Nano-injection in the zebrafish embryo as an alternative exposure route for environmental risk assessment of endocrine disrupting pharmaceuticals

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Pharmaceutical companies are required to perform an environmental risk assessment for each new drug that is launched on the market. Some of the mandatory tests for potential endocrine disrupting (ED) compounds are based on aquatic exposure of juvenile/adult fish. These tests require a lot of time and animals and are thus not consistent with the 3R principle. Therefore, the goal of this study is to develop a zebrafish embryo test, which is not considered an animal test according to the European regulation on the use of laboratory animals. However, it is difficult to perform aquatic exposure experiments with ED pharmaceuticals because they are often lipophilic. Nano-injection is therefore proposed as an alternative route of administration for these pharmaceuticals because (1) the yolk of zebrafish embryos contains a high amount of lipids and (2) it mimics maternal transfer which is a relevant exposure route for lipophilic compounds. To use nano-injection as an alternative exposure route it needs to be characterised and compared to the classical exposure route via water. As a case-study, 5 ED pharmaceuticals with each a different mode of action (MoA) were selected (i.e., estrogen receptor (ER) agonism and antagonism, androgen receptor (AR) agonism and antagonism and aromatase inhibition).

Wildtype and transgenic (5xERE-GFP, obtained from the Carnegie Institution of Washington) zebrafish embryos were injected before they reached the 64-cell stage. After evaluation, dimethyl sulfoxide (DMSO) was chosen as a solvent, because it caused low mortality and no sublethal effects. 0.5 nl droplets of the different concentrations were injected after which the embryos were monitored until 120 hours post fertilization. Using a profile based on multiple biological responses, including survival, hatching, heart rate, morphological deviations, swimming activity, length and GFP expression both exposure routes were compared.

GFP expression analysis showed that 17α-ethinylestradiol (ER agonist) was still present and active in the embryos at the end of the exposure period (5 days post fertilization). The exposed embryos gave a significantly higher signal compared to the vehicle control embryos (100% DMSO) and the order of magnitude of the signal was comparable between both routes. Nano-injection or aquatic exposure also caused a lot of morphological and physiological effects when the embryos were exposed to 17α-ethinylestradiol (ER agonist) or to 17β-trenbolone (AR agonist). This was the case for both exposure routes but the effects were different. When the embryos were exposed to fulvestrant (ER antagonist) or flutamide (AR antagonist) we observed only mortality or no effects at all. The aromatase inhibitor, letrozole, only caused physiological effects for both routes: a decreased swimming velocity after aquatic exposure and an increased heart rate after nano-injection.

Nano-injection can be used as an alternative exposure route because (sub)lethal effects were detectable and the pharmaceuticals were still active in the embryo 5 days after the injection. While the effects caused by the pharmaceuticals were comparable between both exposure routes when

they were grouped in broad categories (i.e. morphological and physiological effects), detailed effects were different. The reason(s) for this will be investigated in the near future (e.g. bioanalysis).

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Nano-injection can be used as an alternative exposure route because (sub)lethal effects were detectable and the pharmaceuticals were still active in the embryo after 5 days. While the effects caused by the pharmaceuticals were comparable between both routes when they were grouped in broad categories (i.e. morphological and physiological effects), detailed effects were different. The reason(s) for this will be investigated in the future (e.g. bioanalysis).