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Flusilazole induces spatio-temporal expression patterns of retinoic acid-, differentiation- and sterol biosynthesis-related genes in the rat Whole Embryo Culture

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ABSTRACT

Embryotoxic responses are critically dependent on the timing of exposure during embryo development. Here, we examined the time- dependent developmental effects in rat embryos exposed to flusilazole (FLU), and their link to retinoic acid (RA) mediated pathways. To this end, we assessed the effects of 4 h exposure of rat embryos *in vitro* to 300 μM FLU during four developmental time windows (0–4, 4–8, 24–28 and 44–48 h), evaluating morphological parameters, expression and localization of five genes directly or indirectly linked with the RA pathway. These were RA- (*Cyp26a1* and *Dhrs3*), differentiation- (*Gbx2* and *Cdx1*) and sterol biosynthesis- (*Cyp51*) related genes. Extended exposure for 48 h to 300 μM FLU resulted in morphological changes, typical for triazoles and RA, while the 4 h exposure times did not. Time dependent significant upregulation of the five selected genes was observed. These results corroborate that the embryotoxic responses to FLU are correlated with the regulation of the RA pathway. Thus, these gene expression markers can be considered early biomarkers of FLU-induced potential developmental toxicity later in the development.

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1. Introduction

On 1 July 2007, the Regulation on Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) was implemented by the European Union (EU) to decrease chemical risks for both humans and the environment. Consequently, there is a demand to assess the safety of all current and future chemicals, which will utilize millions of laboratory animals. The assessment of possible developmental toxicants through the application of alternative techniques may reduce animal use and enhance mechanistically driven risk assessment [1–4].

According to ECVAM (European Centre for the Validation of Alternative Methods), the rat post-implantation whole embryo culture (WEC) is a standardized alternative *in vitro* method for identifying developmental toxicants after exposure during early organogenesis [2,5–7]. The advantage of this method is that early organogenesis and neurulation can be continuously monitored during this 48 h critical period [2,6], while there is a high level of concordance between human and rodent *in vivo* development during this embryonic stage [6,8,9]. On the other hand, possible limitations of this model could be the lack of maternal metabolism and the restricted 48- hour experimental window. However, validation studies have proved that by using the WEC assay, the developmental toxicity of chemical substances can be studied taking into consideration a variety of both morphological and molecular endpoints [5,6,10–12].

Triazoles are a category of fungicides widely used in both medicine and agriculture [13,14]. The triazoles' mechanism of pharmacological action is based on inhibiting the fungal *Cyp51*, the catalyst for converting lanosterol to ergosterol. This inhibition causes increased fungal cell wall (exterior membrane) permeability and cell death [15,16]. In mammalian systems some triazoles may induce developmental toxicity [17,18]. One of the triazoles, Flusilazole (FLU), has been extensively used as a model compound in the study of induced developmental toxicity [18–20].

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In vivo and *in vitro* studies have suggested that the mechanism of developmental toxicity of FLU is associated with the expression changes of genes, such as *Cyp26a1* and *Dhrs3*, which participate in the mechanisms of growth and differentiation [21,22]. In detail, data from *in vitro* techniques have suggested that the embryotoxicity of triazoles is promoted through the retinoic acid (RA) pathway, on account of similarities in both morphological embryotoxic outcome and read-outs of selected biomarkers after exposure to either triazoles or RA [2,23–25]. Furthermore, recent animal studies demonstrated commonly regulated morphological characteristics, which support the aforementioned hypothesis concerning the involvement of the RA pathway [22]. The most common malformations introduced by FLU and RA are craniofacial and posterior axis defects, disturbance of neural crest cell (NCC) migration, altered branchial arch development and abnormal hindbrain segmentation [26,27].

Given that developmental toxicity may be highly dependent on the embryonic stage when exposure occurs, it is of interest to compare different exposure time windows, and compare the differences in the magnitude of gene expression responses that may underlie developmental toxicity upon exposure during these different time windows. This allows determination of time windows which would be most sensitive to detect gene expression changes as biomarkers of developmental toxicity induced by specific groups of compounds, such as the triazole group. The two selected genes for this study, *Cyp26a1* and *Dhrs3*, are actively involved in balancing embryonic RA concentration and, therefore, respond to RA- like exposures [2,28,29]. Furthermore, the next two RA-related genes, *Gbx2* and *Cdx1*, are involved in craniofacial development, posterior axis, early embryonic patterning and cardiovascular formation [2,30–34], while the last selected gene, *Cyp51*, is linked to steroid biosynthesis [21]. Changes in expression of these genes have been shown to be associated with a malformation when there was an over-accumulation of RA in the embryonic body [35]. The role of these genes in the toxicological mechanism of FLU's action could be the key for explaining FLU-induced teratogenicity in a stage-dependent manner during early embryonic development. In this study, we assess the time- dependent induced teratogenicity after exposure of rat WEC to FLU during different time windows by monitoring phenotypic and genotypic alterations, the latter with RT-PCR and *in situ* hybridization.

