INTRODUCTION

1,3,7-trimethylxanthine, commonly known as caffeine, is one of the most popular drugs in the world. It comes from coffee beans, tea leaves, kola nuts, cacao pods, and so on (Gilbert et al., 1976). People ingest caffeine in one form or another every day, and it has become very popular in our daily lives. Recently, caffeine consumption has been increasing even among teenagers. Caffeine is known to enhance physiological functions in humans including sharpening our mind, improving athletic performance, and many other health improvements (Reviewed in Weinberg and Bealer, 2002). However, caffeine is an addictive drug. Although its effects are milder than those of other addictive drugs such as amphetamines, cocaine, and heroin, over-lapping channels mediate the signal (Nehlig, 1999), which means that caffeine can also cause medical concerns.

Research revealed that caffeine consumption both before and during pregnancy increased the risk of spontaneous abortion (Cnattingius et al., 2000) and births classified as 'small for gestational age' (Hoyt et al., 2014). Maternal caffeine intake of over 300 mg/day doubled the risk of miscarriage compared with that of 151 mg/day (Giannelli et al., 2003). Such results suggest that intake of high doses of caffeine during pregnancy is a risk factor for fetal growth retardation. Therefore, understanding the basis of developmental defects caused by caffeine intake is an emerging issue, considering the fact that caffeine is now intertwined in the daily life of most people. Caffeine is metabolized in the liver by cytochrome P450 (CYP450) and excreted as urine in mammals (Kot and Daniel, 2008). The half-life of caffeine ranges from 3 to 7 h in adult plasma. Interestingly, the half-life is prolonged during pregnancy, in which it can be more than 10 h (Knutti et al., 1981). In addition, neonates have a greatly reduced capacity to metabolize caffeine, and it is excreted into the urine in largely unmetabolized form until hepatic metabolism becomes activated (Brent et al., 2011). Mammals contain 51 CYP family members, which are grouped into 10 subfamilies (Nelson et al., 2004). Among them, CYP1 to CYP4 are the drug-metabolizing families, and caffeine has been shown to be metabolized by CYP1A2 in mammals (Kot and Daniel, 2008). CYP450s render drugs more water-soluble so they can be excreted more easily in the urine or bile, and thereby, the drugs are processed by oxidative metabolism. Thus, CYP450s are able to promptly reduce the effects of drugs. The reduced capacity of neonates to metabolize caffeine therefore suggests that insufficient neonatal cyp gene expression may be the cause of developmental defects after caffeine intake during pregnancy. Besides detoxification effect of cyp-35A family genes, it was reported that CYPs could convert non-toxic chemicals to
Caffeine Metabolism in *C. elegans*

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MATERIALS AND METHODS

*C. elegans* strain and maintenance

Methods for the maintenance and handling of *C. elegans* were as previously described (Brenner, 1974). Strain N2 was used as the wild type for all analyses. Worms were maintained at either 15°C or 20°C on Nematode Growth Medium (NGM) agar plates containing *Escherichia coli* strain OP50 supplemented with 5 μM of cholesterol. For caffeine treatment, appropriate amounts of caffeine (Sigma, USA) were included in the NGM agar plates before autoclaving to achieve final concentrations from 10 to 30 mM.

Analysis of brood size, embryonic lethality, and percent larval development

L4-stage N2 hermaphrodites were individually cloned onto either caffeine-containing or control NGM agar plates and grown at 20°C. They were transferred to new plates in 24 h intervals for three days to allow embryo production. Laid embryos were considered dead if they did not hatch after 48 h at 20°C. Brood size was calculated as the total number of non-hatched and hatched embryos produced by a single mother hermaphrodite. Embryonic lethality was calculated as the percentage of non-hatched embryos among the total number of embryos produced. Percent larval development was calculated as the percentage of larvae that reached the adult stage among the total number of hatched embryos, as previously described (Kawasaki et al., 2013). The broods of 10 mother hermaphrodites were examined to calculate the above values at each concentration of caffeine treatment.

