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Abstract Methods for quantitative oral administration of various substances to *Caenorhabditis elegans* are needed. Previously, we succeeded in oral administration of hydrophilic substances using liposomes. However, an adequate system for delivery of hydrophobic chemicals was not available. In this study, we developed a method for oral administration of lipid-soluble substances to *C. elegans*. γ-cyclodextrin (γCD), which delivers hydrophobic chemicals, was used to make micro-particles of inclusion compounds that can be ingested by bacteriophagous nematodes, which do not distinguish these micro-particles from their food bacteria. Successful oral delivery of the hydrophobic fluorescent reagent 3,3'-dioctadecyloxacarbocyanine perchlorate into the intestines of *C. elegans* was observed. Oral administration of the hydrophobic antioxidants tocotrienol, astaxanthin, or γ-tocopherol, prolonged the nematode lifespan; tocotrienol rendered them resistant to infection with the opportunistic pathogen *Legionella pneumophila*. In contrast, older conventional delivery methods that involve incorporation of chemicals into the nematode growth medium or pouring chemicals onto the plate produced weaker fluorescence and no longevity effects. Our method efficiently and quantitatively delivers hydrophobic solutes to nematodes, and a minimum effective dose was estimated. In combination with our liposome method, this γCD method expands the usefulness of *C. elegans* for the discovery of functional food factors and for screening drug candidates.

Keywords Longevity · Nematodes · Oral administration · Antioxidant · Cyclodextrin · Innate immunity

Introduction

Caenorhabditis elegans is a free-living bacteriophagous nematode that plays an important role in biological research. Despite increased use of *C. elegans* in a variety of studies, no efficient method for oral administration of chemicals exists. Previously, we succeeded in oral administration of hydrophilic substances using liposomes (Shibamura et al., 2009). However, an adequate system for delivering hydrophobic chemicals is unavailable. Currently, these chemicals are generally dissolved in the nematode growth medium (NGM) using dimethyl-sulfoxide or ethanol (Ishii et al., 2004; Pun et al., 2010). Alternatively, the solution is spread onto the surface of NGM with the *Escherichia coli* strain OP50 (OP50), which is the international standard food of nematodes (Gruber et al., 2007; Srivastava et al., 2008). However, estimating the amount of chemicals taken into nematodes with these methods is difficult unless the worms are physico-chemically analyzed. Further, the amount of organic solvents must be increased when higher amounts of hydrophobic chemicals need to be added to the NGM.

We hypothesized that nematodes could efficiently ingest hydrophobic chemicals if they were made into particles similar to bacteria, as we previously reported (Shibamura et al., 2009). Here, we used γ-cyclodextrin (γCD) to deliver hydrophobic substances. γCD forms inclusion compounds with hydrophobic molecules due to the unique nature of its structure. γCD is topologically represented as a toroid in which the interior is considerably less hydrophilic than the aqueous environment and thus can host other hydrophobic molecules. In contrast, the exterior is sufficiently hydrophilic to render the complexes water soluble. These properties enable inclusion compounds containing γCD and hydrophobic molecules to penetrate tissues and release biologically active compounds (Bhagavan et al., 2007). The fluorescent reagent 3,3'-dioctadecyloxacarbocyanine perchlorate (DiO) was used to test oral

administration of lipid-soluble substances to *C. elegans*. We compared the efficiency of γCD-mediated delivery with conventional methods.

Dietary supplements of antioxidants are reported to have positive effects on longevity (Brown et al., 2006; Kampkötter et al., 2008; Melov et al., 2000; Wilson et al., 2006; Wu et al., 2002), but other studies have reported controversial results (Bass et al., 2007; Goldstein et al., 1993; Keaney et al., 2004; Larsen and Clarke, 2002). As an example of application of our method, hydrophobic antioxidants were administered using both the new and conventional methods to compare the effect on the nematode lifespan and on host defense against bacterial infection. Using our new method, we showed clear effects of the hydrophobic antioxidants tocotrienols astaxanthin and γ-tocopherol on the lifespan and host defense of *C. elegans*.

Materials and methods

Ingestion of γCD inclusion compounds

C. elegans Bristol strain N2 worms were propagated on NGM with standard techniques using OP50 as food bacteria (Stiernagle, 1999). Inclusion compounds of γCD and hydrophobic chemicals were prepared as shown in Fig. 1. To monitor nematode ingestion of γCD (MW 1297) bound to hydrophobic chemicals, the lipid-soluble fluorescent compound DiO (MW 881.7) was used. A sterile γCD solution was prepared by filtering a nearly saturated γCD (230 mg/ml) solution. Sterile DiO ethanol solution (100 μg/ml) was prepared by filtration through an organic solvent-resistant filter (SLLG013SL, Millipore, Carrigtwohill, Ireland). One milliliter γCD solution was mixed with 0.1 ml DiO solution and stirred for 12-24 h with a rotary converter. The solid complex (inclusion compounds) was collected by centrifugation and weighed. The amount of DiO contained in the compounds was measured based on the

fluorescence intensity. Finally, the inclusion compound (approximately 14 mg wet weight) containing 8.0 μg DiO was added to M9 buffer and vortexed so that the nematodes could ingest the compounds. For comparison, 80 μl DiO ethanol solution was dissolved in a peptone-free NGM (mNGM) plate (10.0 ml mNGM, 5 cm diameter) directly, or the solution was poured onto the mNGM plate with OP50 (10 mg/plate). Nematodes were fed on OP50 *ad libitum*, and then collected at various time points, counted and washed three times with M9 buffer. The nematodes were placed in a 0.65-ml graduated microtube (Scientific Specialties, Lodi, CA, USA) containing 20 μl M9 buffer and mechanically disrupted with a microtube pestle. The volume was adjusted to 100 μl with M9 buffer, and fluorescence intensity of the supernatant was measured with a spectrofluorophotometer (Wallac 1420 ARVOsx, PerkinElmer, Waltham, MA, USA). Standard curves were generated by plotting fluorescence intensity against the concentration of DiO. The amount of DiO ingested and absorbed by a worm was calculated by dividing the total DiO recovered from the worms by the number of worms.

