Effects of phytosterols on zebrafish reproduction in multigeneration test

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“Capsule”: A multigeneration test is used to show disruption of the reproductive system by phytosterols.

Abstract

Zebrafish from mixed sex populations were exposed continuously across three generations to two phytosterol preparations both containing β-sitosterol. The phytosterols were isolated from wood and soy beans. Blood vitellogenin levels and sex ratio changes were used as intermediate indicators of the reproduction failures. Both sterol preparations caused vitellogenin induction in the exposed fish. The wood sterol changed the sex ratio of the exposed fish. In generation F1, the predominant sex was male, and in generation F2 it was female. The soy sterol in the used test concentration was lethal to the exposed fish in generation F1. This multigeneration test evidenced that phytosterols containing β-sitosterol disrupt the reproduction system of zebrafish by changing the sex ratios and by inducing the vitellogenin production in the exposed fish.

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1. Introduction

The phenomenon endocrine disruption has focused attention on the need for suitable test methods for the identification of this occurrence. The potential estrogenicity of chemicals has been demonstrated using in vitro systems (Soto et al., 1991; Pelissero et al., 1993; Jobling et al., 1995; Arnold et al., 1996) like also by injecting chemicals into organisms (MacLatchy et al., 1995; Christiansen et al., 1998). However, it remains unclear whether chemicals are able to change the reproductive status of parental fish and their offspring in vivo.

The endocrine disruption effects can be triggered during embryonic or larval development, but clearly significant effects can be expressed during adulthood or in subsequent offspring generations (Kavlock et al., 1996; Allen et al., 1999). Exposure at an early life stage may lead to alterations in key developmental processes such as sexual differentiation. Exposure at maturity can disrupt normal reproductive parameters.

The purpose of this study was to investigate the effects of phytosterols containing β-sitosterol on parental fish and on the two following generations using a multigeneration in vivo-protocol as a tool. Used test concentrations were representative of those observed in natural waters (Hassett and Lee, 1977; Lehtinen et al., 1999). The zebrafish was chosen to be the test fish, because it is universally used in standard tests. The species is an excellent model organism for studying the endocrine disruption effects of chemicals and waste waters in parental fish as well as in subsequent generations. The zebrafish breeds regularly in optimal conditions, and is fully mature within 3–4 months of hatching, and the developmental period from fertilization to hatching is approximately 96 h at 26°C (Laale, 1977).

Phytosterols containing β-sitosterol were chosen as the test chemicals. Phytosterols are widespread within the plant kingdom, especially among pine trees used in the pulping industry (Rydholm, 1965; Pollak and Kritchevsky, 1981). β-sitosterol is one of the most common ones (Cook et al., 1996), also, in Finnish water
bodies (Mellanen et al., 1996). It is also one ingredient in soy bean extracts used for many commercial fish diets (Pelissero et al., 1991). It is known that \( \beta \)-sitosterol may be broken down by microorganisms to produce androgenic compounds, and thus cause masculinization of female fish (Charney and Herzog, 1967; Marcheck et al., 1972; Davis and Bortone, 1992). But also, the estrogenicity of \( \beta \)-sitosterol in fish has been confirmed (Mellanen et al., 1996; Tremblay and Van Der Kraak, 1998).

2. Material and methods

The mixed sex populations of zebrafish, *Danio rerio*, were exposed continuously across three generations to two phytosterol preparations. One preparation was isolated from wood and the other from soy beans. The wood sterol (a gift from Finnish Environment Research Group) contained 80\% \( \beta \)-sitosterol, 10–15\% \( \beta \)-stigmasterol, about 8\% campesterol and 1\% campestanol. The soy sterol (Sigma, S 5753) contained about 50\% \( \beta \)-sitosterol, the rest being campesterol and dihydrobrassicasterol. Untreated water from lake Päijänne (raw water of Helsinki city tap water) was used both as a control and as dilution water. Both plant sterol groups had their own control group.

Water quality and test conditions followed the ISO 12890 standard (1999) and the OECD guideline 305 (1996). Tests were performed under flow through conditions. Only fertilised eggs and sag-fry larvae were kept in semi-static conditions (test waters changed once daily, not on weekends). The light rhythm was 12L/12D, and the test temperature 24±2 °C. The stainless steel exposure vessels (15 l) were of two-piece. The removable inner vessel was riddled letting the eggs pass through. It was used for spawning. After spawning the inner vessel with the adults was transferred into a new exposure vessel, and the exposures were continued. Fish of the offspring generations were sampled at the stage of maturity, in generation F1 after about 6 months’ exposure and in generation F2 after about 5 months’ exposure.

