, 500 U/kg Epo IV results in peak circulating concentrations similar to those achieved in rat pups given 5000 U/kg IP, but area under the curve (AUC) is most similar when 1000 U/kg in a preemie is compared to 5000 U/kg in a rat [18,19].

In human studies, we do not consider a therapy proven until there are many hundreds (or even thousands) of subjects that have shown benefit. Yet in animal studies, we expect to show meaningful differences comparing small groups. This may not be a reasonable expectation. At this point, more studies are needed, ideally, in multiple large and small animal models to establish whether hypothermia and Epo will prove to be of additional benefit relative to hypothermia alone.

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