Longevity effect

After hatching, nematodes were grown on OP50 for 3 days, and then the adult worms were divided into groups that were supplemented with vitamin E (Oryza Oil & Fat Chemical, Ichinomiya, Aichi, Japan). The product was composed of 4.9% α-tocotrienol (T3), 0.5% β-T3, 61.3% γ-T3, 4.2% δ-T3, 1.2% α-tocopherol, 0.6% β-tocopherol, 4.5% γ-tocopherol, and 0.7% δ-tocopherol; the total amount of T3s was more than 70% of the product, whereas tocopherols comprised 7%. The inclusion compound was prepared as described above and spread onto mNGM plates so that each plate contained 26, 86, or 259 μg T3s; these amounts were based on the assumption that all T3s were included in the inclusion compound by the addition of a sufficient amount of γCD. Astaxanthin (Wako, Tokyo, Japan) or γ-tocopherol (Sigma, St.

Louis, MO, USA) was also used as another well-known antioxidant.

Each group of 30 worms was added to a plate and incubated at 25 °C. Live and dead worms were determined every 24 h. A worm was considered dead when it failed to respond to gentle touch with a worm picker. Worms that died from getting stuck to the wall of the plate were not scored. Worms were transferred every other day to fresh plates to avoid contamination from progeny. Each assay was carried out in duplicate and repeated two or three times to confirm the reproducibility. The same amount of γCD only was administered to control worms.

Mean life span was estimated using the formula (Wu et al., 2006):

$$\mathrm{MLS} = \frac{1}{N}\sum_{j}\frac{x_j + x_{j+1}}{2}d_j$$

where *dj* is the number of worms that died in the age interval (x_j, x_j+1), and *N* is the total number of worms. The standard error of the mean life span estimate was calculated using the equation:

$$\mathrm{SE} = \sqrt{\frac{1}{N(N-1)}\sum_{j}\left(\frac{x_j + x_{j+1}}{2} - \mathrm{MLS}\right)^2 d_j.}$$

Maximum life span was calculated as the mean life span of the longest-living 15% of each group.

Nematodes were also examined for lipofuscin, the so-called “age pigment” that accumulates with aging, with fluorescence microscopy (Gerstbrein et al., 2005).

Effect of tocotrienols on innate immunity

After hatching, nematodes were grown on OP50 for 3 days. The adult worms were then divided into two groups that were given γCD only or were supplemented with a γCD inclusion compound containing T3s. Seven- or 8-day-old worms were fed *Salmonella enterica* subsp.

enterica serovar Enteritidis strain SE1 or *Legionella pneumophila* serogroup 1 strain JR32, respectively, instead of OP50 as reported previously (Ikeda et al., 2007; Komura et al., 2010). Their survival was measured as described above.

Results

Inclusion compounds (approximately 14 mg wet weight) composed of γCD and 8.0 μg DiO were spread on the surface of a mNGM plate with OP50, and nematodes were allocated onto the plate. In 20 min, the amount of fluorescent dye taken up was high compared with worms administered dye by conventional methods (Fig. 2A). Nematodes showed fluorescence in the lumen of the mouth to the pharynx, and beyond the pharyngeal bulb to the anus (Fig. 2B). DiO fluorescence was observed not only in the intestinal lumen but in the cytoplasm of the intestinal cells (Fig. 2C). Nematodes appeared to ingest the inclusion compounds in the presence of OP50, showing that nematodes did not selectively avoid the compounds.

Using this method, tocotrienols (T3s) were administered to worms (Fig. 3). The lifespan of nematodes that ingested γCD containing T3s was longer than that of control worms maintained on mNGM containing γCD alone. Survival curves of worms maintained on mNGM containing the same amount of T3s were indistinguishable from the control. γCD-mediated oral supplementation with the antioxidants did not clearly alter lipofuscin accumulation (data not shown). Similarly, astaxanthin (6.0 μg/plate) and γ-tocopherol (43 μg/plate) also prolonged the lifespan of the worms (Fig. 4A and B). However, astaxanthin (6.0 μg/plate) that was directly spread onto the mNGM plate failed to prolong the lifespan to the same extent as the inclusion compound.

To study the effects of T3s on host defense, 7- or 8-day-old worms were fed *Salmonella enterica* subsp. *enterica* serovar Enteritidis strain SE1 or *Legionella pneumophila* serogroup 1

strain JR32, respectively, instead of OP50. Oral supplementation with T3s failed to enhance the host defense to *Salmonella* (data not shown). However, when nematodes were exposed to the opportunistic pathogen *Legionella* (Komura et al., 2010), T3s protected against death from the infection (Fig. 5).