In each generation, 20 females and 20 males were sampled for physiological and biochemical analysis. Sex ratios were determined by microscopic examination of the gonads of the individual fish and were based on the sample of 100 or 150 fish per treatment.

The wood sterol concentration of 20 \( \mu \)g/l proved to be lethal to zebrafish. Fish started to die on the fifth test day. On the seventh day, when about half of the fish had died, those fish still living were transferred into the clean water. After the transfer, the fish started to recover and also to spawn. Fertilised eggs were taken for further rearing in clean water and the tests were continued until the sex ratio of the offspring in generation F2 could be determined. This experiment at the concentration of 20 \( \mu \)g/l of the wood sterol mixture was repeated exactly according to the first experiment. Only the stock of the zebrafish in this repetition test was different.

Fish were sampled for plasma vitellogenin, calcium and cholesterol analyses and for gonadal histology. Blood was drawn from the cut fish tail vein into heparinized hamatocrit capillaries, and the plasma was separated by centrifugation (2 min, 12000 rpm). Samples for the analyses were frozen immediately in liquid nitrogen, and also stored therein until analysed. The gonads from each fish were prepared for histological studies.

Plasma vitellogenin was assayed with ELISA, according to the method developed by Biosense Laboratories AS, Bergen, Norway (Nilsen et al., 1998). The monoclonal anti-salmon vitellogenin BN-5 was...
used as an antibody. In preliminary tests, it gave a positive cross-reaction with plasma samples of female zebrafish. Purified rainbow trout vitellogenin was used as a standard. Plasma total cholesterol and calcium concentrations were assayed with Boehringer test-kits. All measurements were performed in duplicates. Gonads were fixed by buffered formalin, embedded in paraffin wax, and sectioned at 7 μm. The sections were stained with Masson-Gomori.

Results between each control and test group were statistically analysed by ANOVA post hoc test, the mean difference from the control being significant at the 0.05 level.

3. Results

Compared to the control fish the wood sterol compound at concentration of 10 μg/l had no significant effects on the spawning success of the exposed fish, and only a slight effect on the mortality and hatching of the eggs of both offspring generations. In generation F1 the total mortality (eggs and hatched larvae) of the exposed fish was 12.2±3.9%, and generation F2 13.6±4.9%, whereas none of the fish of the control groups died during the experimental period. Most (95%) of the eggs in the test group (both generations) hatched on the fifth day, while in the control group on the sixth.

In the first wood sterol test at the concentration of 20 μg/l (test A in the figures) exposed parents started to die on the fifth test day. On the seventh day, when about half of the fish had died (18 males and 15 females), those fish still living were transferred into the clean water. After the transfer, the fish started to recover and also to spawn. The spawning efficiency of these fish was about 25% lower than that of the control group fish. The spawning efficiency of the offspring generations (F1 and F2) was nearly the same than that of the control group fish. No significant effects could be observed in the mortality of eggs or hatched larvae either.

In the repetition test (test B in the figures) at the concentration of 20 μg/l the total mortality of the parental fish was only 30%. After the fish had been transferred into the clean water no significant differences could be observed in the spawning efficiency or in the mortality of the eggs and hatched larvae when compared to the control fish.

Sex ratio changes of the exposed fish were significant. The short-term exposures (A, B) of the parental fish (generation F0) at the concentration of 20 μg/l, resulted in a significant masculinisation of the F1 generation but a reversal of this trend in the second generation (F2) (Table 1). The same result was got also from the chronic wood sterol exposure at the concentration of 10 μg/l (Table 1). In histological examinations, no androgynous fish were observed.

Table 1
<table>
<thead>
<tr>
<th>Sex ratios (%) of the exposed zebrafish</th>
<th>Generation F1</th>
<th>Generation F2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Males</td>
<td>Females</td>
</tr>
<tr>
<td>Control</td>
<td>53</td>
<td>47</td>
</tr>
<tr>
<td>Wood sterol 10 μg/l</td>
<td>69</td>
<td>31</td>
</tr>
<tr>
<td>Offspring test A</td>
<td>59</td>
<td>41</td>
</tr>
<tr>
<td>Offspring test B</td>
<td>72</td>
<td>28</td>
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</tbody>
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Compared to the control fish the soy sterol mixture at the concentration of 10 μg/l seemed to have no significant effects on the spawning success of the parental fish or on the mortality or hatching of the generation F1. However, it appeared to be toxic to the newly hatched zebrafish in generation F1. Fish started to die after 4 weeks’ exposure. Some of the surviving fish were transferred into clean water to try to aid survival but after 5 weeks all the fish had died, and the test had to be stopped at this point.

