Estrogenicity of Phytosterols Evaluated In Vitro and In Vivo

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The estrogenic activity of two phytosterol preparations was evaluated in vitro and in vivo. For the in vitro evaluation, freshly separated hepatocytes of rainbow trout were used. By contrast, the in vivo evaluation was performed by injecting the phytosterols intraperitoneally into juvenile rainbow trout. Both assays confirmed the estrogenic activity of the phytosterols. The in vitro screening technique, based on the synthesis and secretion of vitellogenin from the isolated liver cells, produced a clear, significant curve in response to the presence of both phytosterol mixtures. In the in vivo tests, the phytosterol preparations caused significant increases in plasma vitellogenin concentrations of juvenile fish. These short-term assays proved to be suitable for assessing the estrogenic activity of phytosterols.

1. Introduction

In aquatic environments, a number of substances may interfere with the endocrine system of animals and disrupt their reproduction and development. (1–4) Of primary concern are substances with estrogenic activity. (2,5) These environmental estrogens include compounds such as natural and synthetic estrogens, natural phytoestrogens, and xenobiotics with estrogenic activity. (4,6–8)

Research has focused on the development of reliable assays to detect the estrogenic activity of chemicals. (9) The capacity of the teleost liver to respond specifically to estrogens by producing vitellogenin (10) provides a very useful screening assay for compounds with potential estrogenic activity. Vitellogenin is a sex-limited precursor protein which is produced by the liver in all oviparous vertebrates in response to estrogens; it is then rapidly transformed into the yolk proteins of the oocytes. (11–13) The synthesis of vitellogenin can also

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be induced by estrogen treatment of nonvitellogenic females and normal males. Thus, vitellogenin is a very sensitive indicator in male fish, because its production is an abnormal process.

In view of the above, it is not surprising that a number of \textit{in vivo} and \textit{in vitro} assays using vitellogenin as an estrogen marker have been developed and applied for assessing endocrine disruption activity of chemicals or waste waters. For example, primary hepatocytes of male fish, cultured human breast cancer cells, and recombinant yeast cell cultures containing the human estrogen receptor gene have successfully been employed to detect the estrogenic potency of waste waters and chemicals \textit{in vitro}. \textit{In vivo} tests, by contrast, the method has been the measurement of vitellogenin production in male fish which have either been exposed to waste waters and chemicals or injected with chemicals.

\textit{In vitro} methods are useful for assessing the estrogenic activity of large numbers of compounds. The experimental parameters can be more precisely controlled than is possible in \textit{in vivo} studies. Moreover, \textit{in vitro} tests employing primary material can be used for detecting compounds which have estrogen potentiating activity or which can be transformed into estrogenic compounds. One advantage of primary material is that it maintains a wider range of differentiated functions when compared to cell lines or subcellular fractions.\textsuperscript{(37)}

In primary hepatocyte assays, important processes such as uptake, bioaccumulation and metabolic activation or degradation of test compounds are taken into account.\textsuperscript{(3)} Furthermore, the inter-individual variability is minimized since the cells of one fish can be exposed to various concentrations of different compounds.

By contrast, although \textit{in vitro} methods are very useful for assessing the intrinsic activity of large numbers of chemicals, most of the metabolic steps which normally occur \textit{in vivo} are absent \textit{in vitro}. Therefore, the \textit{in vivo} markers for confirming the effects on long-term reproductive success are required and should be identified, but because these studies are expensive and time-consuming, rapid, reliable \textit{in vitro} and \textit{in vivo} assays for the screening and determination of the endocrine disrupting effects of compounds are needed.

Against this background, this work studied the suitability of short-term \textit{in vivo} and \textit{in vitro} assays for the evaluation of the estrogenic potency of two phytosterol mixtures, which in multigeneration tests of zebrafish have proved to be estrogenic. The main compound in both phytosterols was $\beta$-sitosterol, which is thought to have endocrine disrupting activity.

2. Materials and methods

Two phytosterol preparations (purchased as $\beta$-sitosterol) were used for the tests. One $\beta$-sitosterol mixture (a gift from the Finnish Environmental Research Group) was isolated from wood (by-product of pulp manufacture), and it contained $>80\%$ $\beta$-sitosterol, the rest being $\beta$-sitostanol, campesterol and campestanol. The other $\beta$-sitosterol preparation was a commercial product (Sigma), isolated from soy beans, and it contained approximately $50\%$ $\beta$-sitosterol, the rest being campesterol and dihydrobrassicasterol. Estradiol-17$\beta$ was used as a positive control compound.
2.1 In vitro test

Hepatocytes were isolated from rainbow trout males by collagenase perfusion according to a slightly modified method of Moon et al. (44) Medium 199 (Sigma) with added L-glutamine, NaHCO₃, Na₂HPO₄ and an antibiotic-antimycotic solution (Sigma) was used for the washing and incubation medium. The viability of isolated hepatocytes was assessed by the Trypan Blue exclusion method. (44) Only preparations with over 90% viability were accepted for the tests. The hepatocytes were diluted to a concentration of 1×10⁶ cells ml⁻¹ and distributed in disposable Petri-dishes in a final volume of 5 ml. Test compounds were dissolved in ethanol. For each dose, cells were incubated in duplicate for 72 h at 12°C ± 1°C. Estradiol-17β was run alongside each test as a positive control, and its concentrations were chosen with reference to Kwon et al. (45) The same concentrations were also chosen for the phytosterols.

