

Caenorhabditis elegans Responses to Specific Steroid Hormones

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In this paper, *Caenorhabditis elegans* (*C. elegans*) is proposed as a model organism for studying chemical effects over multiple generations. We investigated whether *C. elegans* responds to vertebrate steroid hormones. We found that estrogenic steroids, especially estradiol (E2), have a cholesterol-like potency in supporting the reproduction of *C. elegans*. In contrast, testosterone (TS) and diethylstilbestrol (DES) did not display this potency. On the other hand, E2, TS and DES suppressed the fecundity rate of *C. elegans*, when culture carried out with cholesterol. Moreover effect of TS accumulated over generation, in contrast to the other chemicals tested. These data suggested that with convenient biomarkers such as fecundity, *C. elegans* might be an effective model organism for studying chemical actions, including the disruption of reproduction.

Key words — *Caenorhabditis elegans*, reproduction, fecundity, endocrine disrupting chemical, steroid hormone

INTRODUCTION

Endocrine disrupting chemicals (EDCs) are known affect not only human health, but also that of wild animals.¹⁾ Of the vast number of chemicals released into the environment, many are suspected to have endocrine-disrupting activity. The identification of EDCs is, however, hindered by a lack of convenient screening methods. Recently, the estrogen-like activity of EDCs has served as the basis for assays: two-hybrid assays using estrogen receptors and cell proliferation assays using ovarian cancer cells.^{2–4)} However, these assays can not detect any chemicals that have no binding affinity for estrogen receptors. Hence we expect to develop an appropriate assay to detect and evaluate EDCs.

Caenorhabditis elegans (*C. elegans*), a free-living soil nematode, is probably the most thoroughly characterized multicellular organism with regard to genetics, development, behavior and anatomy. *C.*

elegans has been widely used in molecular biology, for studying the nervous system and so on, due to its ease of culture, short life span, cellular simplicity and genetic tractability.^{5–6)} Recently, genomic sequencing of *C. elegans* was completed.⁷⁾ The *C. elegans* genome encodes more than 200 predicted nuclear receptor superfamily genes, which includes the steroid receptor family, as shown by the sequence analysis.⁸⁾ In many cases, very little is known about the specific ligands of these nuclear receptors.

Williams and Dusenbery proposed an aquatic toxicity test using *C. elegans*.⁹⁾ Subsequently, many researchers have used it for aquatic, sediment, and soil toxicity tests.^{10–13)} Power and Pomerai developed a transgenic nematode, carrying a stress-inducible β -galactosidase reporter, for a toxicity test in soil biomonitoring.¹⁴⁾ More recently, we reported that treatment of *C. elegans* hermaphrodites with estradiol or certain EDCs increases the expression level of vitellogenin (VTG), the egg yolk protein precursor synthesized by female oviparous animals.¹⁵⁾ Although *C. elegans* lacks an endocrine system, it is known to respond to some EDCs by a mechanism that is poorly understood. Meanwhile *C. elegans* requires dietary cholesterol, apparently during all

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developmental stages¹⁶⁾ and it has been reported that *C. elegans* has an ecdysteroid-like substance.¹⁷⁾ Cholesterol is an important lipid as a component of cell and organelle membranes and it is a raw material for steroids, cholates and vitamin D in higher organisms. *C. elegans* cholesterol metabolism is relevant to EDCs, and an assay for EDCs can be based on this fact.

In this study, we investigated the effects of vertebrate steroid sex hormones and certain synthetic hormones on *C. elegans* reproduction. We discuss the application of *C. elegans* as a model organism for EDC assays.

MATERIALS AND METHODS

Chemicals — Estrone (E1), estradiol (E2), estriol (E3), testosterone (TS), ethynylestradiol (EE2), methyltestosterone (MTS), pregnenolone, and diethylstilbestrol (DES) used in this study were purchased from Wako Pure Chem. (Japan).

Animals and Culture Conditions — Worms used in this study were wild-type hermaphrodite *C. elegans*. General procedures for working with *C. elegans* were according to Brenner.¹⁸⁾ Briefly, 5 to 10 worms were grown on a nematode growth medium (NGM) plate with a lawn of *Escherichia coli* (*E. coli*) DH5 α as a food source and incubated at 20°C. NGM plates used to maintain *C. elegans* contained cholesterol at a final concentration of 25 μ M. Every 4 or 5 days, worms were sub-cultured to new plates.

