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Drug Absorption Efficiency in *Caenorhbditis elegans* Delivered by Different Methods

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Abstract

Background: *Caenorhbditis elegans* has being vigorously used as a model organism in many research fields and often accompanied by administrating with various drugs. The methods of delivering drugs to worms are varied from one study to another, which make difficult in comparing results between studies.

Methodology/Principal Findings: We evaluated the drug absorption efficiency in *C. elegans* using five frequently used methods with resveratrol with low aqueous solubility and water-soluble 5-Fluoro-2′-deoxyuridine (FUDR) as positive compounds. The drugs were either applied to the LB medium with bacteria OP50, before spreading onto Nematode Growth Medium (NGM) plates (LB medium method), or to the NGM with live (NGM live method) or dead bacteria (NGM dead method), or spotting the drug solution to the surface of plates directly (spot dead method), or growing the worms in liquid medium (liquid growing method). The concentration of resveratrol and FUDR increased gradually within *C. elegans* and reached the highest during 12 hours to one day and then decreased slowly. At the same time point, the higher the drug concentration, the higher the metabolism rate. The drug concentrations in worms fed with dead bacteria were higher than with live bacteria at the same time point. Consistently, the drug concentration in medium with live bacteria decreased much faster than in medium with dead bacteria, reach to about half of the original concentration within 12 hours.

Conclusion: Resveratrol with low aqueous solubility and water-soluble FUDR have the same absorption and metabolism pattern. The drug metabolism rate in worms was both dosage and time dependent. NGM dead method and liquid growing method achieved the best absorption efficiency in worms. The drug concentration within worms was comparable with that in mice, providing a bridge for dose translation from worms to mammals.

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Introduction

Since *Caenorhabditis elegans* (*C. elegans*) was chosen as a model organism to study genetics [1], the worms have been widely used in many research areas, either as research or drug screening model [2,3], such as in development [4–6], lipid metabolism and obesity [7–9], aging [10,11], neurodegenerative disease [12–14], antimicrobials [15–17], virulence and parasites [18], biomedical and environmental toxicology [19], and cancer [20]. Treating worms with tool drugs was frequently used in these studies. For the tiny nature of the worms, the drug delivery methods were indirect and often varied from one study to another. The drugs were either applied to the LB medium growing the bacteria, the food of worms (LB medium method), or directly spotted onto the surface of NGM plates (spot dead method), or to the NGM with live (NGM live method) [21] or dead bacteria (NGM dead method) spread on the surface of the plates [22–24]. Another method was to keep the worms in liquid medium (liquid growing method) [15,25,26]. These different delivery methods might result in different drug absorption efficiency, causing confusing results between different studies [25–28]. Moreover, the resistance of *C. elegans* to pharmacological perturbation appeal an effective drug delivery approach to make *C. elegans* as a screening tool for novel small bioactive molecules [29].

Currently, the information for drug absorption in worms was scarce. To bridge this gap, we evaluated the drug absorption efficiency in worms of five frequently used drug-delivering approaches with the test compounds resveratrol and 5-Fluoro-2′-deoxyuridine (FUDR). Our data indicated that resveratrol and FUDR administrated with NGM dead method and liquid growing method achieved the best absorption efficiency in worms, while the spot dead method was the economic approach.

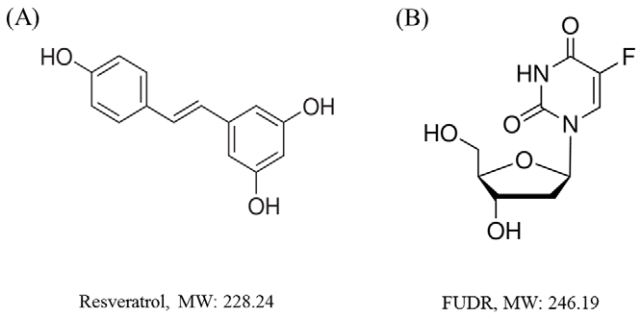

Figure 1. The structure and molecular weight of resveratrol (A) and FUDR (B).
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Materials and Methods

Drug administration, worm culturing and harvesting

The wild type N2 *C. elegans* was provided by the *Caenorhabditis Genetics Center* (CGC). Resveratrol and FUDR (Sigma Aldrich) were used as the test compounds. Resveratrol (3,5,4′-trihydroxy-trans-stilbene) is a type of natural phenol with low aqueous solubility and the molecular weight of 228.24 (Figure 1A). Resveratrol has been shown to extend the lifespan of yeast, fly and *C. elegans* with clear mechanism of action [30–33]. FUDR, with the molecular weight of 246.2 (Figure 1B), was soluble in water at the concentration to 50 mg/mL. FUDR was widely used to inhibit the worms to lay eggs in research with the concentration of FUDR from 40 μM to 50 μM [34,35].

