Behavioural Brain Research 282 (2015) 117–124

Contents lists available at ScienceDirect

Behavioural Brain Research

journal homepage: www.elsevier.com/locate/bbr

Research report

Effects of embryonic cyclosporine exposures on brain development and behavior

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HIGHLIGHTS

- We examined the effects of cyclosporine exposures during zebrafish development.
- Early embryonic exposures led to a reduction in eye size and brain size.
- Late embryonic exposures led to behavioral defects.
- The use of cyclosporine during pregnancy is concerning.

ARTICLE INFO

Article history:
Received 24 November 2014
Accepted 6 January 2015
Available online 13 January 2015

Keywords:
Zebrafish
Brain
Behavior
Calcineurin
Cyclosporine
Cyclosporin

ABSTRACT

Cyclosporine, a calcineurin inhibitor, is successfully used as an immunosuppressant in transplant medicine. However, the use of this pharmaceutical during pregnancy is concerning since calcineurin is thought to play a role in neural development. The risk for human brain development is difficult to evaluate because of a lack of basic information on the sensitive developmental times and the potentially pleiotropic effects on brain development and behavior. In the present study, we use zebrafish as a model system to examine the effects of embryonic cyclosporine exposures. Early embryonic exposures reduced the size of the eyes and brain. Late embryonic exposures did not affect the size of the eyes or brain, but did lead to substantial behavioral defects at the larval stages. The cyclosporine-exposed larvae displayed a reduced avoidance response to visual stimuli, low swim speeds, increased resting, an increase in thigmotaxis, and changes in the average distance between larvae. Similar results were obtained with the calcineurin inhibitor FK506, suggesting that most, but not all, effects on brain development and behavior are mediated by calcineurin inhibition. Overall, the results show that cyclosporine can induce either structural or functional brain defects, depending on the exposure window. The observed functional brain defects highlight the importance of quantitative behavioral assays when evaluating the risk of developmental exposures.

1. Introduction

The discovery of cyclosporine A as an immunosuppressive drug has revolutionized the field of transplant medicine, allowing allograft organ transplants to become commonplace [1]. Cyclosporine acts as an immunosuppressant by inhibiting calcineurin, a calcium-dependent protein phosphatase that plays a critical role in T-cell activation [2,3]. By inhibiting calcineurin and suppressing T-cell activation, cyclosporine effectively reduces the rate of transplant rejection. Following its success in transplant medicine, cyclosporine has been used for the treatment of a wide variety of autoimmune diseases, including psoriasis and rheumatoid arthritis [3].

Cyclosporine is classified as a pregnancy category C drug by the United States Food and Drug Administration [4]. In Pregnancy Category C, 'animal reproduction studies have shown an adverse effect on the fetus, there are no adequate and well-controlled studies in humans, and the benefits from the use of the drug in

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http://dx.doi.org/10.1016/j.bbr.2015.01.006
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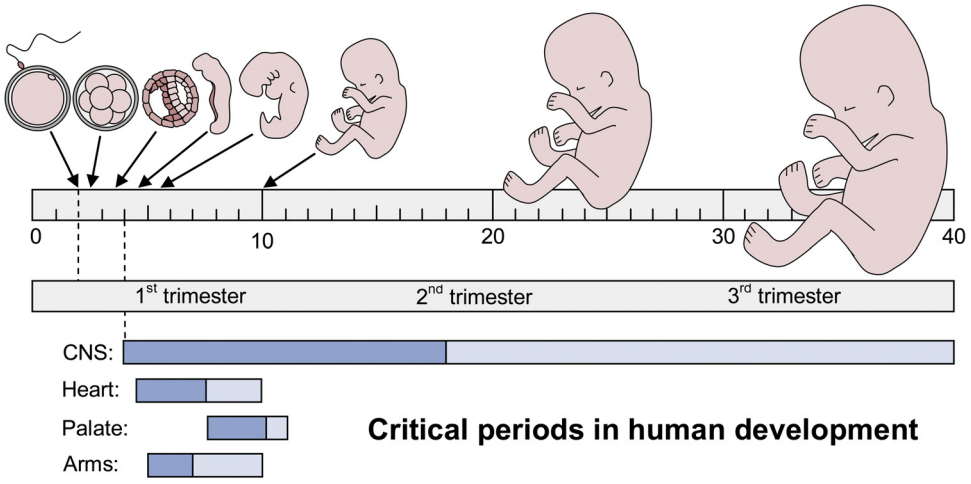

Fig. 1. Critical or sensitive periods in human development. Most developing organs are sensitive to teratogens during the embryonic period from 4 to 10 weeks of gestation. A notable exception is the central nervous system (CNS), which remains sensitive throughout the fetal period from 10 to 40 weeks of gestation. To illustrate the extended period of sensitivity of the CNS, we redrew the textbook model from Moore et al. [9] on a linear 40-week scale (with permission). Dark blue = major structural defects, light blue = minor structural or functional defects, and 40 weeks of gestation = 38 weeks of development.