Microarray analysis

Synchronized L1-stage worms were incubated on the plate containing 0 or 30 mM caffeine in the absence of food, *Escherichia coli* strain OP50, at 20°C for 24 h, and then total RNA was prepared from each worm sample. Poly-A RNA purification, reverse transcription, and labeling were performed as previously described (Reinke et al., 2000; 2004). Microarrays were constructed in Dr. Reinke’s laboratory (Yale University), and the procedures and data analysis were performed as previously described (Reinke et al., 2000; 2004). Three independent microarray analyses were performed. The cyp genes that displayed more than two-fold differences with p-value < 0.05 were selected.

RT-PCR

Synchronized L1-stage worms were grown on NGM agar plates with or without caffeine for 3 days. Total RNA was extracted from the worms with Trizol reagent (Sigma, USA) and purified, after which the first strand cDNA was synthesized by M-MLV reverse transcriptase (Gibco BRL, USA) using oligo-dT primer (Promega, USA). vit-6 cDNA product was then PCR-amplified using vit-6 forward primer, 5’-CAATCAATGGTGAACGCGA-CCACG-3’ and vit-6 reverse primer, 5’-CTCTTCAATTTGTGGTGGT-3’ (NM_088985). PCR was performed in 25 μl reaction volumes with the following parameters: 94°C for 5 min, followed by 25 cycles of 94°C for 1 min, 55°C for 45 s, and 72°C for 1 min, with final extension at 72°C for 10 min. act-3 cDNA product was PCR-amplified as an internal control using act-3 forward primer, 5’-GAGGCCCAATCCAAAGAAG-3’ and act-3 reverse primer, 5’-TGTTGGAGAGTGAGAGG-3’ (NM_073416).

Real-time RT-PCR (qRT-PCR) analysis

Synchronized L1-stage or L4-stage worms were grown on NGM agar plates with or without caffeine for 24 h. Total RNA was extracted from the worms with Trizol reagent (Sigma, USA), purified, and reverse transcribed with M-MLV reverse transcriptase (Gibco BRL, USA) using oligo-dT primer (Promega, USA) to synthesize the first strand cDNA. Respective cDNA products were PCR-amplified using the following primers: cyp-35A2 F primer, 5’-TGAGGTGGTTTGGTTGTTTCAACGAGGATAGA-3’ and cyp-35A2 R primer, 5’-CTCTTCAATTTGTGGTGGT-3’; cyp-35A3 cDNA; cyp-35A4 F primer, 5’-CAATCAATGGTGAACGCGA-CCACG-3’ and cyp-35A4 R primer, 5’-CTCTTCAATTTGTGGTGGT-3’. All PCR was performed in 25 μl reaction volumes using Power SYBR Green PCR Master Mix (Applied Biosystems, USA). The mRNA levels of each gene were averaged from triplicate measurements and normalized against that of act-1. Two or three independent experiments were performed.

RNA interference (RNAi) analysis

RNAi analysis was performed using the “RNAi-by-soaking” method, as described previously (Maeda et al., 2001). N2 worms were synchronized at the L1 larval stage in the absence of food, after which they were soaked for 24 h in each of the double-strand RNA solutions transcribed in vitro from respective cDNA templates, which were PCR-amplified from the corresponding sequences. The double-strand RNA solutions were transcribed in vitro from respective cDNA templates, which were PCR-amplified from the corresponding sequences.
responding L4440 vector feeding RNAi clones using T7 primer, 5'-GTAATACGACTCTATAGGGC-3', and L4440 T7 primer, 5'-ATTAAATACGACTCAGTATAGGGA-3'. Worms were then retrieved and transferred to either caffeine-containing or control NGM plates, where they resumed development at 20°C. After transfer, larval development was observed in 24 h intervals for a few days until most of the control worms reached the adult stage. We distinguished the larval stages as follows: L1, the smallest larvae less than 0.3 mm; L2, larvae larger than L1 (body length, 0.3-0.4 mm) but have no characteristics of L3; L3, larvae containing a white spot in the vulva area (body length, 0.4-0.6 mm); L4, larvae containing a characteristic half-moon-like shape in the vulva area (body length, 0.6-0.8 mm); young adults (YA), worms with an opened vulva with no eggs in the uterus; adults (A), worms with an opened vulva with eggs in the uterus.