Resveratrol was dissolved in DMSO to 100 mM as stock solution. In LB medium method, the resveratrol stock solution was diluted into LB liquid medium containing OP50 *E. coli* bacteria to a final concentration of 100 μM and 1.5 mL of the bacteria solution was applied to each NGM plate (100 mm diameter). In spot dead method, 1.5 mL of 100 μM resveratrol was spotted onto the surface of the NGM plate, then covered with dead bacteria. In NGM live method, the resveratrol stock solution was diluted with NGM (below 65°C after boiled) to the concentration of 100 μM. Then the NGM was poured into petri plates as supporting bed for worms and live OP50 was applied to the surface of NGM plates. In NGM dead method, the plates were made as the NGM live method, except that the food OP50 bacteria was killed by incubating in 65°C for 30 minutes [24]. The drug administration of above four methods was summarized in Figure 2. The maintenance of *C. elegans* in liquid medium was described as previously [23] with slight modification. Briefly, the synchronized N2 adult day 1 worms were cultured in 50 mL centrifuge tubes that contained 35 mL S medium [23], the concentration of resveratrol in S medium was 100 μM. Dead bacteria were added to S medium as food. FUDR was dissolved in H_2O to 50 mM as stock solution. The procedures of treatment of worms with FUDR were the same with resveratrol, except that the final concentration of FUDR in the five treatment methods was 50 μM.

About 5,000–10,000 adult day 1 wild type worms were transferred to each NGM plate (100 mm diameter). For the liquid growing method, the worms were cultured in several 50 mL centrifuge tubes with each containing about 35 mL S medium [23]. Worms in each method were harvested by using cold M9 buffer at the 10 min, 30 min, 1 hr, 3 hr, 6 hr, 12 hr, day 1, day 2, day 4, day 7, day 14 and day 20 after treated with the compounds [23], and collected to 15 mL centrifuge tubes. The control group without treatment with compound was also harvested. The tubes were putted into ice for 10 minutes, then spin for 2 minutes at 1,150× g to precipitate the worms. The worm pellets were rinsed three times with cold M9 buffer, air dry, and weighed. The worm pellets were resuspended by using 1 mL methyl ethanol (HPLC grade) (for resveratrol) or H_2O (for FUDR) and sonicated 50 times (200 V, operation 5 seconds every 5 seconds). Then, the worm solution was centrifuged under 12, 000× g for 3 minutes. The supernatant of the worm solution containing resveratrol or FUDR was transferred to a 1.5 mL centrifuge tube.

To test the drug metabolism, the worms treated with 400 μM, 200 μM, 100 μM, 50 μM, 25 μM, and 12.5 μM of resveratrol and FUDR, respectively, for 6 hours under NGM dead method were transferred to NGM plates without resveratrol and FUDR. Then, the worms were harvested at the 10 min, 30 min, 1 hr, 2 hr, 3 hr, 4 hr, 6 hr, 8 hr, 12 hr, and 16 hr time points. Subsequent sample preparation was the same as described above.

Measurement of the concentration of resveratrol and FUDR using HPLC

The Agilent 1200 with auto-injector and dual absorbance UV detector was used for sample analysis. All samples including standard resveratrol solutions were filtered with 0.45-μm organic