Discussion

Our results show that γCD is an excellent vehicle for quantitative oral administration of hydrophobic chemicals to *C. elegans*. Because nematodes showed fluorescence in the lumen of the mouth to the pharynx and beyond the pharyngeal bulb to the anus in 20 min, we assessed the amount of inclusion compounds ingested by the nematodes in 20 min. When inclusion compounds (14 mg wet weight) containing 8 μg DiO (calculated based on its fluorescence intensity) were spread onto mNGM with 10 mg OP50, the amount of DiO ingested by a single nematode in 20 min was calculated to be 70 pg because 50 worms ingested 3500 pg DiO (Fig. 2A). Thus, to intake 70 pg DiO from the inclusion compound (8 μg of DiO/14 mg of compound), a single worm must ingest 120 ng of the inclusion compound. The increase in DiO recovered 24 h later suggests that the fluorescent dye was absorbed from the digestive tract and accumulated in the cytoplasm. The experiment with DiO-γCD inclusion compounds clearly showed that worms did not selectively ingest OP50, and therefore did not distinguish the bacteria from the inclusion compounds. Assuming that the worm would ingest compounds at the same rate if other chemicals were bound by γCD, this method enables us to estimate the amount of test substances ingested by a worm over a particular time. It is easy to determine if worms ingested an inclusion compound as well as they ingested DiO-γCD inclusion bodies by counting the number of worms remaining in both

suspensions spotted separately onto mNGM plates in a preparatory experiment.

We showed that DiO-γCD inclusion compounds were ingested by worms. Then, lipid-soluble antioxidants were used to examine if the γCD could carry and release chemicals that retain their function. The lifespan of nematodes that ingested γCD containing T3s was longer than that of control worms. As described above, a worm ingested 120 ng of the inclusion compound when 14 mg of the inclusion compound was present with OP50 on mNGM. Based on the rule-of-three calculation, we estimated that a worm would ingest 57 ng out of 6.6 mg of T3-γCD inclusion compound in 20 min. Because 6.6 mg of T3-γCD inclusion compound included at most 86 μg of T3 if we assumed that all added T3 was included, a worm would intake 740 pg of T3 included in the 57 ng of the inclusion compounds, proportionally. Thus, our method efficiently delivered hydrophobic antioxidants to nematodes with significant effects on lifespan. The clear pro-longevity effect of T3 was consistent with a previous report (Adachi and Ishii, 2000). Because our method is very efficient at delivering hydrophobic antioxidants to nematodes, significant pro-longevity effects were observed at a concentration of 86 μg per mNGM plate, which is a 10-fold lower concentration than that used by Adachi and Ishii (2000). Oral administration of astaxanthin (6 μg/10 ml of mNGM plate) and γ-tocopherol (43 μg/10 ml of mNGM plate) also prolonged the lifespan at a very low dose compared to previous reports in which 0.1-1 mM (59.7-597 μg/ml) astaxanthin or 200 μg/ml γ-tocopherol were added to the medium (Yazaki et al., 2011; Zou et al., 2007). This delivery mechanism should therefore facilitate clarification of the effects of hydrophobic compounds such as T3 on longevity in future studies.

The age-dependent accumulation of lipofuscin in the intestinal cells of worms has been demonstrated (Klass, 1977), but antioxidants and lifespan extension are not always associated with reduction in this age pigment (Braeckman et al., 2002; Kampkötter et al., 2007). T3 may play major roles in tissues other than intestinal cells, resulting in increased longevity.

Previously we showed that nematodes fed bifidobacteria or lactobacilli for 4 days were clearly resistant to subsequent *Salmonella* infection compared with nematodes fed OP50 before the infection (Ikeda et al., 2007). T3s protected the worms from infection with the opportunistic pathogen *Legionella*, but T3s did not prevent infection with *Salmonella* unlike the lactic acid bacteria. The discrepancy is probably due to the higher virulence of *Salmonella* organisms (Hoshino et al., 2008; Komura et al., 2010). Mechanisms of how T3s protected worms against death from *Legionella* infection still remain to be elucidated. Protein damage occurs specifically at the sites of host-pathogen interactions, and reactive oxygen species produced by the host are a source of protein damage during infection (Mohri-Shiomi and Garsin, 2008). T3s may reverse reactive oxygen species-mediated protein damage due to the opportunistic pathogen *Legionella*, whereas the lactic acid bacteria probably enhance the endogenous host defense.

γCD is theoretically likely to cause cholesterol depletion by forming inclusion compounds with cholesterol. Cholesterol depletion can cause brood size reduction and internal egg hatching, so-called worm bagging. However, no effects on the lifespan or brood size were observed when we administered saturated γCD solution (50 μl/plate) to nematodes. In addition, our method involves administration of γCD after it has been transformed into inclusion bodies with chemicals. This is probably the reason why the γCD did not cause cholesterol depletion.

A few limitations of γCD application are as follows: 1) a few organic solvents, methylene chloride for example, cannot be used because they form inclusion compounds with γCD, inhibiting the reaction between γCD and test chemicals; 2) some test chemicals would be perfectly included within the cavity of γCD and be dissolved in water without forming insoluble particles; and 3) some chemicals that are larger than the γCD cavity cannot be included in γCD inclusion bodies.

In conclusion, γCD is an excellent vehicle for oral delivery of hydrophobic substances to *C. elegans*. Using this method, oral supplementation with antioxidants prolonged the lifespan of worms more efficiently than conventional delivery methods. In combination with our liposome method (Shibamura et al., 2009), this γCD method provides an alternative choice to administer chemicals orally to *C. elegans*, particularly when test chemicals are limited or the amount of organic solvents must be restricted. Our method will expand the usefulness of nematodes not only in biogerontology but also for screening drugs, health-promoting chemicals, and toxic substances.

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Figure legends

Fig. 1. Flow chart of the system to prepare γCD inclusion compounds

Fig. 2. Nematode fluorescence after oral administration of DiO. (A) The amount of DiO recovered from worms. Bars indicate the average of five experiments. The amount of fluorescent dye recovered from worms orally administered with DiO-γCD inclusion compounds was significantly higher than that from the other two groups in which DiO was spread onto or dissolved into the medium. *$p < 0.05$, **$p < 0.01$. At 40 min, only the difference between DiO-γCD and DiO in NGM was significant. (B) A nematode fed OP50 and γCD bound with DiO shows clear fluorescence along the digestive tract. (C) The intestinal lumen is between the arrows. Faint fluorescence is visible in the cytoplasm of intestinal cells.

