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Review article

Application of advanced preclinical models and methods in anesthetic neurotoxicity research

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A B S T R A C T

Recently, there has been increasing concern regarding the potential of anesthetics to disturb the long-term function of the central nervous system (CNS). The field of anesthesia-related toxicology, therefore, has engaged multiple scientific disciplines and utilized a variety of pre-clinical research models in an attempt to identify the basic characteristics of the anesthetic agents that may produce acute and/or chronic adverse effects on the CNS. This review discusses how the application of advanced research approaches and models, such as the nonhuman primate, neural stem cell-derived organotypic slice cultures and/or organs-on-chips systems, can serve as translational models of infantile anesthetic exposure. Utilization of these models may expeditiously decrease the uncertainty in the risk posed to children by postnatal anesthetic exposure.

1. Introduction

General anesthesia is routinely utilized in prolonged and complex procedures of pediatric and obstetric surgeries. A recent review article (Walters and Paule, 2017) summarizes the major findings from most pre-clinical studies and indicates that concerns have recently arisen regarding the effects of anesthetic drugs on the central nervous system (CNS), with some evidence suggesting that long-term cognitive changes may occur after surgery with general anesthesia in a pediatric population (DiMaggio et al., 2009; DiMaggio et al., 2011; Ing et al., 2012; Sprung et al., 2012). The clinical relevance of the long-term impact of anesthetic neurotoxicity is an urgent public health matter.

While it is difficult to verify the adverse anesthetic effects on human infants and children, advanced research models, both *in vivo* and *in vitro*, including the nonhuman primate (Slikker et al., 2007; Wang et al., 2012), neural stem cell-derived organotypic slice cultures (Bai et al., 2013; Liu et al., 2014a), and/or organs-on-chips models (Esch et al., 2015), have proven invaluable for informing aspects of human physiology, pathology, pharmacology, toxicology and systems biology.

This review presents an overview regarding how the utilization of highly relevant preclinical models, *e.g.*, nonhuman primates, might serve as translational models to evaluate the vulnerability of the immature brains. This article also discusses the potential in the application of advanced techniques, as effective tools, to dissect mechanisms underlying anesthetic-induced neurotoxicity. In addition, biological combination of appropriate preclinical models with advanced techniques is critical to explore possible protective strategies and to decrease the uncertainty associated with extrapolating the preclinical findings to humans.

Over the last several years it has been established that anesthetic exposure *in vivo* will cause neurotoxicity in a variety of preclinical species (Zhang et al., 2013a, 2013b; Liu et al., 2014a). Similarly, powerful *in vitro* research models are now readily available, such as three-dimensional (3D) screening and micro-engineered chips from tissues to organs (Hartung et al., 2017; Stoppini et al., 2017). When combined, these cutting edge research approaches make it possible to unravel the complexity of anesthetic-induced genetic changes, phenotype variations, morphological and biochemical alterations, as well as dynamic pathological changes and long-term behavioral deficits. This review provides some examples of how these combined approaches can improve the clarity of studies examining the impact of anesthesia on the developing brain, and uncover the mechanisms contributing to neurotoxicity.

2. Developing nonhuman primate models, molecular imaging and anesthetic neurotoxicity

The study of the potential neurotoxic effects of anesthetic agents on the pediatric population (clinically) is very difficult due to safety and ethical issues. Very recent reports from clinical studies, such as the Pediatric Anesthesia Neurodevelopment Assessment (PANDA) and the General Anesthesia compared to Spinal anesthesia (GAS) trial (Davidson et al., 2016; Sun et al., 2016) have indicated that single anesthesia exposure or short sevoflurane exposure (less than 1 h) to

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healthy children younger than 3-year old [the definitive outcome will be measured at 5 years of age (in 3 years from now)] may not cause significant adverse effects. Although these findings are a relief to some patients and anesthesiologists, the potential adverse effects of general anesthetics on pediatric population who have complicated clinical conditions still need further investigation. Moreover, an inevitable difficulty for clinical studies is lack of control population that can accurately match anesthetic-exposed groups. Therefore, any conclusion drawn from a clinical study has some limitations; and no straightforward advice from clinical studies can be provided to anesthesiologists for their practice on pediatric anesthesia.