Vitellogenin in the culture medium was the measured end-point of the estrogenic activity of the test substances. Vitellogenin was assayed with an ELISA, according to the method developed by Nielsen et al. (46) Monoclonal anti-salmon vitellogenin BN-5 (Biosense Laboratories AS, Bergen, Norway) was used as an antibody. All samples were tested on three separate test occasions. The differences between the test results from the unexposed and the exposed cells were analyzed statistically with the ANOVA test.

2.2 In vivo test

Juvenile rainbow trout were injected intraperitoneally (one injection, 6 mg/kg) with either wood sterol or soy sterol mixtures or with estradiol-17β. Controls were sham injected with olive oil, in which also the test compounds were dissolved. There were ten fish of both sexes in each group. Before being injected, the fish were anaesthetised with MS222 (0.01%). To minimize the test animal usage, only one concentration was chosen with reference to McLatchy and Van der Kraak. (47)

After 14 days the fish were killed (one at a time) by a blow to the head, then measured and weighed. A blood sample was taken from the dorsal vein with a heparinized syringe (ammonium heparinate) and centrifuged at 8000 g for three min. The liver was dissected and weighed for calculation of the organosomatic index (% of body weight). The isolated plasma and liver samples were frozen without delay in liquid nitrogen and stored therein until analyzed.

The plasma was assayed for vitellogenin, testosterone, estradiol-17β and calcium concentrations. Liver samples were assayed for ethoxyresorufin-O-deethylase (EROD) activity and for protein content.

The plasma vitellogenin was assayed as described above. Purified rainbow trout vitellogenin (Biosense Laboratories AS, Bergen, Norway) was used as a standard. Plasma calcium concentration was assayed with a Sigma test kit. The plasma testosterone and estradiol-17β concentrations were assayed by RIA, as described by Scott et al. (48) Tritium-labelled hormones were purchased from the Radiochemical Centre, Amersham, and antisera from Sigma.

The liver EROD activity was measured according to a slightly modified method of Klotz et al. (49) Hodson et al. (50) and van den Heuvel et al. (51) The final concentration of 7-ethoxyresorufin in Tris-buffer was 2.5 µM, and of NADPH 250 µM. The liver protein
concentration was assayed by the method of Lowry et al.\textsuperscript{(52)} using bovine serum albumin as a standard.

The differences between the test results from the control group fish and the test group fish were statistically analyzed by ANOVA.

3. Results

3.1 In vitro tests

The \textit{in vitro} screening technique based on the synthesis and secretion of vitellogenin from the isolated liver cells produced a clear, significant curve in response to the presence of both phytosterols (Fig. 1). Compared to the control hepatocytes, the potency of the soy sterol mixture for inducing the vitellogenin production was slightly stronger ($p<0.001$) than that of the wood sterol mixture ($p<0.01$) (Fig. 1). The estrogenic potency of both phytosterol mixtures was, however, much weaker than that of the positive control compound, estradiol-17\textbeta{} (Fig. 2).

![Graph of absorbance units vs test concentration (ug/l)](Fig. 1. Vitellogenic response (mean \pm SD) of rainbow trout hepatocytes to wood sterol (---) and soy sterol (—) mixtures in three tests at different times. 

\#Significant difference between unexposed and exposed hepatocytes at $p<0.01$; *significant difference between unexposed and exposed hepatocytes at $p<0.001$.}
The decline in vitellogenin production at high test concentrations seems to be characteristic of this assay and obviously is due to the toxic effect of the test compounds.

3.2 In vivo tests

Both phytosterol preparations raised the fish plasma vitellogenin concentration (Table 1). Similar observations to those made in the in vitro tests were also made in the in vivo tests: the estrogenic potency of the tested phytosterols was weaker than that of estradiol-17β.

In all test groups, the fish plasma calcium concentration correlated positively (p<0.01) with the plasma vitellogenin concentration (Table 1).

The estradiol-17β injections significantly increased fish plasma testosterone and estradiol-17β concentrations (Table 1). By contrast, phytosterols had hardly any significant effect on these plasma variables. Only in males injected with soy sterol was the plasma estradiol-17β concentration significantly raised.

Compared to the control fish, the wood sterol mixture increased the liver EROD activity of males, whereas the soy sterol mixture lowered it (Table 1). Also, in fish injected with estradiol-17β, the liver EROD activity was significantly reduced (Table 1).

The liver weight was significantly increased in fish injected with estradiol-17β, but
Table 1
Results of the *in vivo* tests.