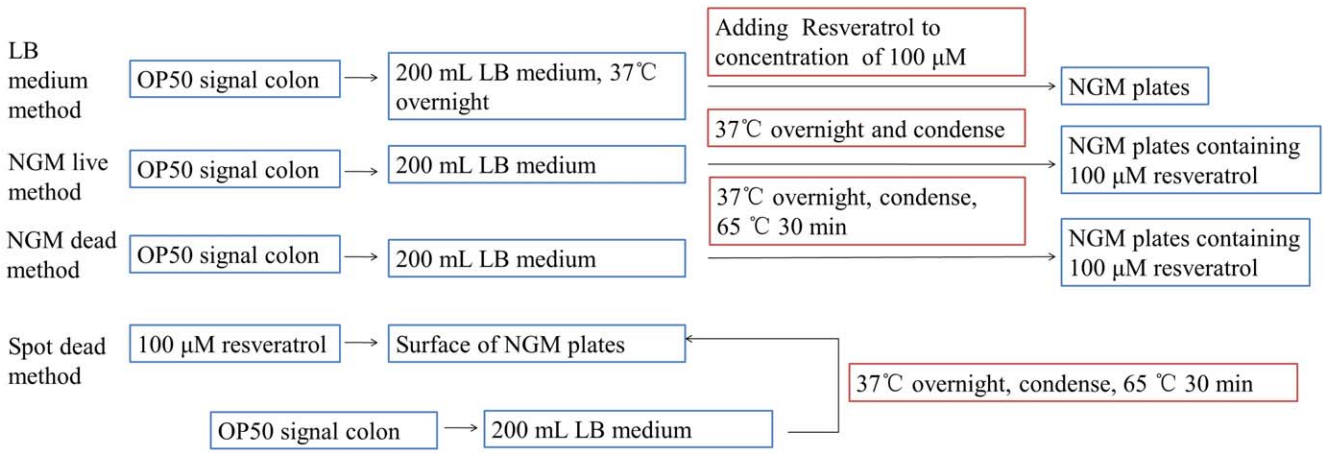

Figure 2. The summary of drug administration for LB medium method, NGM live method, NGM dead method, and spot dead method.
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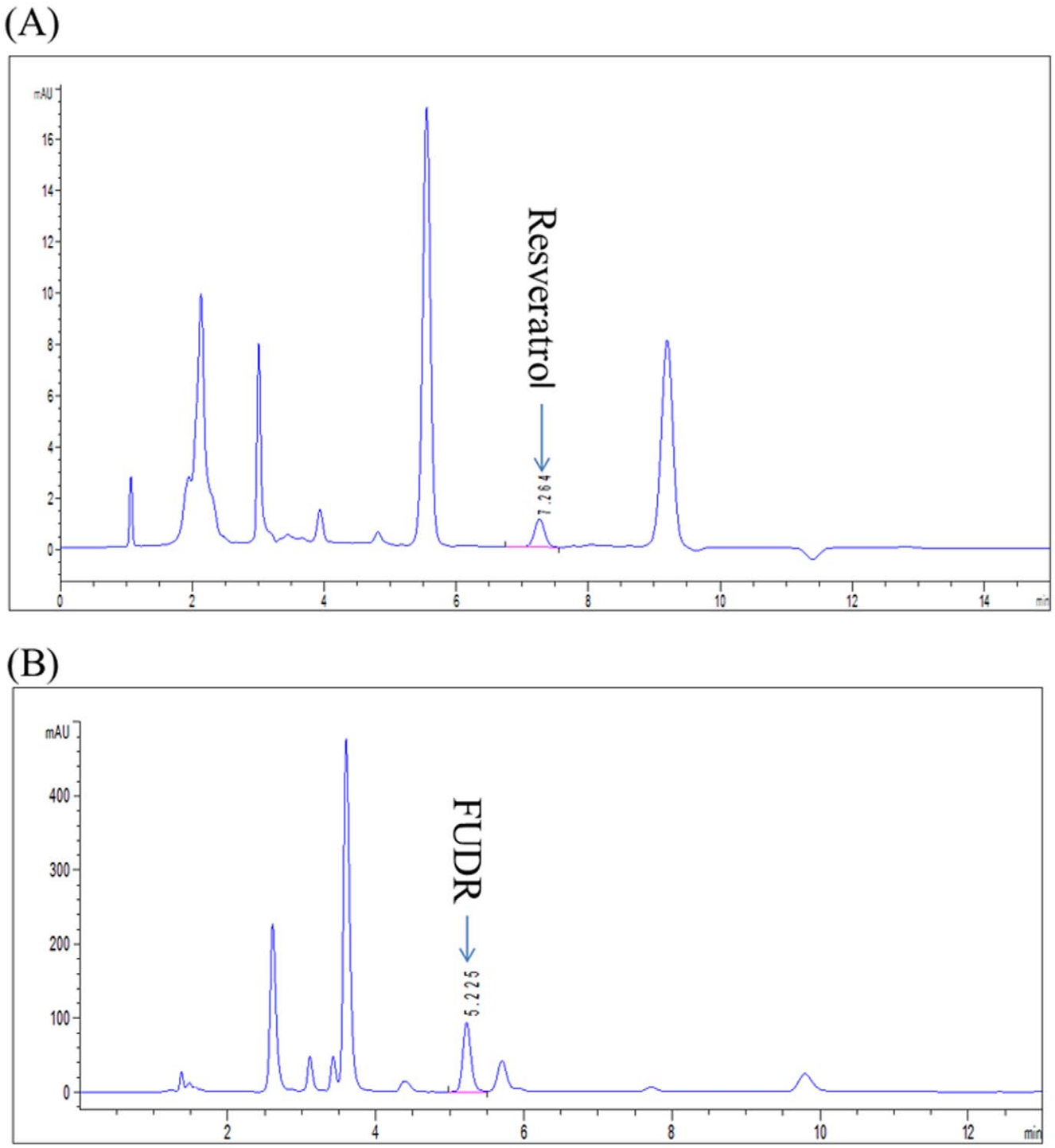

Figure 3. The HPLC profile and retention time of resveratrol and FUDR extracted from the day 1 worms. The retention time of resveratrol (A) and FUDR (B) in the worms was 7.2 min and 5.2 min, respectively.
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filter. Then, 10 μL of each sample was injected into the system and separated in a reversed-phase Agilent C18 column (ZORBAX, SB-C18, 4.6×250 mm, 5 μM) containing 27% of C_2H_3N and 73% of H_2O at mobile phase, with flow rate of 1 mL/min and temperature at 30°C, and detected at 303 nm wavelength. For FUDR, the solutions passing through the mobile phase with flow rate of 1 mL/min at 20°C were 10%–12% of MeOH in H_2O for over 5 min, followed by 12%–15% of MeOH in H_2O for 5 min, finally with 50% of MeOH for 3 min. FUDR was detected at 268 nM wavelength.

To calculate the concentration of resveratrol and FUDR in worms, the standard concentration curve of resveratrol and FUDR were made first. A series of standard solution of resveratrol were made freshly before analysis by diluting the methyl alcohol

Table 1. The repeatability and stability of the HPLC method.