It has been reported that commonly used general anesthetics induce neurotoxicity in developing rodent brains (Choi, 1988; Ikonomidou et al., 1999; Jevtovic-Todorovic et al., 2003; Wang et al., 2005b; Wang et al., 2006; Kang et al., 2017). However, whether or not the nature of anesthetic-induced neural damage observed in these rodent models has clinical relevance is still not entirely known. It should be mentioned that current developmental neurotoxicity (DNT) test guidelines are primarily rodent-based: one DNT test requires about 1000 rat pups produced from a minimum of 140 dams. The pups are used for various tests related to brain function until the animals are 60-days of age. Therefore, DNT testing is expensive, time consuming, and raises animal welfare concerns (Moors et al., 2009). Because of the different degree of maturation between rodent fetus and the human fetus, and the different fetal to maternal weight ratio at birth, the findings from neurobehavioral testing in rodents, especially during the development, can only provide gross evaluations; information on mechanisms is limited. In contrast, the non-human primate (NHP, *e.g.*, rhesus monkeys), being 98% genetically similar to humans, can in many instances more accurately predict how pathological conditions arise in the human body. Evidence suggests that the vulnerability of the primate brains to the toxicants/chemicals is closely related to the maturity of brain development. Specifically, it appears that the brain is most vulnerable to the neurotoxic effects of anesthetics during the “brain growth spurt” (Slikker et al., 2007). Because the brain growth spurt in both human and NHPs extends over a much longer time period than in rodents, matching the timing of a developmental event in humans and NHPs is less problematic than matching the same periods between humans and rodents. It is also generally believed that the NHP fetus (especially that of the rhesus monkey) and the human fetus have a more similar degree of maturation at birth as compared to rodents. No other commonly used animals have a functional fetal placental unit, a propensity for a single birth and a fetal-to-maternal weight ratio comparable to that of human. Thus, NHPs may be more efficient, mechanism-focused, cost-effective and appropriate models for the studies of the adverse effects and mechanisms of pediatric anesthesia due to their complicacy of brain structure and the durable period of brain development.

It has been reported that intravenous infusion of a general anesthetic agent, ketamine (noncompetitive NMDA receptor antagonist), can induce reversible neuronal damage, including apoptosis and necrosis in infant monkeys (Slikker et al., 2007). This study in monkeys demonstrated that prolonged exposure to ketamine *in utero* [gestational day (GD) 122] or during infancy [postnatal day (PND) 5] caused clear neuronal cell damage. In contrast, ketamine exposure at PND 35 did not result in overt signs of toxicity. These data indicate that GD 122 or infant monkeys are more vulnerable to ketamine exposure than PND 35 monkeys, potentially because of decreased synaptic formation at the later time point of development. Since this initial demonstration of anesthetic-induced neuroapoptosis in the developing NHP, other studies have confirmed and extended these findings (Wang et al., 2006; Hotchkiss et al., 2007; Slikker et al., 2007; Zou et al., 2009; Paule et al., 2011; Zou et al., 2011; Liu et al., 2012; Zhang et al., 2013a, 2013b; Liu et al., 2015). Moreover, after comparing the vulnerability of neurons to anesthetics at different ages, it is demonstrated that, neurons in rhesus monkeys were less sensitive to anesthetic after 20-day of age, but oligodendrocytes became more sensitive at that age (Brambrink et al., 2012; Schenning et al., 2017). Their findings were consistent with ours in the view of anesthetic-induced neuronal apoptosis in NHP.