**Statistical analysis**

All experiments were repeated more than three times for statistical evaluation of data. P values were calculated by Student's t-test.

**RESULTS**

**Caffeine affected multiple developmental processes of C. elegans**

As a preliminary study, we examined growth rates of worms after treatment of 0, 5, 10, 15, 20, and 30 mM caffeine. We found that effects of caffeine were dosage-dependent, and determined three different concentrations of caffeine, 10, 20, and 30 mM as mild, moderate, and critical doses, respectively. To examine the effects of caffeine on the developmental process of C. elegans, the L4-larval stage wild-type N2 hermaphrodites were treated with 10, 20, and 30 mM of caffeine, after which the brood size of the progeny, embryonic lethality, and percent larval development were scored. As a result, brood size and percent larval development were decreased while embryonic lethality was increased, indicating that caffeine indeed inhibited the developmental processes of C. elegans at both embryonic and post-embryonic stages (Table 1). Treatment with 30 mM caffeine caused more severe defects than 10 mM or 20 mM. At 30 mM caffeine, brood size was decreased to 31.6% of the non-treated control, and 50.2 ± 13.7% of the progeny were arrested as embryos. After hatching, only 22.7 ± 5.7% of the larvae could develop into adults. These results indicate that caffeine affected multiple developmental processes of C. elegans in a dosage-dependent fashion.

**High dose of caffeine inhibited the early-stage larval development**

The effect of caffeine on the larval development of C. elegans was further examined by treating the larvae with 0, 10, or 30 mM of caffeine starting at the L1 stage (Fig. 1A). The development of about 900 L1 worms were observed at each caffeine concentration under dissecting microscope in 24 h intervals for 96 h at 20°C, and the developmental stage of each individual was determined at each time point. The results were displayed as percent distribution of worms in each developmental stage in the population at each caffeine concentration and each time point, either as line graphs or as a table (Fig. 2 and Supplementary Table S2). This confirmed the dose-dependent effect of caffeine. That is, although 98.9 ± 0.4% of the larvae eventually developed into adults after 96 h at 10 mM caffeine, 86.1 ± 3.4% of the larvae remained in the L1 stage after 96 h at 30 mM caffeine (Figs. 1A and 2; Supplementary Table S2). The effect of caffeine on larval development was irreversible when worms were treated with 30 mM caffeine for over 2 days (data not shown). These results indicate that high doses of caffeine treatment are fatal to the development of early-stage larvae.

To confirm that the larvae arrested with the 30 mM caffeine treatment indeed did not reach an adult stage, the expression of vit-6, a vitellogenin gene, was examined, as it is known that vit-6 is expressed exclusively during the adult stage (Spieth and Blumenthal, 1985). Expression of vit-6 mRNA was examined by RT-PCR with total RNA isolated from the worms grown for 72 h after hatching at 20°C, with or without 30 mM caffeine (Fig. 1B). A PCR-amplified vit-6 cDNA band was detected only from the worm preparation grown without caffeine, not from that grown with 30 mM caffeine, confirming that the 30 mM caffeine was not present in the progeny of the treated worms.