		Resveratrol		FUDR	
	Injected volume (μL)	Worm weight (mg)	Peak area (mAU*s)	Worm weight (mg)	Peak area (mAU*s)
Experiment 1	10	150	3.32298	30	34.2
Experiment 2	10	150	3.25668	30	36.4685
Experiment 3	10	150	3.32298	30	34.2
Experiment 4	10	150	3.36413	30	34.7917
Experiment 5	10	150	3.55011	30	34.5687
Experiment 6	10	150	3.56435	30	34.2349
Average			3.39687		34.7439
Standard Deviation			0.128978		0.878403
Relative Standard Deviation (%)			3.8		2.5

The day 1 worms cultured by using the NGM dead method were used in these tests. The table showed the six individual experiments.
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solution of resveratrol from stock solution of 2 g/L to working standard solutions of 0.5, 20, 80, 500, 600, 800, 1,200 mg/L, respectively. FUDR was diluted with H_2O to 1, 5, 20, 100, 300, 800 mg/L, respectively. Calibration curve was plotted with the standard concentrations as the x-axis and the detected peak area signals as y-axis. The parameters of slope, intercept and correlation coefficient were carried out by linear regression.

The detection limits were evaluated according to the criteria that the signal to noise ratio should be >3. The precision was evaluated by the relative standard deviation (RSD) of the recovery test. To carry out the recovery tests, the standard samples of drugs with three known concentrations were spiked into the blank worm samples, subsequent drug isolation procedure and detection conditions were the same as other test samples. The recovery rate was indicated by the ratio of the detected concentration of the standard drugs to the spiked concentration. The intra-day precision was evaluated by conducting three times of the recovery test at different time point within one day. The inter-day precision was evaluated by conducting three times of the recovery tests on each day, with total of three days within one week. The repeatability and stability of drug absorption efficiency in worms was also analyzed by repeating the NGM dead method for six times.

Results

Validation of HPLC method to detecting the concentration of resveratrol and FUDR in worms

The retention time of the standard resveratrol and FUDR was 7.2 and 5.2 minutes, respectively. The regression equation of the standard curve for resveratrol and FUDR were $y = 0.029x+0.517$ and $y = 12.131x-29.491$, respectively. The parameters of the linear curve were carried out by analyzing three independent experiments with the determination co-efficiency of $r^2 = 0.999$ for resveratrol and $r^2 = 0.9995$ for FUDR. This calibration curve showed an excellent linearity in the concentration range of 20–1,200 mg/L for resveratrol and of 1–800 mg/L for FUDR.

The relative standard deviation (RSD) of the six repeated experiments of resveratrol and FUDR absorption efficiency of NGM dead method was 3.8% and 2.5%, respectively (Figure 3, Table 1). The recovery rate in the recovery test of resveratrol standard samples with concentration of 35, 60, and 100 mg/L were 100.6%, 98.6%, and 101.0%, respectively. The relative standard deviation for intra-day and inter-day in the recovery test of resveratrol standard samples with concentration of 35, 60, 100 mg/L were 2.99 and 3.76, 1.68 and 1.21, 1.42 and 1.49, respectively (RSD$<$3.8%, Table 2). The recovery rate in the recovery test of FUDR standard samples with concentration of 20 and 35 mg/L were 99.4% and 99.8%, respectively. The relative standard deviation for intra-day and inter-day in the recovery test

Table 2. The precision of the HPLC detection of resveratrol and FUDR.

	Standard	Detected	Recovery rate	Relative Standard Deviation (%)	
	(mg/L)	(mg/L) ±SD	(%)	Intra-day	Inter-day
Resveratrol	35	35.2±1.05	100.6	2.99	3.76
	60	59.2±1.00	98.6	1.68	1.21
	100	101.0±1.44	101	1.42	1.49
FUDR	20	19.88±0.18	99.4	0.92	1.02
	35	34.92±0.26	99.8	0.73	0.85