Determination of Fertility — A L4 larva step worm was picked up and transferred to an NGM plate, cholesterol-free or containing 0.5 or 25 μ M cholesterol, with other chemicals as specified. Incubation was at 16°C. (At this temperature, *C. elegans* grows from egg to adult in approximately 5 to 6 days, requiring about a day per molt step.) The day, that first offspring was identified, was defined first day. The numbers of worms and eggs on the plates were counted under a dissecting microscope at a fixed time every day.

Multi-Generation Test — The worms which was transferred from an NGM plates to the 0.5 μ M cholesterol with chemicals, was defined the first generation. The second generation worms allowed to grow to L4 larvae on the same plate of the first generation, then one worm was picked up and sub-cultured to a new plate containing the same ingredients. The above steps were repeated until the fifth

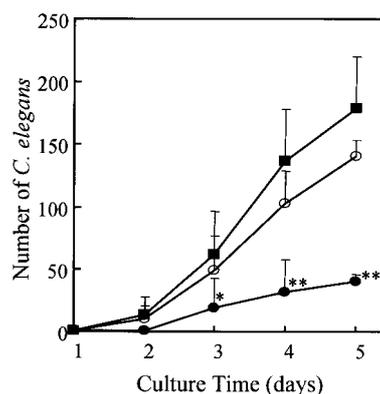


Fig. 1. Effects of Cholesterol Concentration on the Fecundity of *C. elegans*

A L4 larva step worm was picked up and transferred to a NGM plate, cholesterol-free (●) or containing 0.5 (○) or 25 μ M (■) cholesterol. Culture was carried out at 16°C. The day, that first offspring was identified, was defined first day. The numbers of worms on the plates were counted under a dissecting microscope at a fixed time every day. *, **: significant difference from the parallel data of 25 μ M cholesterol (* p < 0.05, ** p < 0.01).

generation cultured or the extinction of the *C. elegans*. The fertility levels were determined every generation according to the above method.

These experiments were carried out at least three times and all results were expressed as means \pm S.E.M. Statistical evaluation was done by analysis of variance (ANOVA). Values of p < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

In this study we tested the effects of cholesterol-like molecules on *C. elegans* reproduction. First, we determined the minimum amount of cholesterol that supports *C. elegans* growth. Single nematodes were transferred to plates with various cholesterol concentrations, and the numbers of offspring of the initial nematodes were monitored over five days (Fig. 1). On cholesterol-free plates, *C. elegans* lost fecundity, and we could not culture them after the third generation in the absence of cholesterol. However, when 0.5 μ M cholesterol was supplied in the culture medium, *C. elegans* maintained normal fecundity through the fifth generation. At cholesterol concentrations below 0.5 μ M, *C. elegans* lost fecundity in a dose-responsive manner (Data not shown).

Some researchers have indicated that certain steroid precursors and sitosterols can be used in place of cholesterol.¹⁹⁾ In vertebrates, steroid sex hormones are made from cholesterol, and they have structural

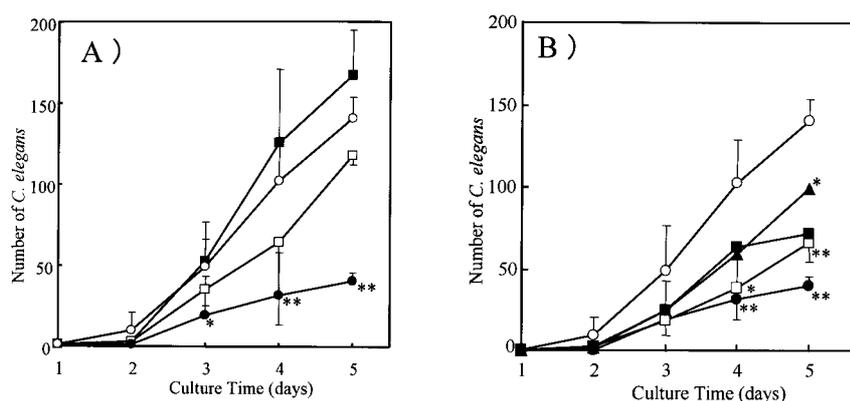


Fig. 2. Effects of Various Steroids on the Fecundity of *C. elegans* in the 2nd Generation