**Table 1. Effects of caffeine on C. elegans development are dosage-dependent**

<table>
<thead>
<tr>
<th>Caffeine treatment</th>
<th>Brood size</th>
<th>Embryonic lethality (%)</th>
<th>Percent larval development (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mM</td>
<td>250 ± 28.1 (100%)</td>
<td>2.54 ± 3.00</td>
<td>95.2 ± 5.63</td>
</tr>
<tr>
<td>10 mM</td>
<td>185 ± 22.2 (74.0%)</td>
<td>1.64 ± 9.83</td>
<td>76.9 ± 15.1</td>
</tr>
<tr>
<td>20 mM</td>
<td>167 ± 57.5 (66.8%)</td>
<td>27.2 ± 11.2</td>
<td>66.0 ± 11.3</td>
</tr>
<tr>
<td>30 mM</td>
<td>79.1 ± 27.3 (31.6%)</td>
<td>50.2 ± 13.7</td>
<td>22.7 ± 5.78</td>
</tr>
</tbody>
</table>

10 synchronized L4-stage hermaphrodites were treated with different concentrations of caffeine after which brood size, F1 embryonic lethality, and F1 percent larval development were measured, as described in “Materials and Methods”.

**Fig. 1. C. elegans larval development in the presence or absence of caffeine. (A) Wild-type N2 worms synchronized at the L1 larval stage were cultured on NGM agar plates containing 0, 10, or 30 mM caffeine, and their development was observed in 24 h intervals for 96 h at 20°C. Scale bar, 40 µm. (B) Absence of adult-stage-specific vit-6 gene expression in 30 mM caffeine-treated larvae. N2 worms synchronized at the L1 larval stage were cultured on NGM agar plates with or without 30 mM caffeine for 72 h at 20°C. Total RNA was extracted from the worms and expression of vit-6 was examined by RT-PCR, with act-3 as an internal control. NTC, no template controls.”
treatment arrested development at the larval stage.

L1-stage larvae were more susceptible to caffeine than later-stage larvae

Next, the effect of caffeine during different stages of larval development was examined by treating the worms with 30 mM caffeine starting from the L1, L2, L3, L4, or young adult stage (Fig. 3). Worms treated with 30 mM caffeine since the L1 stage were found to be mostly arrested at the L1 larval stage, as observed above. In contrast, the majority of the worms treated with the same concentration of caffeine after the L2 stage eventually developed into the adult stage, although their growth rate was significantly slower than the non-caffeine-treated controls and some worms also arrested as larvae (Fig. 3). These results indicate that although L1-stage larvae were the most susceptible to caffeine treatment, later-stage larvae were also partially susceptible.

To clarify that the larval developmental arrest after caffeine treatment was not caused by starvation due to blocking of pumping, we measured the pharyngeal pumping rate. Both L1-stage worms and L4-stage worms cultured in the presence of 30 mM caffeine for 24 h still maintained pumping although the rate was reduced to 69% and 64%, respectively, of non-caffeine-treated controls (Supplementary Fig. S1). These results clearly indicate that the larval arrest after 30 mM caffeine treatment was not caused by starvation due to blocking of pumping although this reduction of pumping rate might have affected the slow growth rate of worms treated with caffeine since the L2, L3, and L4 stages (Fig. 3).

cyp-35A family genes were highly induced in the early-stage larvae upon caffeine treatment

To study the effects of caffeine treatment on global gene expression changes in C. elegans, microarray analysis was performed using the RNAs isolated from L1-stage larvae grown with or without caffeine. Among 65 cyp (cytochrome P450) genes tested, 29 were up- or down-regulated with \( p < 0.05 \) (Supplementary Table S1), and 24 were up-regulated more than 2-fold (Table 2). Among them, expression of cyp-35A family genes was most prominently induced (more than 28.5-fold). Therefore, further analysis was carried out on the effect of caffeine treatment on the gene expression of cyp-35A family genes, C49G7.8 (cyp-35A4), K07C6.5 (cyp-35A5), K09D9.2 (cyp-35A3), and C03G6.15 (cyp-35A2) by qRT-PCR analysis (Fig. 4, Table 3). It was confirmed that although cyp-35A family genes were not highly expressed in the worms grown in normal media without caffeine, the expression levels of the cyp-35A family genes were highly induced in the worms grown in the presence of 30 mM caffeine, as shown by the microarray analysis (Fig. 4, Table 3). Notably, the levels of induction were more prominent in the L1 stage than in the L4 stage. From 217-fold (cyp-35A2) to 4,042-fold (cyp-35A5) induction was observed compared with the non-caffeine-treated controls in the L1 stage.
Table 3. Relative expression levels of cyp-35A genes in caffeine-treated L1 and L4 worms