The precision was evaluated through the relative standard deviation (RSD) of the recovery test by determining the recovery signal of the standard samples with three different concentrations each mixed into one of control samples.
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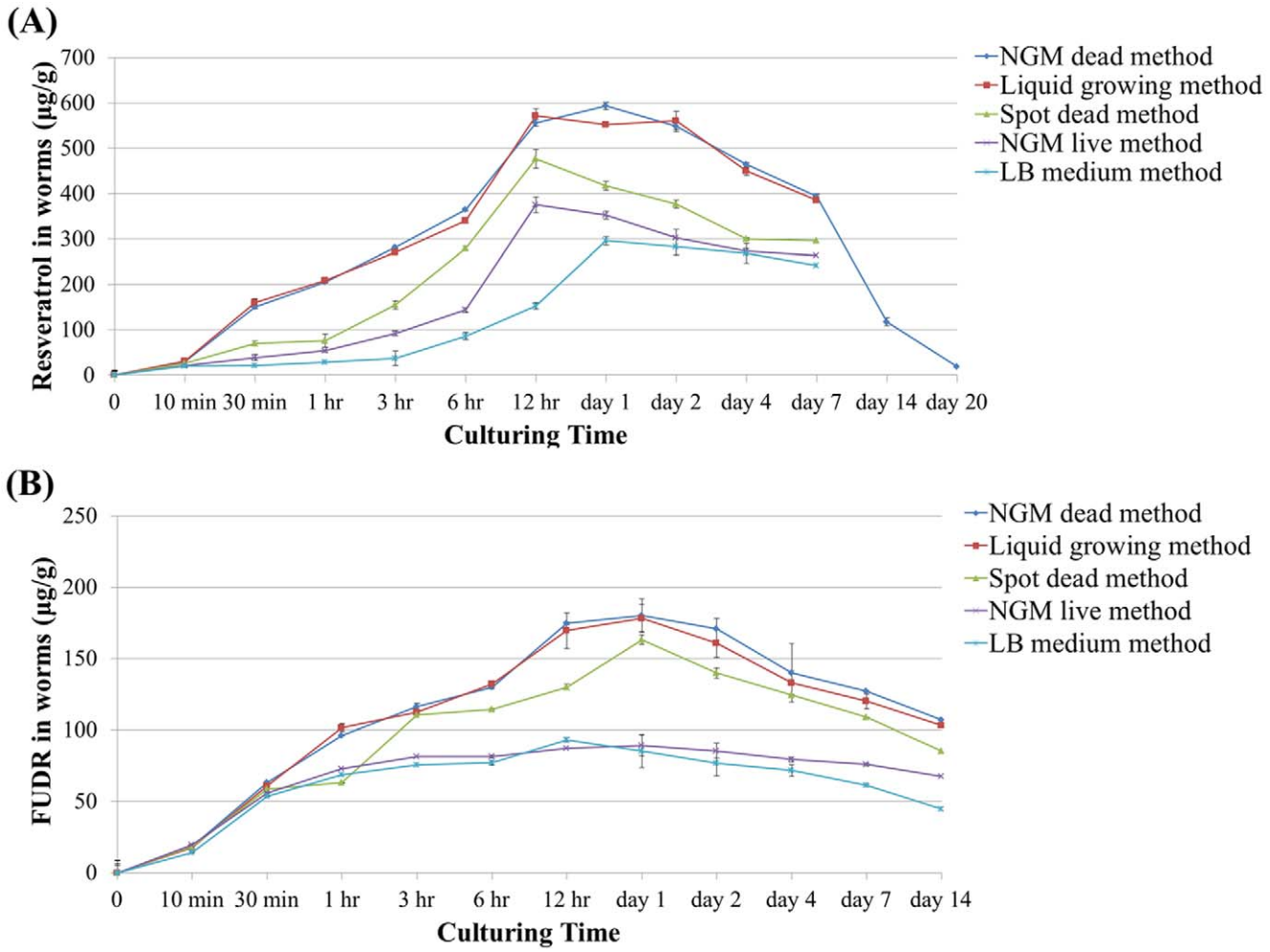

Figure 4. The drug absorption efficiency of worms administrated with 100 μM resveratrol (A) or 50 μM FUDR (B) by five delivering methods (μg/g). The concentration of drugs in worms was presented as μg/g. The figure showed the average of three repeated experiments for each method. The details of data were summarized in Table S1 and S2.
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of FUDR standard samples with concentration of 20 and 35 mg/L were 0.92 and 1.02, 0.73 and 0.85, respectively (RSD<1.1%, Table 2).

Drug absorption efficiency of the five drug delivering methods

We tested the concentration of resveratrol and FUDR in worms administrated with drugs by five delivery methods at the time point of 10 min, 30 min, 1 hr, 3 hr, 6 hr, 12 hr, day 1, day 2, day 4, day 7, day 14, and day 20. The concentration of resveratrol and FUDR in worms accumulated steadily as time passing on and reached the highest at day one, then decreased gradually (Figure 4, Tables S1 and S2). The absorption rate of resveratrol and FUDR in worms during the whole treatment process from high to low was NGM dead, liquid growing, spot dead, the NGM live, and LB medium method. Therefore, the best absorption efficiency of worms was the NGM dead method ($P<0.05$, t-test).

To investigate the relationship between absorption efficiency and drug dosage, we determined the concentration of drugs in

Table 3. The absorption efficiency of worms compared between the NGM dead method and the liquid growing method.