Both phytosterol mixtures caused vitellogenin induction in the exposed parental males (generation F0). In F0 females elevated vitellogenin induction was observed only in those fish exposed to wood sterol (Fig.1a). In generation F1 plasma vitellogenin concentration of the exposed fish was significantly increased both in males and in females. Elevated plasma vitellogenin levels were detected even in those offspring that originated from the lethally exposed fish, but only in test A (Fig. 1b). The result of the generation F2 was quite similar (Fig. 1c), although no changes could be observed in females exposed to the wood sterol mixture at the concentration of 10 μg/l.

In all tests, there was a significant positive correlation (at the 0.01 level) between plasma calcium and vitellogenin concentrations both in females and males (Fig. 2a–c).

The soy sterol mixture increased significantly the plasma cholesterol concentration of the fish in generation F0 (Fig. 3a). In generation F1 the wood sterol had the same effect (Fig. 3b). Also, in generation F2 plasma cholesterol concentrations were significantly increased but only in the exposed females, and in the offspring of the lethally exposed parents (Fig. 3c).

4. Discussion

In short-term tests the phytosterol, β-sitosterol, has been shown to alter the endocrine status of cold water fish (MacLatchy & Van Den Kraak, 1995; Mellanen et al., 1996; MacLatchy et al., 1997; Lehtinen et al., 1999). Now, with this multigeneration test with zebrafish we have shown that changes can be seen not only in the parental fish, but also in the following generations. The chronic exposure to wood sterol mixture at the concentration
relevant to the natural waters resulted in sex ratio changes in subsequent generations and in the physiological induction of vitellogenin production in the fish.

The masculinization of parental female fish by exposure to plant sterols, mainly $\beta$-sitosterol has been shown by Bortone and Cody (1999), Denton et al. (1985) and Krotzer (1990). In these short-term tests the used chemical concentrations were higher than those used in our studies. Perhaps this was the reason we did not observe any masculinization of the parental females. On the contrary the exposure effect was estrogenic. Plasma vitellogenin levels of the exposed parent both females and males were significantly increased. This vitellogenin induction by $\beta$-sitosterol is in accordance with the studies of MacLatchy et al. (1995), Mellanen et al. (1996) and Tremblay and Van Der Kraak (1998).

Fig. 1. Plasma vitellogenin concentration of zebrafish (mean±SD) in the generations of (a) F0, (b) F1 and (c) F2. * Denote significant difference from the controls ($P<0.05$).
In our study the masculinization effect of the wood sterol preparation was observed in the next generation (F1). The sex ratio of the exposed fish was significantly biased in favour of males, although plasma vitellogenin levels of these fish (both females and males) were significantly increased. As to generation F2 the sex ratio was significantly biased in favour of females. In these fish plasma vitellogenin levels were also significantly elevated but only in males. This 2-phase response was also observable in the offspring of lethally exposed parents, which indicates the possibility that hormonal dysfunctions are transmittable by DNA. We have confirmed this 2-phase response later again (manuscript under preparation). However, at the moment we have no explanation for this phenomena, but it evidences the importance of long-term
life cycle studies when assessing the effects of chemical mixtures.

Structurally, β-sitosterol closely resembles cholesterol (Mellanen et al., 1996), which is a precursor to the reproductive sex steroid hormones. It has been shown that β-sitosterol reduces the plasma cholesterol concentration, which leads to a decrease in plasma sex steroids and hereby alters the reproduction function of fish (Pollak and Kritchevsky, 1981; Tremblay and Van Der Kraak 1998). In our study plasma cholesterol concentration of the exposed fish was increased. This supports the fact that sterol mixtures may affect through multiple mechanisms, and further work is needed on the bioconcentration and metabolism of the separate components of the sterol mixtures if we are to understand the mechanisms responsible for both masculinisation and feminisation.

Fig. 3. Plasma cholesterol concentration of zebrafish (mean±SD) in the generations of (a) F0, (b) F1 and (c) F2. * Denote significant difference from the controls ($P<0.05$).
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References


