Reproductive Toxicology 51 (2015) 79–89

Contents lists available at ScienceDirect

Reproductive Toxicology

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

Effects of 2,5-hexanedione on angiogenesis and vasculogenesis in chick embryos

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ARTICLE INFO

Article history:
Received 13 April 2014
Received in revised form 5 December 2014
Accepted 10 December 2014
Available online 27 December 2014

Keywords:
2,5-Hexanedione
Chick embryos
Blood islands
Vasculogenesis
Angiogenesis

ABSTRACT

n-Hexane is widely used in industry and its metabolite, 2,5-hexanedione (2,5-HD), has been implicated as a neural toxin in the developing fetus. Using the chick embryo model, we have previously revealed the neurotoxicity of 2,5-HD during development and established that high dose of 2,5-HD was embryo lethal. In view of the close linkage in biology for neurogenesis and angiogenesis, we speculated that it was most likely caused by cardiovascular dysplasia, therefore in this study, we investigated the effects of 2,5-HD on the development of the vasculature, which involves vasculogenesis and angiogenesis. Using gastrulating chick embryos as a model, we demonstrated that the hemangioblasts (precursor of hematopoietic and endothelial cells) migrated to the area opaca where they form the blood islands. However, this process was impaired when the embryos were treated with 2,5-HD, suggesting that 2,5-HD is capable of impairing vasculogenesis. To study the effect of 2,5-HD exposure on angiogenesis, we used the chick yolk-sac membrane (YSM) and chorioallantoic membrane (CAM) models. We found that, at low (0.02 M) concentration, 2,5-HD stimulated angiogenesis while at higher concentrations (>0.1 M) it inhibited this process. This biphasic response of angiogenesis to 2,5-HD exposure was found to be associated with altered expression of the VEGF-R, FGF-2 and angiogenin. Moreover, we also determined that 2,5-HD exposure increased the reactive oxygen species (ROS) production. In conclusion, 2,5-HD could induce dysplasia in the developing vasculature, which in turn could cause extravascular hemolysis and the embryos to die.

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1. Introduction

n-Hexane has been widely used as an organic solvent for cleaning electronic and printing devices, such as circuit board, the screen of mobiles and so on. Furthermore, the solvent is also used in a variety of commercial manufacture, including furniture, leather, shoes, viscose and rubber. Despite its apparent low toxicity, there is now growing concern that it might be occupationally high risk to health because of poorly ventilated systems in those factories [1–4]. *n*-Hexane is absorbed by inhalation or transcutaneous permeation and can accumulate in the body. Being highly volatile and highly fat-soluble, *n*-hexane could lead to neuropathy, which is presented as peripheral sensory and motor nerve degeneration [5,6]. In the body, *n*-hexane is catalyzed by CYP2E1 to form the metabolite, 2,5-hexanedione (2,5-HD) [7]. It is believed that the metabolite of *n*-hexane was producing the toxic effect on the nervous system [8]. 2,5-HD ($CH_3COCH_2CH_2COCH_3$) is classified as a small molecule with molecular weight of 114. It has been reported that 2,5-HD could increase the permeability of the blood–nerve barrier [9]. Potentially, this could have dire implications on pregnant women exposed to 2,5-HD as it might be able to penetrate the blood–placenta barrier too. Using the chick embryo model, we have recently investigated the neurotoxicity of 2,5-HD during development [10]. We established that high dose of 2,5-HD was embryo lethal and speculated that it was most likely caused by cardiovascular dysplasia, because the close linkage in biology for neurogenesis and angiogenesis [11], however, the malformation in nerve system does not always result in embryolethality, while the serious disruption of angiogenesis does. Furthermore, the vascular system is

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http://dx.doi.org/10.1016/j.reprotox.2014.12.006
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the first functional organ to form during embryogenesis [12]. Any disturbance of this biological process can lead to varying degrees of adverse consequences, ranging from congenital angiodysplasia to fetal malformations and embryo death.

The vasculature is one of the most important and complex organs in the body. Development of the vasculature occurs in two distinct morphological stages which include vasculogenesis and angiogenesis [13]. Both vasculogenesis and angiogenesis are mainly accomplished during embryo development, while the maintenance of blood vessel integrity and the control of vessel physiology and hemodynamics have important consequences throughout embryonic and adult life. Vasculogenesis involves mesoderm-derived hemangioblasts, from inside and outside the embryo, which joins the embryonic and yolk sac circulation, and forms the first primitive vascular plexus. This process is accompanied by the differentiation and coalescence of endothelial cells (ECs). While angiogenesis involves the remodeling and expansion of the vascular plexus through ECs sprouting and intussusceptive microvascular growth, regarded as the formation of new blood vessels from pre-existing vasculature [14,15].

Signals regulating vasculogenesis and angiogenesis come from a variety of molecules linked to ECs biological behavior, extracellular matrix remodeling and the local production of cytokines, chemokines and growth factors. The signaling genes closely associated with this process are vascular endothelial growth factor (VEGF), vascular endothelial cadherin (VE-cadherin), fibroblast growth factor (FGF), angiogenin (Ang), and TGF-β, which must be expressed in a precise spatiotemporal fashion for development and pattern formation properly [16–19]. Currently, toxicity testing has been progressed toward using high-throughput screening (HTS) assays to rapidly test thousands of chemicals against molecular targets and biological pathways, and to identify the sensitive targets and relevant pathways. The blood vessel development has been revealed as a target for lots of environmental chemicals shown by HTS of a 200-chemical small-molecule library in transgenic zebrafish embryos, which benefits to understand developmental health risks associated with the diverse compounds entering the environment [19,20].