Resveratrol concentration	50 μM	100 μM	150 μM
	test resveratrol concentration (μg/g)	test resveratrol concentration (μg/g)	test resveratrol concentration (μg/g)
NGM dead method	175.83±14.75	397.20±20.10	495.86±19.20
Liquid growing method	158.60±20.00	385.16±9.50	492.26±15.25

The day 7 worms treated with resveratrol at three concentrations cultured by using NGM dead method and liquid growing method. The table showed the average of three individual experiments for each concentration in each method.
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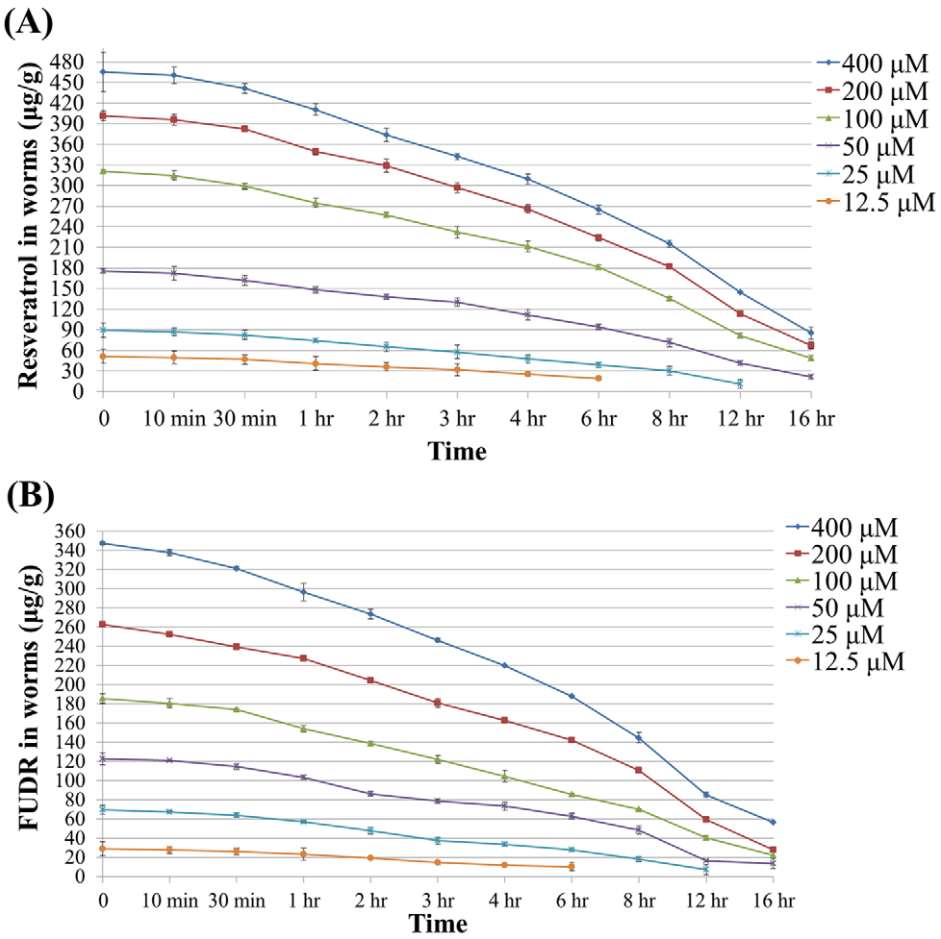

Figure 5. The resveratrol (A) and FUDR (B) catabolism rate inside the worms within 16 hours (μg/g). The worms were cultured by using NGM dead method for 6 hours, then transferred to NGM plates containing no resveratrol or FUDR. The worms were harvested at the 10 min, 30 min, 1 hr, 2 hr, 3 hr, 4 hr, 6 hr, 8 hr, 12 hr and 16 hr after transferring respectively. The details of data were summarized in Tables S3 and S4.
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worms administrated with the series of dosages for seven days. The drug absorption efficiency at dosage of 50 μM, 100 μM, and 150 μM administrated was 175.83±14.75, 397.20±20.10, and 495.86±19.20 with NGM dead method, and 158.60±20.00, 385.16±9.50, and 492.26±15.25 with liquid growing method, respectively (Table 3).

The metabolism of resveratrol and FUDR in *C. elegans*

To measure the metabolism of resveratrol and FUDR in *C. elegans*, the concentration of resveratrol and FUDR within *C. elegans* were determined at multiple time points after treatment of drugs with concentrations varied from 400 μM to 12.5 μM for 6 hrs. As indicated in Figure 5, the slopes between the adjacent time interval represent the metabolism rate. The resveratrol and FUDR share the same metabolite pattern (Figure 5, Tables S3 and S4). The metabolism rate increased gradually as time passed on and reached the highest at the time interval of 8 to 12 hours, then decreased followed by the decrease of drug concentration within worms. Generally, the metabolism rate was dose respondent. The higher concentration of drugs has higher metabolism rate at the same time interval.

Live *E. coli* OP50 lowered the overall drug concentrations in LB medium method and NGM live method

We suspect that live OP50 *E. coli* would metabolize the drug and lower the overall drug concentrations in the media. To test our hypothesis, we determined the concentrations of resveratrol and FUDR in the medium of LB medium method, NGM live method, and NGM dead method at 0.5 hr, 1 hr, 3 hr, 6 hr and 12 hr after applying live or dead bacteria. The concentration of drug in the medium with dead bacteria degraded slowly over time (Figure 6, Table S5). However, the concentration of drugs in medium with live bacteria incubated for 12 hours were only half of the original concentration. The concentration of drugs in medium with live bacteria decreased much faster than with dead bacteria (Figure 6, Table S5). Above results indicated that the live bacteria could metabolize the drug and lower the drug absorption rate of worms.