A L4 larva step worm in the 2nd generation was picked up and transferred to a NGM plate, A) cholesterol-free (●) or containing 0.5 μM cholesterol (○), 0.5 μM E2 (□) or 5 μM E2 (■), B) cholesterol-free (●) or containing 0.5 μM cholesterol (○), 5 μM pregnenolone (▲), 5 μM MTS (□) or 5 μM EE2 (■) plate at 16°C. The day, that first offspring was identified, was defined first day. The numbers of worms on the plates were counted under a dissecting microscope at a fixed time every day. *, **: significant difference from the parallel data of 0.5 μM cholesterol (* $p < 0.05$, ** $p < 0.01$).

similarity. In *C. elegans*, details of vertebrate sex steroid hormone metabolism are unknown. To test the effects of the vertebrate sex steroids and synthetic steroids on *C. elegans*, we tested whether these hormones could replace cholesterol in supporting *C. elegans* growth.

Figure 2 shows the typical reproductive rates, starting at the second generation of culture with the hormones. On plates with 5 μM E2, *C. elegans* maintained the same reproductive rate as on the normal plates through the fifth generation. Even on 0.5 μM E2 plates, the number of *C. elegans* increased significantly (Fig. 2A). These results indicate that E2 has a cholesterol-like potency for *C. elegans*, and this potency increases with dose. Dropkin *et al.*²⁰⁾ reported that *C. elegans* growth is inhibited by E2 and other vertebrate sex hormones, then they concluded that a variety of mammalian sex hormones are non-specifically inhibitory to nematode reproduction. But the concentration of these chemicals (more than 100 μM) was higher than in our study (0.5–5 μM). The phenomenon that we observed in this study may indicate a low-dose effect of these chemicals on *C. elegans* physiology.

C. elegans could reproduce on plates containing pregnenolone, which is an intermediate vertebrate steroid, and EE2, which is a synthetic estrogen (Fig. 2B), and the nematodes could be cultured through five generations (Data not shown). However, reproduction was very slow after the second generation in 5 μM pregnenolone (Fig. 2B). Thus, EE2 has a cholesterol-like potency, and pregnenolone may have a weak potency.

TS, MTS, E1, E3, and DES are typical verte-

brate androgen, synthetic androgen, estrogen, steroid intermediate and synthetic non-steroid estrogen hormones, respectively. On plates containing these chemicals, *C. elegans* could not be cultured after the third generation, similar to the cholesterol-free condition. Thus, these chemical had no cholesterol-like potency. In addition, E3 and DES showed toxicity for *C. elegans*, since fecundity of the second generation in the presence of these chemicals (0.5 and 5 μM) was lower than in the cholesterol-free condition.

Next, we determined effects of these chemicals on *C. elegans* in the presence of 0.5 μM cholesterol. Several concentrations (0.005, 0.05, 0.5, 5 μM) of E1, E2, TS, EE2, MTS or DES were examined in NGM plates containing 0.5 μM cholesterol. Fig. 3A shows that a high dose (5 μM) of E2 suppressed fecundity. However, fecundity was not affected by 0.5 μM E2. This implies that the cholesterol-like potency of E2 is not additive with cholesterol's effect, but rather that E2 at high doses competes with cholesterol. DES at 5 μM also suppressed fecundity on NGM plates containing 0.5 μM cholesterol, and this effect appeared from the first to fifth generations. However, 0.5 μM DES showed only weak effects on fecundity (Fig. 3B). The combination of EE2 and MTS with 0.5 μM cholesterol showed no effects on *C. elegans* fecundity. TS at 5 μM reduced fecundity on NGM plates containing 0.5 μM cholesterol. Comparing the first to third generations under these conditions with the fourth and fifth generations, the average fecundity decreased significantly after longer exposure (Fig. 4). Thus, TS had an effect that accumulated over generations, in contrast to the other