Fig. 3. Survival curves of nematodes supplemented with γCD-tocotrienol inclusion compounds. Young adult worms were divided into groups that were supplemented with T3-enriched vitamin E. The inclusion compound was spread onto mNGM plates so that each plate contained 26, 86, or 260 μg T3s. The same amount of γCD only was administered to control worms and those maintained on mNGM containing 86 μg T3s. Nematode survival was calculated with the Kaplan-Meier method, and survival differences were tested for significance using the log rank test. *$p < 0.05$, ***$p < 0.001$, compared to the control. The mean lifespans (in days) of worms supplemented with 26, 86, or 259 μg T3s were 16.1 ± 0.43 (8.6%), 18.0 ± 0.46 (21%), and 16.7 ± 0.35 (12%), respectively. The numbers in parentheses are the percent differences in the mean relative to control. Mean lifespans of control worms and worms supplemented with 86 μg T3s with the conventional method were 14.8 ± 0.39 and

14.5 ± 0.41 (−2.6%) days, respectively. Similarly, the maximum lifespans of worms supplemented with 26, 86, or 259 μg T3s were 19.6 ± 0.13 (3.5%), 22.3 ± 0.24 (18%), and 21.1 ± 0.29 (11%), respectively. Maximum lifespans of control worms and worms supplemented with 86 μg T3s with the conventional method were 19.0 ± 0.38 and 19.6 ± 0.29 (3.2%) days, respectively.

Fig. 4. Survival curves of nematodes supplemented with γCD-antioxidant inclusion compounds. (A) Young adult worms were divided into groups that were supplemented with 6.0 μg astaxanthin as the inclusion compound (AX-γCD) or by directly spreading the DMSO solution (AX-DMSO). The same amount of DMSO was administered to a group of worms to compare with the control worms. Nematode survival was calculated with the Kaplan-Meier method, and survival differences were tested for significance using the log rank test. *$p < 0.05$, ***$p < 0.001$, compared to the control. The mean lifespans (in days) of the groups given AX-γCD, AX-DMSO, and DMSO were 20.9 ± 0.59 (20.8%), 18.6 ± 0.55 (7.5%), and 17.7 ± 0.63 (2.3%), respectively. The numbers in parentheses are percent differences in the mean relative to control. The mean lifespan of the control was 17.3 ± 0.64 days. Similarly, the maximum lifespans (in days) of each group were 25.9 ± 0.3 (14.6%), 24.4 ± 0.77 (8.0%), and 23.9 ± 0.53 (5.8%), respectively. The maximum lifespan of the control was 22.6 ± 0.48 days. (B) Young adult worms supplemented with 43 μg γ-tocopherol as the inclusion compound (γTP-γCD) survived longer than control worms ($p < 0.05$). The mean lifespan (in days) was 18.5 ± 0.49 (10.1%), whereas that of the control was 16.8 ± 0.39. The numbers in parentheses are percent differences in the mean relative to control. Similarly, the maximum lifespans of worms treated with γTP-γCD and control worms were 25.5 ± 0.44 (12.3%) and 22.7 ± 0.43, respectively.

Fig. 5. Effect of supplementation with T3s on the survival of nematodes infected with *Legionella pneumophila*. Nematodes supplemented with T3s were significantly more resistant to the pathogen than controls (**$p < 0.01$). The mean survival times of worms after *Legionella* infection were 13.5 ± 0.23 days for control worms and 15.0 ± 0.32 days for those supplemented with 86 μg T3s. The percent difference in the mean relative to control was 11%. Similarly, maximum survival times were 17.1 ± 0.46 days for control worms and 19.6 ± 0.72 (14%) days for those supplemented with 86 μg T3s.