<table>
<thead>
<tr>
<th>Caffeine (mM)</th>
<th>Treated stage</th>
<th>cyp-35A2</th>
<th>cyp-35A3</th>
<th>cyp-35A4</th>
<th>cyp-35A5</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>L1</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>30</td>
<td>L1</td>
<td>217 ± 0.6*</td>
<td>2168.0 ± 18.8*</td>
<td>2180.9 ± 4.9*</td>
<td>4042.2 ± 45.4*</td>
</tr>
<tr>
<td>0</td>
<td>L4</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>30</td>
<td>L4</td>
<td>8.4 ± 0.3*</td>
<td>168.8 ± 4.2*</td>
<td>1029.5 ± 3.7*</td>
<td>97.9 ± 1.7*</td>
</tr>
</tbody>
</table>

Real time RT-PCR was performed with total RNA extracted from synchronized L1-stage or L4-stage worms grown with 30 mM caffeine or without caffeine for 24 h as described in "Materials and Methods". *p < 0.05.

Fig. 3. N2 worms synchronized at each post-embryonic stage (n = 10) were cultured on NGM agar plates with or without 30 mM caffeine, and the developmental stage shown by the majority of worms in each population was determined every 24 h for 120 h at 20°C, then plotted as a line graph. L1 to L4, L1 to L4 larval stages; YA, young adult stage in which egg production had not yet started; Ad, adult stage in which egg production had already commenced; F1, F1 progeny were generated. Worms treated with 30 mM caffeine since the L1 stage were developmentally arrested, whereas worms treated with the same concentration of caffeine after the L2 stage eventually reached the adult stage.
larval development. Although the effect was not as prominent as that observed in 30 mM caffeine, cyp-35A RNAI-treated larvae also showed partial recovery from growth retardation caused by treatment with 10 mM caffeine (Supplementary Fig. S3). It was also confirmed that cyp-35A RNAI treatment did not affect larval development under normal culturing conditions without caffeine (Supplementary Fig. S2). These results suggest that the high-level induction of cyp-35A family genes in response to caffeine treatment is fatal to the early larval development of C. elegans.

We also examined RNAI of cyp-14A family genes, but caffeine effects on the larval development were not suppressed unlike RNAI of cyp-35A family genes (Supplementary Fig. S4). These results indicate that cyp-35A family genes have a specific function on caffeine response.

**DISCUSSION**

In humans, caffeine is mainly metabolized by CYP1A2 in the liver (Kot and Daniel, 2008), while neonates have a significantly reduced capacity for caffeine metabolism (Brent et al., 2011). This raises the question of whether insufficient expression of cyp genes is the major cause of developmental defects observed after caffeine intake during pregnancy, and suggests a possible correlation between developmental stage and capacity to induce cyp genes.