<table>
<thead>
<tr>
<th></th>
<th>Plasma vitellogenin concentration (µg/ml)</th>
<th>Plasma calcium concentration (µmol/ml)</th>
<th>Plasma estradiol concentration (pg/ml)</th>
<th>Plasma testosterone concentration (pg/ml)</th>
<th>Liver EROD activity (pmol/mg prot/min)</th>
<th>Liver weight (g)</th>
<th>Liver somatic index (%)</th>
<th>Weight of fish (g)</th>
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*aSignificance between control and test group fish at the 0.05 level.
decreased in fish injected with the wood sterol mixture (Table 1). The soy sterol preparation seemed to have no effect on the size of the fish livers.

4. Discussion

Both assays, in vitro and in vivo, demonstrated the estrogenicity of the phytosterol mixtures tested. The in vitro screening technique based on the synthesis and secretion of vitellogenin from the isolated liver cells of male rainbow trout in the presence of these plant sterols repeatedly produced, in three separate tests, a clear dose response curve. However, compared to the equivalent amounts of the model estrogen, estradiol-17β, the estrogenic response of these phytosterols was about three times weaker.

A similar response was observed in vivo, i.e. when these phytosterols were injected intraperitoneally, they increased the plasma vitellogenin concentration of the fish. This fact coupled with the synchronous increase in the fish plasma calcium concentration confirms the vitellogenin-inducing potency, because one atom of calcium is associated with every protein phosphate group in the vitellogenin complex. The response arising from injected phytosterol preparations was again weaker than that arising from an equal amount of injected estradiol-17β, but this time the difference was about 200-fold less.

In the in vitro test, the concentration required to produce the response was almost the same as that needed to produce the response in zebrafish multigeneration tests. In addition, the test concentrations were representative of those observed in natural waters. The dome-shaped response curve of the dilution series indicates a degree of toxicity in the samples. The toxic effect of both waste waters and chemicals has been shown by determining the EROD activity of the exposed cells when validating the hepatocyte assay. It was observed that when the test concentration gets high enough, the detoxification capacity of the cells starts to decrease. This decrease in the detoxification activity of the cells is obviously the reason for the simultaneous decrease seen in the vitellogenin concentrations of the cell media. Anderson et al. confirmed this observation by showing that the potency of vitellogenin inhibition in hepatocyte cultures is directly related to the strength of the inducer of EROD activity.

Metabolism of steroids by the liver is important for the maintenance of plasma steroid concentrations. The liver EROD activity of the males injected with the wood sterol preparation increased, while it fell in males injected with the soy sterol preparation or with estradiol-17β. MacLatchy and Van Der Kraak observed that β-sitosterol reduced plasma testosterone and estradiol-17β concentrations when injected into goldfish. In the present study, no decreases were observed in fish plasma steroid levels irrespective of whether the fish were injected with wood sterol or soy sterol mixtures. On the contrary, there seemed to be an increasing trend in the plasma estradiol-17β concentration following the injections. The decreased detoxification metabolism, i.e. decreased liver EROD activity, of the fish injected with estradiol-17β or soy sterol must have been one reason for the raised levels of plasma reproductive hormones.

Although the estrogenicity of the phytosterol mixtures was demonstrated by both assays, the mixtures reacted a little differently in the tests. This had been previously observed in the fish multigeneration tests. In the in vivo tests, the liver EROD activity increased in males
injected with the wood sterol preparation, but decreased in males injected with the soy sterol preparation, in turn giving rise to the increase in the plasma hormone levels. Differences were also observed in the liver weights. The wood sterol preparation decreased the liver weight of the fish. In the in vitro tests, the response of the soy sterol preparation was a little stronger than that of the wood sterol mixture. Previously, chronic exposure to the wood sterol mixture\(^{(40)}\) had shown a two-phase response. Masculinization dominated in the first generation, while in the second generation it was reversed to feminization. By contrast, the soy sterol preparation produced only feminization effects.

In part, these differences can be explained by the different compositions of these phytosterol mixtures, both purchased as \(\beta\)-sitosterols. The wood sterol preparation contained \(>80\%\) \(\beta\)-sitosterol, the rest being \(\beta\)-sitostanol, campesterol and campestanol, whereas the soy sterol mixture contained only \(50\%\) \(\beta\)-sitosterol, the rest being campesterol and dihydrobrassicasterol. Also, the reason for the differences may lie in the toxicities of these sterol mixtures. It has been shown that the soy sterol mixture is more toxic than the wood sterol mixture.\(^{(40)}\)

The estrogenic potency of substances can be measured in different tests, but the assays must be suitable and sensitive, and they must give reproducible results.\(^{(19)}\) Both the in vitro test and the in vivo test gave reliable results, because the results were comparable to those of the long-term in vivo tests.\(^{(40,41)}\) In the in vitro tests, the concentrations used were representative of those observed in nature, and in separate tests the assay gave reproducible results. Although the response producing vitellogenin was lower in the in vitro tests, freshly separated hepatocytes of male fish can provide an excellent means to screen the estrogenic activity of compounds. Compared to the in vivo test, the hepatocyte assay can be used to obtain reproducible empirical data in a time-, animal- and cost-effective manner. However, in vitro models have limitations as predictive tools in risk assessment. Moreover, because the models fail to account for the complexity of the whole animal, not to mention several important mechanisms inherent to in vivo systems, genuine endocrine disruption effects should be ascertained with longer-term in vivo tests.

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References


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