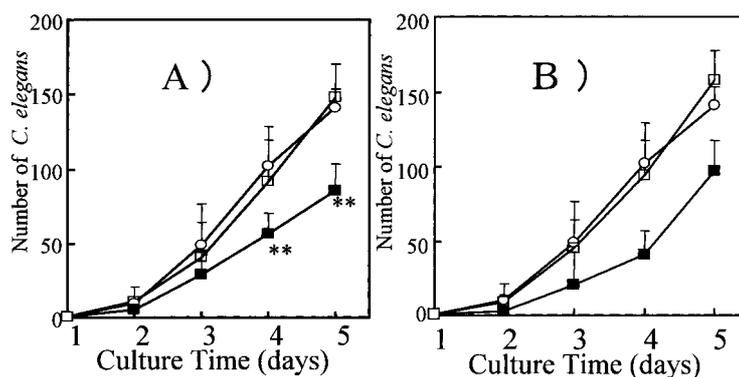


Fig. 3. The Fecundity of *C. elegans* after Long-Term Steroid Exposure

The worms which was transferred from an NGM plates to the 0.5 μM cholesterol with E2 (A) or DES (B), was defined the first generation. The second generation worms allowed to grow to L4 larvae on the same plate of the first generation, then one worm was picked up and sub-cultured to a new plate containing the same ingredients. The above steps were repeated until the fifth generation. A) \square : 0.5 μM E2 (average of generations 1–5); \blacksquare : 5 μM E2 (average of generations 1–5); \circ : 0.5 μM cholesterol, B) \square : 0.5 μM DES (average of generations 1–5); \blacksquare : 5 μM DES (average of generations 1–5); \circ : 0.5 μM cholesterol. *, **: significant difference from the parallel data of 0.5 μM cholesterol (* $p < 0.05$, ** $p < 0.01$).

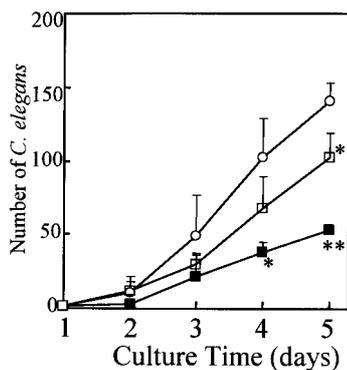


Fig. 4. The Fecundity of *C. elegans* after Testosterone Exposure

The worms which was transferred from an NGM plates to the 0.5 μM cholesterol with TS, was defined the first generation. The second generation worms allowed to grow to L4 larvae on the same plate of the first generation, then one worm was picked up and sub-cultured to a new plate containing the same ingredients. The above steps were repeated until the fifth generation. \square : 5.0 μM TS (average of generations 1–3); \blacksquare : 5.0 μM TS (average of generations 4–5); \circ : 0.5 μM cholesterol. *, **, significant difference from the parallel data of 0.5 μM cholesterol (* $p < 0.05$, ** $p < 0.01$).

chemicals tested. All chemicals used in this study have no acute lethal toxicity against *C. elegans* at 5 μM (Data not shown). These results suggested that the mechanisms of effects of DES and TS were quite different and we could distinguish effects of chemicals by using multi-generation test. Goldstein *et al.*^{21,22} reported that 100 μM DES is fatal to *C. elegans*. Our experiments confirmed that invertebrates, like vertebrates, are more strongly affected by DES than by E2.

In this study, we focused on the cholesterol replacement activity of vertebrate sex steroids in *C. elegans*. In *C. elegans*, we have already reported that

E2 can induce a vitellogenin.¹⁵ Interestingly, Matyash *et al.*²³ suggested that vitellogenin is one of the major proteins used for sterol transport in *C. elegans*. It is possible that some sterol-like substances may be transformed into steroids after extensive enzymatic modification in *C. elegans*. Chitwood *et al.*¹⁹ suggested that minute amounts of sterol may be metabolized to regulatory substances, such as steroid hormones. Our findings from this study may support this hypothesis. It is possible that some sterol may be supplied by the *E. coli* that is the normal diet for *C. elegans*. The results of this study indicate that some vertebrate steroids and synthetic steroids affect sterol metabolism or hormonal regulation in *C. elegans*. From gene analysis, *C. elegans* has no obvious estrogen receptors. Further study is in progress on the mechanisms of the response to these hormones.

However, we think that an assay to evaluate the EDCs needs a potency to distinguish specific hormones and hormonal responses. This study confirms that *C. elegans* can response specific hormones and be used as a model organism for assaying chemical effects over multiple generations. Dhawan *et al.*¹² reported that behavioral and reproductive responses are both much more sensitive indicators of toxicity in *C. elegans* than is lethality, and Anderson *et al.*²⁴ also described various sublethal endpoints for toxicity measures with the nematode. Williams *et al.*²⁵ proposed *C. elegans* as an alternative animal test species in toxicity studies of pharmaceuticals.

In conclusion, the free-living nematode *C. elegans* is becoming an effective model organism for testing chemical toxicity including reproductive

disruption.

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