The chick yolk-sac membrane (YSM) model has been extensively employed for studying angiogenesis [18,21]. The yolk sac is located extra-embryonically and normally functions to provide nutrition to the developing embryo. It is also the first site where blood vessels and angioblasts develop. Another good model for studying angiogenesis is the blood vessels on chorioallantoic membrane (CAM) of older chick embryos. CAM is a highly vascularized membrane found directly underneath the inner surface of the egg shell. It is formed by the fusion of the chorionic membrane and allantois during embryo development. Both YSM and CAM are excellent models for studying the formation of blood vessels because they are highly vascular and the experimental results produced are highly reproducible [22].

In this study, we used the early stage chick embryos, YSM and CAM models to investigate the adverse effects of 2,5-HD on vasculogenesis and angiogenesis during the development.

2. Materials and methods

2.1. Treatment of chick embryos

Fertilized Leghorn eggs were purchased from Avian Farm of the South China Agriculture University (Guangzhou, China). The eggs were incubated in an incubator (Yiheng Instruments, Shanghai, China) at 38 °C with 70% humidity until the embryos reached the desired developmental Hamburger and Hamilton (HH) stage [23]. Embryos at HH10 were then treated with different concentrations of 2,5-HD (Sigma–Aldrich, MO, USA) or PBS (serving as control) as described previously [10]. Briefly, 100 μL PBS or 2,5-HD (0.01, 0.1, or 1 M) was directly injected, at the blunt end of the egg, into the air chamber of the fertilized egg. After treatment, the embryos were further incubated at 38 °C for 4.5 days before being harvested. Then each embryo was photographed using a stereomicroscope (Olympus MVX10, Japan), and 10 embryos were employed for each treatment group.

2.2. Evaluation of 2,5-HD effect on mesodermal cell migration and in situ hybridization

Briefly, culture dishes were tailor-made with a plastic barrier running down the midline of the dish, which could separated the culture medium into two different parts [24]. Then we placed a HH3 chick embryo along the barrier. The gastrulating embryo was carefully orientated so that the primary streak was equally divided along its middle line on the barrier. This permitted one side of embryo to be exposed to different concentrations of 2,5-HD (0.02 and 0.05 M) while the other side exposed to PBS control. To follow mesodermal cell migration, we used DiI fluorescent dye (Molecular Probes, Inc.) to label a small group of the posterior primitive streak cells of HH3 embryos. A 2.5% stock of DiI (in ethanol) was diluted 1:10 in 0.3 M sucrose and was then injected into the posterior primitive streak of HH3 chick embryo using a micropipette. Approximately, 10–30 cells were labeled. The embryos were incubated at 38 °C for up to 30 h and the extent of mesodermal cell migration was monitored using a fluorescent microscope [25]. 10 embryos in each concentration group were examined.

Chick embryos were harvested after DiI labeling and fixed in 4% paraformaldehyde at 4 °C overnight. In situ hybridization was performed on whole-mount chick embryos according to standard in situ hybridization protocols [26]. Digoxigenin-labeled riboprobes specific for VE-cadherin were synthesized to demonstrate the development of the blood islands [27]. All stained embryos were photographed and the VE-cadherin$^+$ blood island areas were automatically quantified and analyzed using an IPP 5.0 system (Image Pro-Plus 5.0, Media Cybernetics) [18].

The whole mount immunocytochemistry against Caspase 3 (1:100, BD, USA) was performed on some other chick embryos harvested after being exposed to 2,5-HD, followed by specific secondary antibody mixture coupled with Alexa Fluor 555 anti-rabbit IgG (1:1000, Invitrogen, USA). All the embryos were counterstained with DAPI (4′-6-diamidino-2-phenylindole, 1:1000, Invitrogen, USA) for 1 h at room temperature. At least 4 embryos were analyzed in each treatment group. The whole mount images were visualized under fluorescence stereomicroscope. And then the embryos were embedded in OCT and frozen section with 10 μm thickness was serially done with a cryostat microtome (Leica CM 1900, Germany). The transverse sections of these embryos at the level of trunk portion were photographed by fluorescence microscope (Olympus IX51, Japan). Quantitative analysis of Caspase 3$^+$ cells in 2,5-HD treated side compared with control side was carried out as previously described [28]. Cell counting was carried out on at least 30 different sections of four different embryos after each experimental condition.

2.3. Assessment of 2,5-HD effect on angiogenesis in chick YSM

Fertilized eggs were incubated for 2.5 days and then placed into a sterilized glass dish. The YSM containing blood vessels were orientated facing upward. Two silicone rings were placed directly on top of the leading edge of the blood vessels and used as a reference to determine the extent of vascular plexus growth during embryo development. To avoid differences between different embryos, 50 μL of PBS (control) was introduced into the ring located on the left side of the YSM. While 50 μL of 2,5-HD (0.02, 0.1 and

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