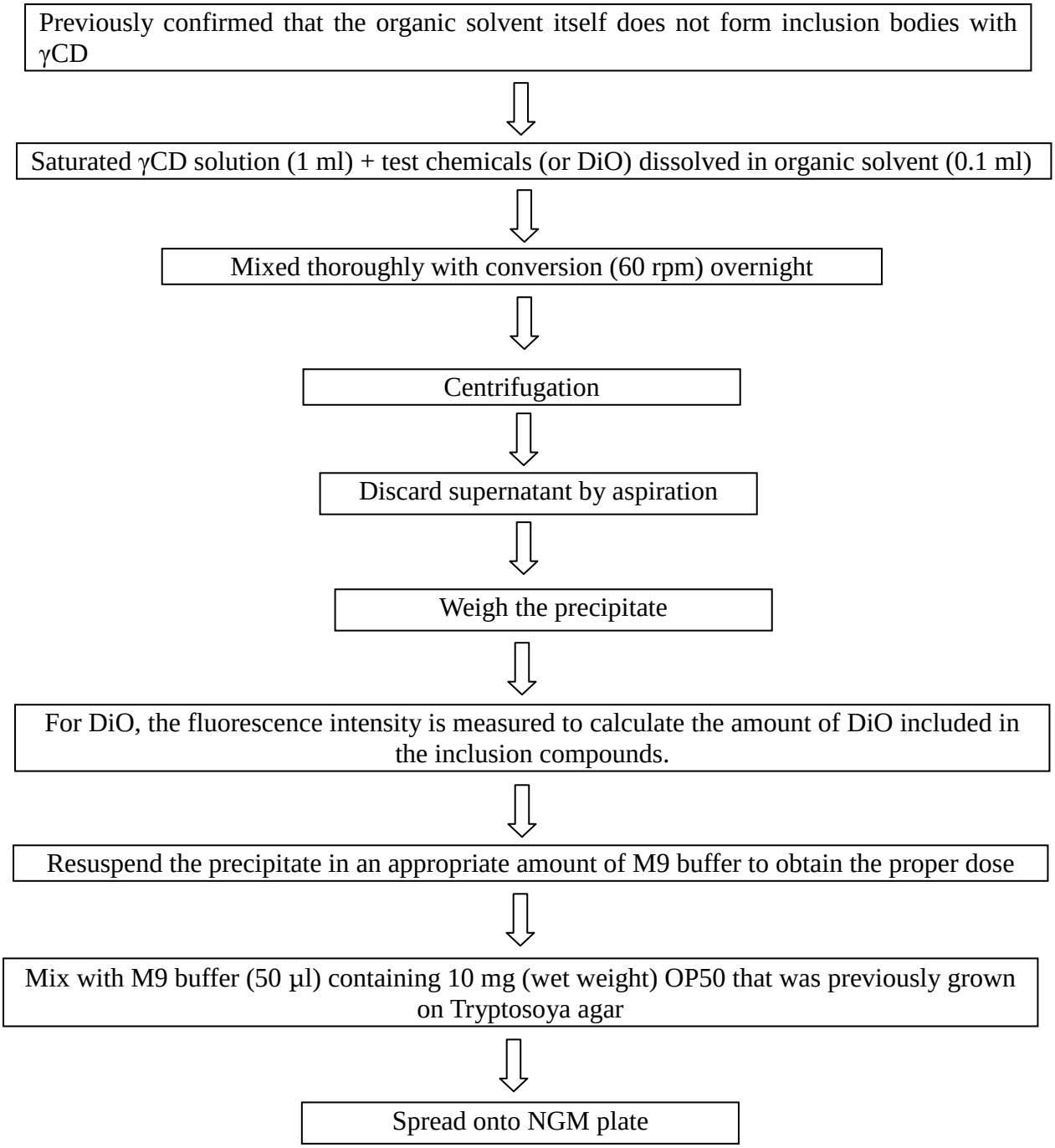
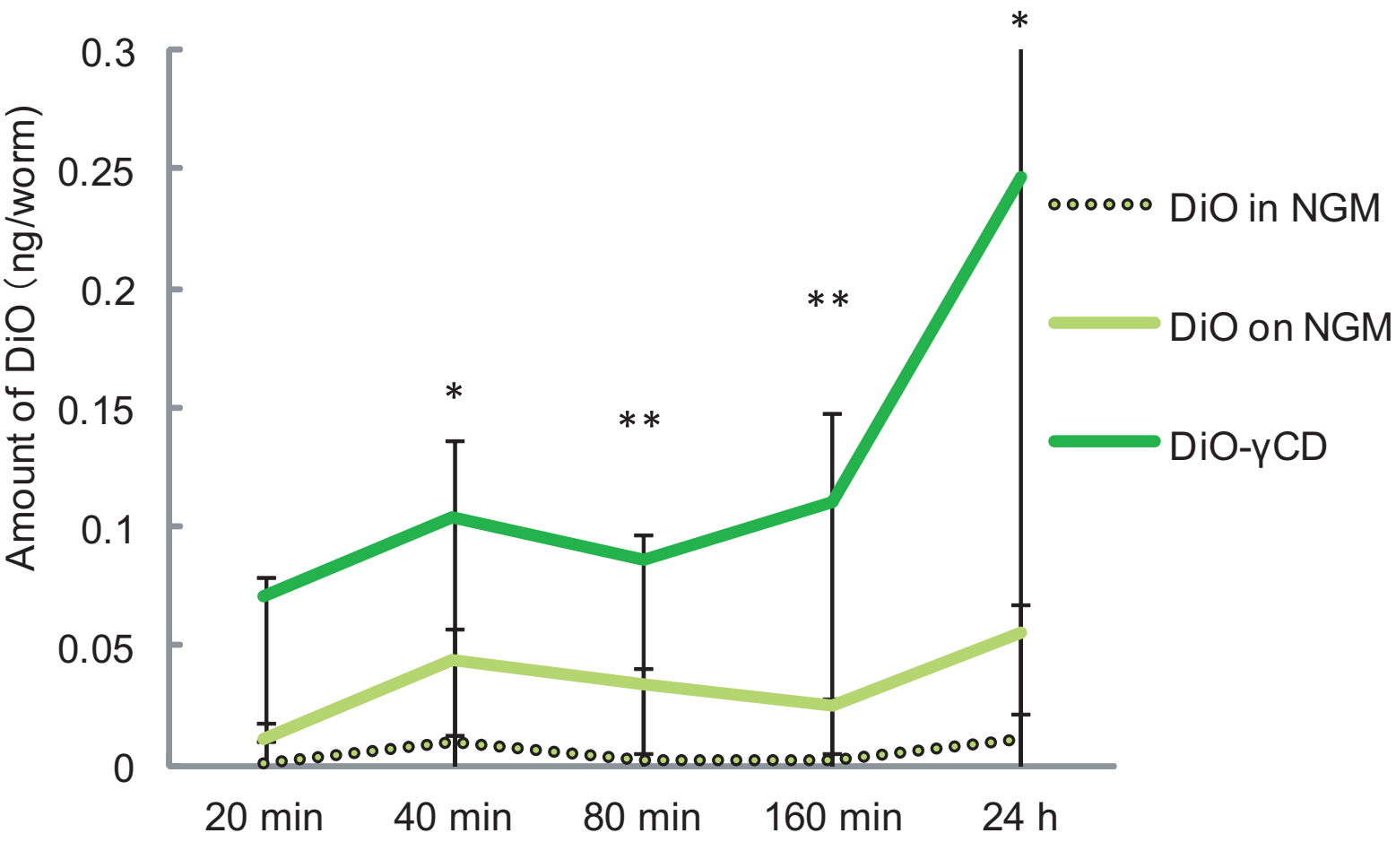