2. Materials and methods

2.1. Animal care

Animal studies were approved and performed in concordance with institutional and federal regulations at the National Institute of Public Health and the Environment (RIVM). Wistar rats (HsdCpd:WU) (Harlan, The Netherlands) were housed at the RIVM Animal Care facility in a climate controlled room with a 12 h light cycle (04:00–16:00 dark). After acclimatizing for 14 days, virgin female rats were mated with male rats for 3 h (9:00-12:00). The presence of copulatory plug in the female rats was considered evidence of pregnancy, and more precisely, as gestational day 0 (GD0). The dams were housed in different cages where their clinical condition was monitored daily.

2.2. Rat whole embryo culture

The whole embryo culture (WEC) technique was performed in accordance with the validated method of Piersma [6]. Previous experiments were conducted in the same laboratory following the same protocol [4,13,36]. On the 10th gestational day, between 9:00 and 12:00 AM, dams were euthanized by intracardiac injection of T61^{R} (Intervet, The Netherlands). Rat embryos were immediately explanted from the mother's uterus. The peripheral trophoblastic cell zone and the parietal yolk sac membrane were removed under the microscope leaving both the visceral yolk sac and ectoplacental cone intact. Embryos with 1–5 somites were cultured for morphological assessment while embryos with 2–4 somites were cultured for gene expression studies. Each embryo was placed individually in a flask with 2 mL culture medium. The medium was a mix of 90% pregnant bovine serum and 10% rat serum (Biochrom, Berlin, Germany), diluted with 14% Hank's solution (Gibco)and supplemented with 1.57 mg/mL D-glucose and L-methionine (Sigma-Aldrich, Zwijndrecht, The Netherlands). The culture flasks with embryos in culture medium were placed in rotating incubators where they were completely protected from light and were stabilized at 37.7 °C. A gas mixture was also provided 5 times for 30 s during the culture period, with an increasing content of oxygen: on the first day (GD10) at 9:00 and 16:00 (5% O_2: 5% CO_2: 90% N_2), on the second day (GD11) at 9:00 and 16:00 (20% O_2: 5% CO_2: 75% N_2) and on the third day at 9:00 (40% O_2: 5% CO_2: 55% N_2).

2.3. Flusilazole exposure

Flusilazole (FLU, CAS number 85509-19-9, Sigma-Aldrich, Zwijndrecht, The Netherlands) was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, Zwijndrecht, The Netherlands) and added to the culture medium at a final concentration of 300 μM FLU and 0.1% DMSO. In agreement with previous studies, DMSO control embryos were also cultured, to enable assurance that the morphological or gene expression alterations were due to FLU exposure. The exposure dose was indicated by a pilot experiment on cultured embryos at 0, 30, 100 and 300 μM of FLU for 48 h. The embryos were further exposed to 300 μM FLU during one of four 4 h timeframes during the whole culture period of 48 h: 0–4 h, 4–8 h, 24–28 h, and 44–48 h. Rat samples, which were cultured for study the morphological outcome after exposing to 300 μM FLU, were collected in the end of the 48 h WEC culture period. Morphological alterations were also assessed for embryos exposed to 300 μM of FLU during the whole culture period (48 h in total). Gene expression signature was studied for embryos that were exposed according to the aforementioned methodology (0–4 h, 4–8 h, 24–28 h, and 44–48 h). These embryos were collected for further studying the expression of the selecting genes immediately after the end of their exposure (4 h, 8 h, 28 h and 48 h). In addition to these samples, we exposed rat embryos to the same concentration of FLU (300 μM) during 0–4 h and we continued their culture into refreshed medium without FLU until the end of the culture period of 48 h. For studying the localization of the genes with *in situ* hybridization technique, additional embryos were exposed to 300 μM FLU following the same methodology as in the gene expression experiments. The embryos were immediately collected after the end of their exposure (4 h, 8 h, 28 h and 48 h).

2.4. Morphological scoring

Embryos, that were cultured for 48 h (whole culture period), were scored according to the Total Morphological Score (TMS) system taking into account a variety of morphological endpoints [37]. The morphological endpoints include growth parameters (crown-rump length, head diameter, number of somites and yolk sac diameter) and developmental parameters, such as yolk sac blood circulation, heart, embryo- turning, caudal neural tube, optic and otic system, fore- and hind- limb, branchial arches, mandibular and maxillary process and the shape and size of somites.

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