pregnant women may be acceptable despite its potential risks; or animal studies have not been conducted and there are no adequate and well-controlled studies in humans' [5,6]. The labeling of Category C pharmaceuticals must state that the drug 'should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus' or 'should be given to a pregnant women only if clearly needed' [5]. Immunosuppressants are clearly needed in transplant medicine to prevent organ rejection and as a result cyclosporine treatment is continued during pregnancy despite the potential risk to the developing fetus [7]. The use of cyclosporine during pregnancy is concerning, since calcineurin is thought to play a role in neural development and axonal growth [8]. However, the risk for human brain development is difficult to evaluate, because of a lack of basic information on the sensitive developmental times and the potentially pleiotropic effects on brain development and behavior. Models of 'critical periods' in human development predict that different types of defects may be induced depending on the exposure window [9], i.e. early developmental exposures can lead to major structural brain defects , while late developmental exposures are likely to induce more subtle or functional brain defects (Fig. 1).

The signaling pathways that regulate brain development and neural function are conserved in vertebrate species [10,11] and the zebrafish has emerged as a powerful model system in behavioral neuroscience [12–14]. Hundreds of synchronously developing embryos can be collected from the bottom of a tank on a daily basis and exposures can be carried out in a culture dish. The embryos are transparent, which makes it possible to image the developing brain in living embryos using various state-of-the-art molecular tools [15–17]. Zebrafish embryos develop rapidly. At 24 h post-fertilization (hpf), the embryos have a beating heart, a moving tail, two large eyes, and a brain with distinct brain regions [18]. The embryos hatch from their chorion between 2 and 3 days post-fertilization (dpf). At 5 dpf, the free-swimming larvae are approximately 4 mm long, have inflated swim bladders, and display a broad range of behaviors, which can be examined in multiwell or multilane plates [19–25].

In the present study, zebrafish embryos were exposed to cyclosporine during different stages in embryonic development. We found that early embryonic exposures led to a reduction in eye and brain size. Late embryonic exposures did not affect the size of the eyes and brain, but did lead to significant behavioral defects.

2. Materials and methods

2.1. Zebrafish embryos and exposures

Adult wild type zebrafish (*Danio rerio*) were originally obtained from Carolina Biological and have been maintained at Brown University as a genetically diverse outbred strain. For the analysis of structural brain defects, we used the *Tg*(*elavl3:EGFP*) line, which expresses the enhanced green fluorescent protein under control of a ubiquitous neuronal promotor [26]. Zebrafish embryos were collected within 1 h after spawning and raised at 28.5 °C in egg water, containing 60 mg/L sea salt (Instant Ocean) in deionized water and 0.25 mg/L methylene blue as a fungal inhibitor. Cyclosporine (cyclosporin A, Enzo Life Sciences) and FK506 (tacrolimus, Enzo Life Sciences) were diluted in egg water from 1000× stocks dissolved in dimethylsulfoxide (DMSO). The corresponding DMSO concentration (0.1% DMSO) was used as a control. Embryos were exposed from 2 to 26 h post-fertilization (hpf), 26–50 hpf, or 50–74 hpf, washed four times in egg water, and grown in egg water for up to 5 days post-fertilization (dpf). The developing zebrafish are referred to as 'embryos' from 0 to 3 dpf and as 'larvae' afterwards [27].

2.2. Analysis of eye and brain defects

To examine eye size, wild type embryos were imaged at 3 dpf in a ventral view by standard bright-field microscopy on a Zeiss Axiovert 200M microscope, using a 10× objective. The eye length was measured in ImageJ, which can be downloaded at http://imagej.nih.gov/ij/download.html. Measurements of the left and right eyes were averaged in Microsoft Excel. These values were subsequently averaged over the number of embryos (n = number of embryos). To examine brain structure, *Tg*(*elavl3:EGFP*) embryos were imaged at 3 dpf by confocal or wide-field fluorescence microscopy. For confocal microscopy, the embryos were grown from 22 to 72 hpf in 0.003% 1-phenyl-2-thiourea (PTU) in egg water to suppress pigmentation. The 3 dpf embryos were oriented in 0.8% low-gelling temperature agarose. Neural patterns were imaged on a Leica SP2 AOBS confocal microscope using a 20× objective for a frontal view (transverse sections) or a 10× objective for a dorsal view (coronal sections). Z-stacks of 125 slices were acquired through 150 μm of the brain using a two Airy unit pinhole, a 488 nm laser for excitation, and a 510–600 nm filter. The data sets were examined by collapsing the stacks as maximum projections