Molecular imaging techniques have been widely utilized to monitor normal physiology, investigate disease processes and assist in drug development (Eckelman, 2003). Previous data (Massoud and Gambhir, 2003; Min and Gambhir, 2004) indicate that exposure of developing NHPs to anesthetics during the period of rapid neuronal growth results in increased rates of neurodegeneration (Zhang et al., 2013a, 2013b; Zhang et al., 2017). Consistent with epidemiological studies (DiMaggio et al., 2009; Istaphanous and Loepke, 2009; Kalkman et al., 2009; Wilder et al., 2009; Nie et al., 2013), these anesthetic-induced adverse (neurodegenerative) effects are also associated with long-term cognitive abnormalities and/or behavioral deficits in developing nonhuman primates (Paule et al., 2011). Since dynamic imaging has a great potential for helping advance our understanding of anesthetic-related toxic process in living animals including rodents and nonhuman primates (Kilbourn et al., 2009; Hillmer et al., 2011; Wooten et al., 2011) as well as human, PET scanning with specific radio-tracer can successfully be applied to repeatedly visualize and quantify aspects of anesthetic-induced neurotoxicity.

For the detection of anesthetic-induced neuronal damage, PET scans provide information about molecular changes associated with cell death, *e.g.*, neuronal apoptosis (Zhang et al., 2009). Effective radioactive tracers for PET scanning are ligands/molecules labeled with short-lived positron-emitting radionuclides such as O-15, N-13, C-11 and F-18. For detecting anesthetic-induced neuronal apoptosis or pathological processes (Myers and Hume, 2002; Lancelot and Zimmer, 2010; Schnockel et al., 2010; Wagner and Langer, 2011), many radioactive tracers, *e.g.*, [^{18}F]-Annexin V, [^{18}F]-DFNSH (^{18}F-labeled dansylhydrazone of *p*-fluorobenzaldehyde), [^{18}F]-FDG (2-deoxy-2-[18F]-fluoro-D-glucose), [^{18}F]-FEPPA (*N*-acetyl-*N*-(2-[^{18}F]-fluoro-ethoxybenzyl)-2-phenoxy-5-pyridinamine) and caspase-3 inhibitors, have been applied in preclinical studies (Liu et al., 2013b; Liu et al., 2014b; Wang, 2016). However, based on our experience, not all tracers used for monitoring neuronal apoptosis can effectively be applied in both developing rodents and NHPs. In addition, it is still not clear why the tracer sensitivity is so different between species. For example, one of the hallmarks or an early event of neural apoptosis is the exposure of phosphatidylserine at the cell surface, where it can be detected by Annexin V conjugate. Previous studies indicated that anesthetic (ketamine)-induced neuronal apoptosis could dynamically and quantitatively be evaluated by translatable biomarker - [^{18}F]-Annexin-V (microPET imaging) in the developing rodent (Zhang et al., 2009). However, anesthetic-induced neuronal apoptosis were not effectively determined by microPET imaging of [^{18}F]-Annexin-V in the developing NHP (Zhang et al., 2013a, 2013b). Here, the possible reasons for this difference in detectability between rodents and NHPs may be due to their maturation levels and the nature of architecture of the blood brain barrier (BBB) during development. Actually, only a few isotope-labeled tracers, *e.g.*, [^{18}F]-FDG and [^{18}F]-FEPPA (Zhang et al., 2012, 2013a, 2013b; Zhang et al., 2016) have successfully been utilized in monitoring anesthetic-induced alterations in monkey brains.

[^{18}F]-FEPPA, a marker of glial activation, can selectively and specifically bind to translocator proteins (TSPOs, 18 kDa), previously known as peripheral benzodiazepine receptors, a group of mitochondrial proteins which are upregulated in expression following brain damage and neuro-inflammation (Choi et al., 2011). Anesthetic-induced alterations/adverse effects have been evidenced by increased radiolabel uptake (tracer accumulation) in specific Regions of Interest (ROIs), *e.g.*, the frontal and temporal lobes (Zhang et al., 2013a, 2013b; Zhang et al., 2016). However, [^{18}F]-FEPPA cannot be counted as a specific marker for neuronal apoptosis but rather for microglial activation. Therefore, microPET imaging of [^{18}F]-FEPPA in the developing nonhuman primate provides only indirect information (surrogate marker) regarding anesthetic-neurotoxicity. Meanwhile, other potential tracers for neuronal apoptosis are being considered, such as ML-10, 2-