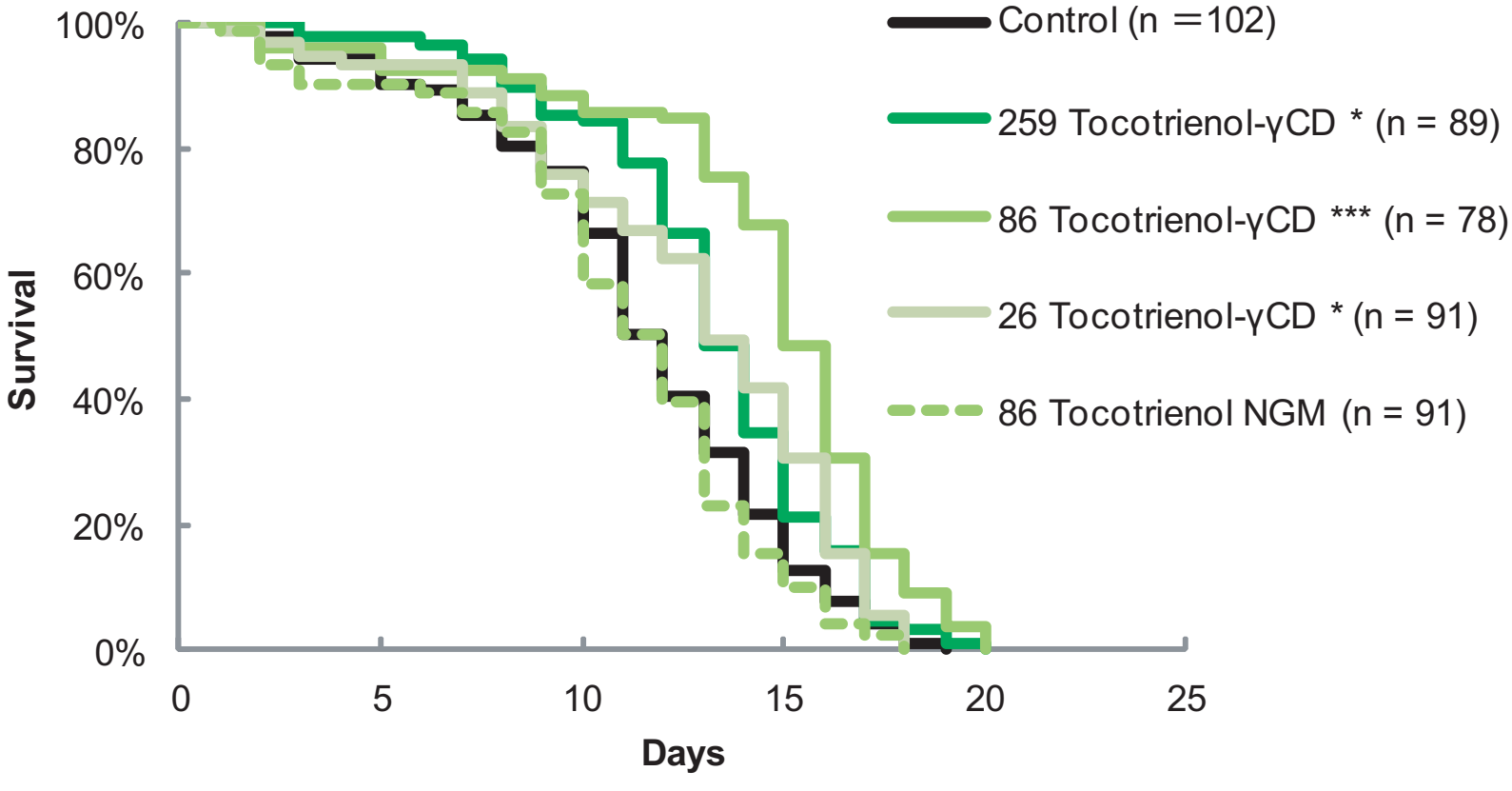