Discussion

As *C. elegans* was more and more widely used in various fields as research and drug screening model, it's important to understand the pharmacological aspect of worms. Here, we established the HPLC method to evaluate the drug concentration in worms administrated by five delivering methods [36–42]. Our results showed that about 20,000 worms treated with 100 μM of

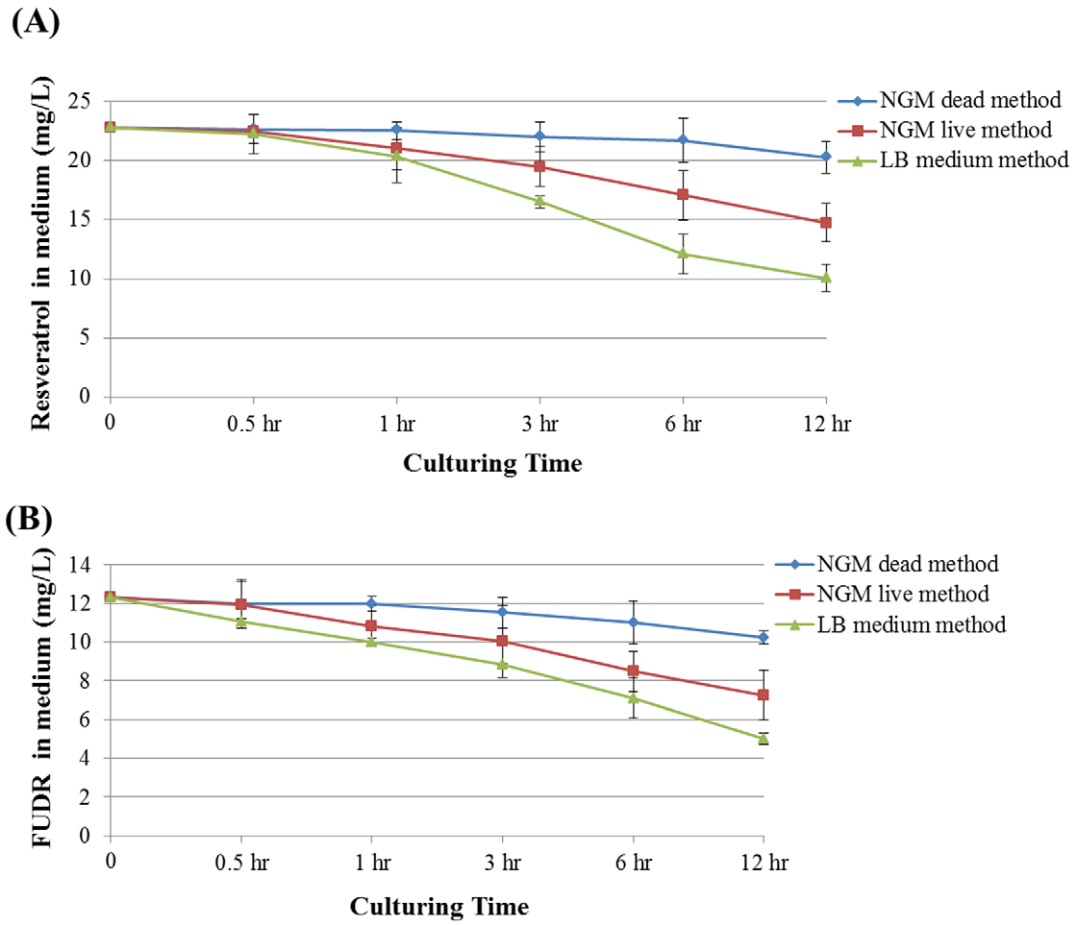

Figure 6. Resveratrol (A) and FUDR (B) concentration in the medium of NGM dead method, NGM live method and LB medium method within 12 hours (mg/L). The initial concentration of resveratrol or FUDR in the medium was 100 μM or 50 μM (0 hr). The medium was crushed and transferred into 15 mL tube at the 0.5 hr, 1 hr, 3 hr, 6 hr and 12 hr after preparing respectively. The same volume of methanol was added into the tube. The mixture was sonicated for 1 hour, then the liquid was collected for HPLC analysis. The details of data were summarized in Table S5.
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resveratrol or 50 μM of FUDR could absorb the fraction well beyond the detecting limit of HPLC. The precision (RSD<3.8% for resveratrol and 1.1% for FUDR, Table 2) and repeatability (RSD = 3.8% for resveratrol and 2.5% for FUDR, Table 1) indicate that the HPLC method was sensitive and reliable in detecting the drug absorption efficiency of worms.