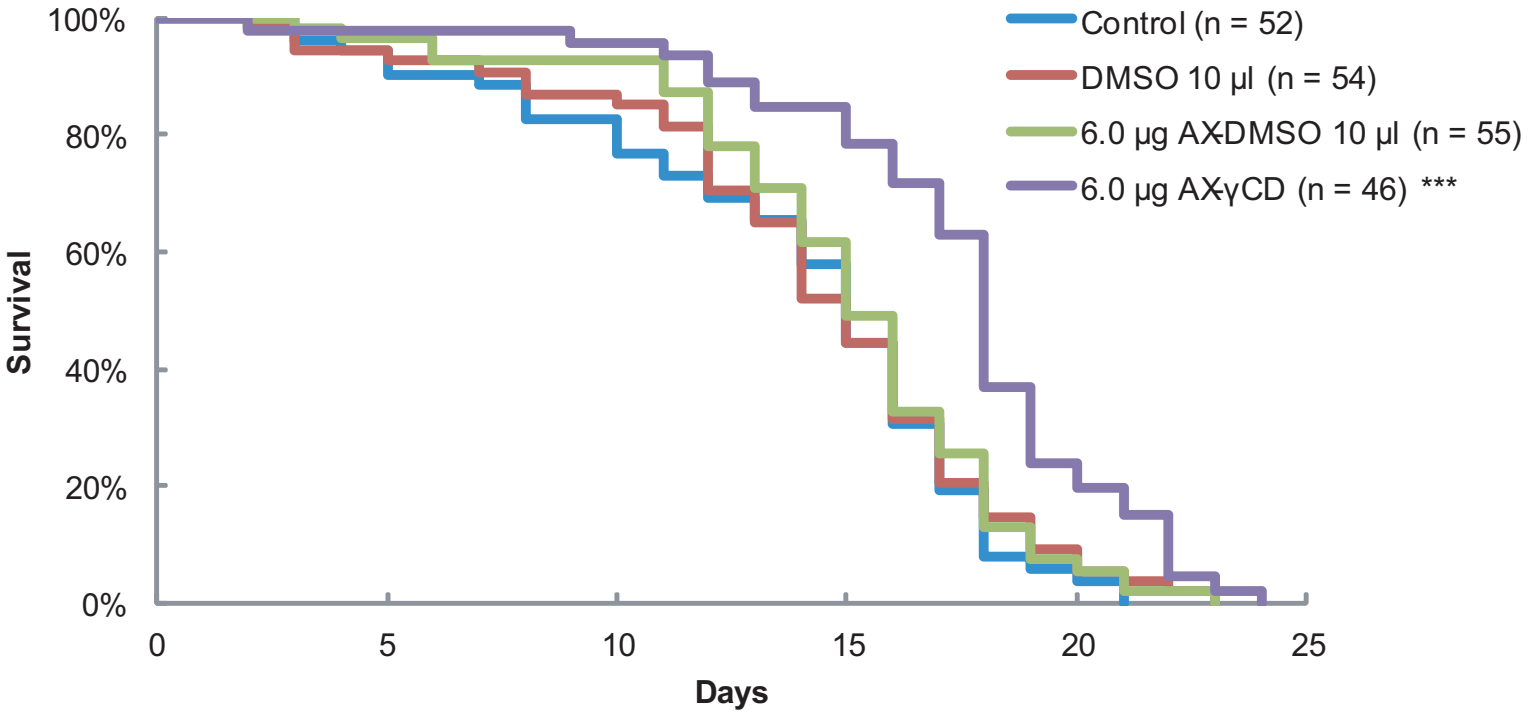
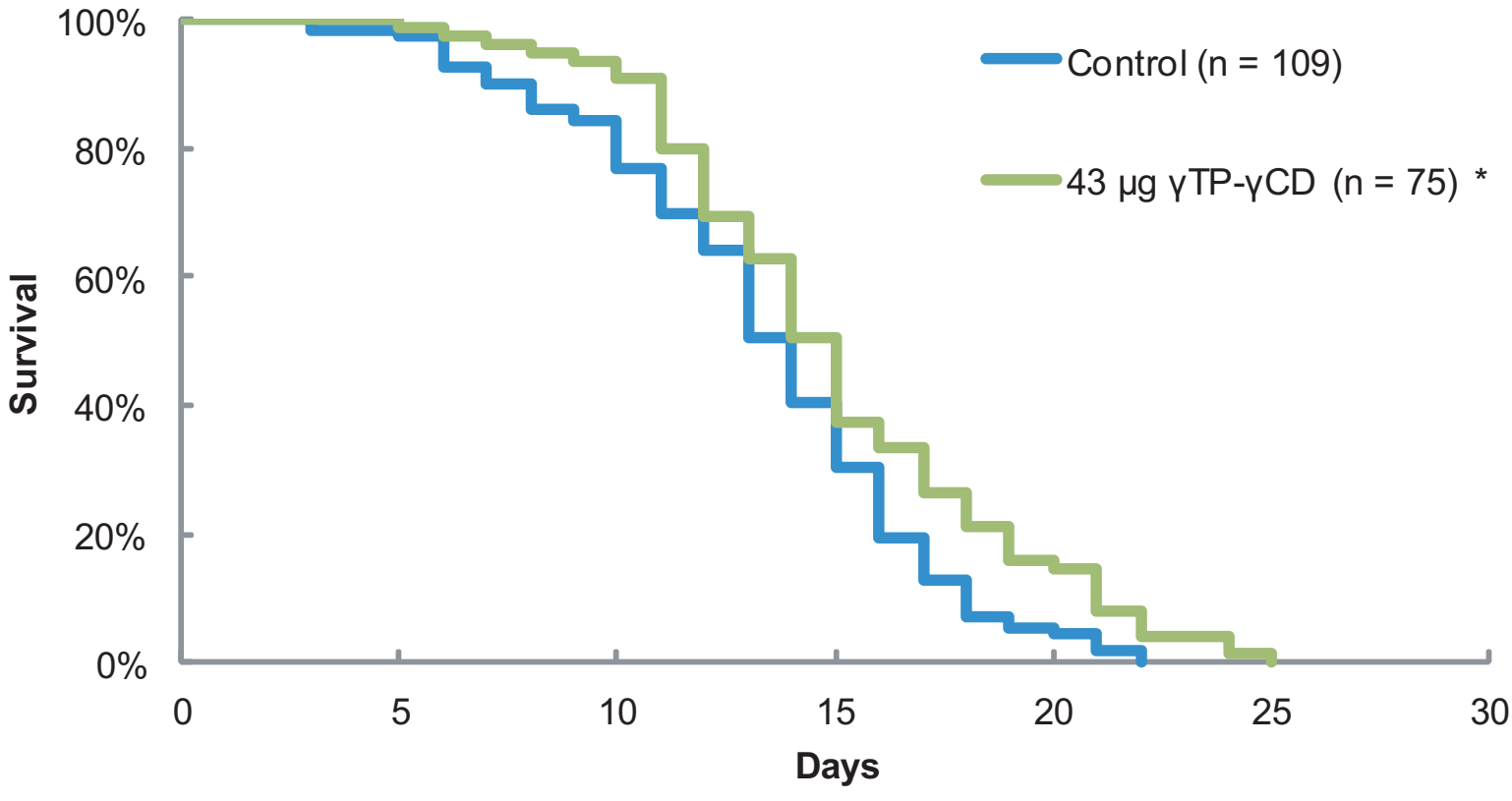

Previously confirmed that the organic solvent itself does not form inclusion bodies with γCD
Saturated γCD solution (1 ml) + test chemicals (or DiO) dissolved in organic solvent (0.1 ml)
Mixed thoroughly with conversion (60 rpm) overnight
Centrifugation
Discard supernatant by aspiration
Weigh the precipitate
For DiO, the fluorescence intensity is measured to calculate the amount of DiO included in the inclusion compounds.
Resuspend the precipitate in an appropriate amount of M9 buffer to obtain the proper dose
Mix with M9 buffer (50 µl) containing 10 mg (wet weight) OP50 that was previously grown on Tryptosoya agar
Spread onto NGM plate

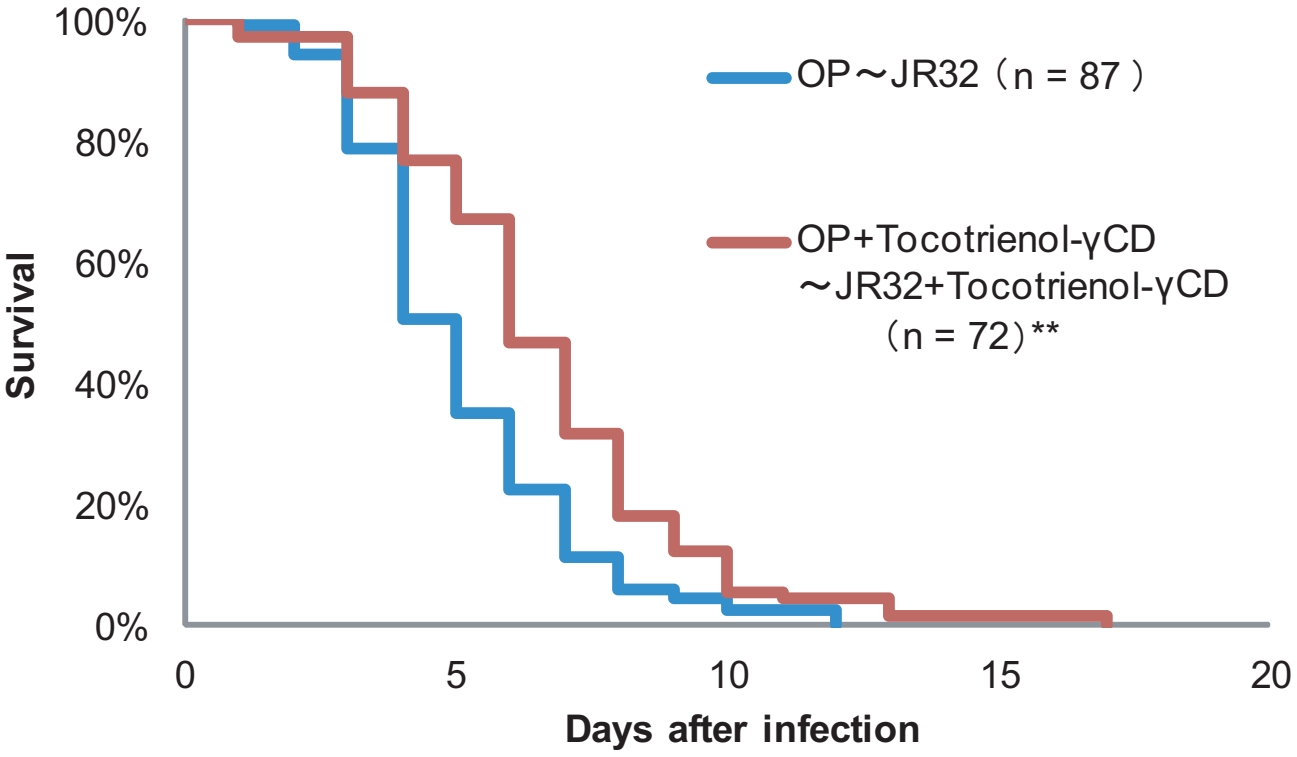
Fig. 1


Amount of DiO（ng/worm）
0.3
0.25
0.2
0.15
0.1
0.05
0
20 min
40 min
80 min
160 min
24 h
*
**
**
*
DiO in NGM
DiO on NGM
DiO-γCD


100%
80%
60%
40%
20%
0%
Survival
0
5
10
15
20
25
Days
Control (n =102)
259 Tocotrienol-γCD * (n = 89)
86 Tocotrienol-γCD *** (n = 78)
26 Tocotrienol-γCD * (n = 91)
86 Tocotrienol NGM (n = 91)


100%
80%
60%
40%
20%
0%
Survival
0
5
10
15
20
25
Days
Control (n = 52)
DMSO 10 µl (n = 54)
6.0 µg AX-DMSO 10 µl (n = 55)
6.0 µg AX-γCD (n = 46) ***


100%
80%
60%
40%
20%
0%
Survival
0
5
10
15
20
25
30
Days
Control (n = 109)
43 µg γTP-γCD (n = 75) *


100%
80%
60%
40%
20%
0%
Survival
0
5
10
15
20
Days after infection
OP～JR32（n = 87）
OP+Tocotrienol-γCD
～JR32+Tocotrienol-γCD
（n = 72）**