The NGM live method [22,43,44] and the NGM dead method [30,45] were widely used by many laboratories in their researches. The drug absorption efficiency of NGM dead was better than NGM live, indicating the live bacteria might have digested part of the drugs. In consistent with this observation, the drug concentration in medium with live bacteria decreased much faster than with dead bacteria. The bacteria live methods performs poorer than bacteria dead methods, indicating worms absorb the drugs mainly from the solution or surface of NGM directly, rather than from the bacteria. For the spot dead method, although the bacteria was dead on the surface of the NGM plates, when the resveratrol or FUDR solution was spotted onto the surface, the solution could immersed into the NGM agar (data not shown), which reduced the availability. This accounted for the drug absorption lower than NGM dead, but higher than NGM live method. Still, spot dead method could reach considerable concentration with less total compounds compared with other drug delivering methods. Both the water-soluble FUDR and resveratrol with low aqueous solubility accumulated within worms steadily and reach the highest concentration within 12–24 hours, then decreased slowly, indicating they share the same absorption pattern in *C. elegans* regardless of delivery methods (Figure 4).

The resveratrol and FUDR share the same metabolite pattern (Figure 5, Tables S3 and S4). The metabolism rate increased gradually as time passed on and reached the highest at the time interval of 8 to 12 hours, then decreased slowly. The decrease of metabolism rate might be caused by the decrease of drug concentration within worms (Figure 5). The higher concentration of drugs has higher metabolism rate at the same time interval. Metabolism rate was dosage responsive and time dependent, indicating the metabolism rate was drug inducible. It would be interesting to investigate the mechanism of the enzyme system responsible for the xenobiotic metabolism.

Although drug administration was common in studies with worms, the concentration of drugs within the worms was unknown, impeding the comparison of the researches between worm and other research models, such as cell culture and animal models. Our results showed that the concentration of resveratrol in worms was from about 300 to 600 mg/kg worms while treated with 100 μM resveratrol, that was comparable with studies

conducted on mice, ranged from 4.9 to 400 mg/kg body weight per day resveratrol [46–50].

Conclusion

The resistance of *C. elegans* to pharmacological perturbation appeal an effective approach to make *C. elegans* as a screening tool for novel small bioactive molecules [29]. The HPLC method was effective in determine the concentration of drugs in worms. Resveratrol and FUDR administrated with NGM dead method and liquid growing method achieves the best absorption efficiency in worms. Nevertheless, spot dead method could reach considerable absorption efficiency with much less total amount of drugs. The live bacteria could digest part of the drugs, leading to less drug absorption efficiency. The resveratrol with low aqueous solubility and water soluble FUDR share the same bell-shape accumulation pattern within worms and the same metabolism pattern. The drug concentration within worms was comparable with that in mice, providing a bridge for dose translation from worms to mammals. Our results might help investigators to choose appropriate drug delivering method to worms according to pharmacological dynamics as well as to understand some inconsistent results between studies with different drug delivering methods.

Supporting Information

Table S1 The drug absorption efficiency of worms administrated with 100 μM resveratrol by five delivering methods (μg/g). The concentration of resveratrol in worms was presented as μg/g. The table showed the average of three repeated experiments for each method. - represents the contents of resveratrol were under the limit of detection or not determined (Liquid growing method).$^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with NGM dead method (t-test).
(DOCX)

Table S2 The drug absorption efficiency of worms administrated with 50 μM FUDR by five delivering methods (μg/g). The concentration of FUDR in worms was presented as μg/g. The table showed the average of three repeated experiments for each method. - represents the contents of resveratrol were under the limit of detection or not determined (Liquid growing method).$^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with NGM dead method (t-test).
(DOCX)

Table S3 The resveratrol catabolism rate inside the worms within 16 hours (μg/g). The worms were cultured by using NGM dead method for 6 hours, and then transferred to NGM plates containing no resveratrol. The worms were harvested at the 10 min, 30 min, 1 hr, 2 hr, 3 hr, 4 hr, 6 hr, 8 hr, 12 hr and 16 hr after transferring respectively. - represents the contents of resveratrol were under the limit of detection or not determined.
(DOCX)

Table S4 The FUDR catabolism rate inside the worms within 16 hours (μg/g). The worms were cultured by using NGM dead method for 6 hours, and then transferred to NGM plates containing no FUDR. The worms were harvested at the 10 min, 30 min, 1 hr, 2 hr, 3 hr, 4 hr, 6 hr, 8 hr, 12 hr and 16 hr after transferring respectively. - represents the contents of FUDR were under the limit of detection or not determined.
(DOCX)

Table S5 The drug concentration in the medium of NGM dead method, NGM live method and LB medium method within 12 hours (mg/L). The initial concentration of resveratrol or FUDR in the medium was 100 μM or 50 μM (0 hr). The medium was crushed and transferred into 15 mL tube at the 0.5 hr, 1 hr, 3 hr, 6 hr and 12 hr after preparing, respectively. The same volume of methanol was added into the tube. The mixture was sonicated for 1 hour, and then the liquid was collected for HPLC analysis.
(DOCX)

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Author Contributions

Conceived and designed the experiments: SQZ GSW HRL. Performed the experiments: SQZ AJD. Analyzed the data: SQZ HRL. Contributed reagents/materials/analysis tools: AJD GPL. Wrote the paper: SQZ GSW HRL.

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