In this study, the effects of caffeine on developmental process and the expression of cyp genes were examined in C. elegans. Similar to human fetal development (Giannelli et al., 2003), caffeine was found to have a dose-dependent effect on the development of C. elegans, and higher concentrations caused severe developmental defects. It was also found that the first-stage larvae (L1) were more susceptible to caffeine than those in the later stages. In our study, we treated worms with 30 mM caffeine to investigate the effects on larval development. In the previous study with lower concentrations of caffeine, positive effects such as extension of life span and protection against neurodegenerative diseases were reported (Dorsal et al., 2010; Sutphin et al., 2012). However, after 30 mM of caffeine treatment, majority of L1 larvae were arrested at the L1 stage without further development. These results suggest that L1-stage larvae might have not acquired an efficient caffeine metabolic system like young human infants. Approximately 80 different cytochrome P450 genes have been reported in the C. elegans KEGG genes database. They were grouped into three subfamilies based on similarity to mammalian orthologs CYP2, CYP3, and CYP4, which are drug-metabolizing enzymes in mammals (Gotoh, 1998). It was reported that C. elegans cyp genes were highly inducible by treatment with xenobiotics (Menzel et al., 2001; 2005). This implies that the CYP proteins in C. elegans also function for the detoxification of xenobiotic chemicals as mammalian CYP proteins do. It was found herein that genes of the cyp-35A family were highly induced by caffeine treatment. However, the observed levels of induction were much higher in the L1 stage than in the L4 stage (Fig. 4). These results suggest that the significant developmental defects in the L1 stage after caffeine treatment were not due to insufficient metabolic response to caffeine treatment. Rather, high-level induction of genes in the cyp-35A family during the L1 stage could be the cause of early larval stage developmental defects. This hypothesis was supported by the finding that the developmental defects caused by caffeine treatment were partially rescued after RNAI of cyp-35A family genes (Fig. 5). A similar finding was reported by another group (Menzel et al., 2005). They observed that cyp-35A genes were highly induced after treatment with xenobiotics, and furthermore, brood size was more decreased in wild-type N2 treated with xenobiotics than in worms in which all of the cyp-35A gene activities were depleted, either by mutation or by RNAI. These findings suggest two possibilities. First, high-level induction of cyp-35A genes inhibits the development of C. elegans. Expression of cyp-35A genes was also induced by ethanol treatment, whereby stress genes were also induced (Kwon et al., 2004; Peltonen et al., 2012), which supports the view that high-level induction of cyp-35A genes can cause toxic effects. It was also reported that cyp-35A family genes regulate fatty acid metabolism at the transcriptional level (Aarnio et al., 2011). The induction of cyp-35A genes may lead to up-regulation of fatty acid synthesis genes, thus resulting in abnormal fat storage, which subsequently affects the developmental processes. As suggested by the above cases, high-level induction of cyp-35A genes can interfere with the worm development. Second, induction of cyp-35A genes may not be a detoxification step for caffeine, rather, it may be involved in the accumulation of secondary metabolites from caffeine, which are toxic to the development of C. elegans especially when high-dose of caffeine was treated. This possibility needs to be examined further in future studies. The findings that mammalian cytochrome P450 also produces toxic metabolites supports this view. For example, mammalian cytochrome P450 can convert some polycyclic aromatic hydrocarbons and polychlorinated biphenyls to ultimate carcinogenic metabolites (Shou et al., 1996).

We consider that early larval developmental arrest observed after 30 mM of caffeine treatment was not caused by starvation due to blocking of pumping because pumping was slowed but...
not completely blocked in this condition, although slow pumping rate might have affected growth rate of worms.

Two caffeine-resistant mutants were reported previously: osm-3(hf3), which was previously called caf-1(hf3), and che-3(hif5), which was previously called caf-2(hif5). Both OSM-3 and CHE-3 are motor proteins in neurons. OSM-3 is a kinesin-like protein with function in axonal transport in C. elegans (Shakir et al., 1993). CHE-3 is a cytosolic dynein heavy chain functioning in ciliated sensory neurons (Wicks et al., 2000). The effects of caffeine were suppressed in these mutants (data not shown), suggesting that caffeine may not be sensed by sensory neurons in osm-3 or che-3 mutants.

In summary, caffeine can interfere with C. elegans early larval development. Although the cyp genes for caffeine metabolism are conserved between C. elegans and mammals, their functions during development may be different. High-level induction of cyp-35A genes in response to caffeine treatment during the early larval stages appears to be harmful to the development of C. elegans. Mammalian cyto genes are involved in the detoxification of caffeine, but they are expressed at very low levels in neonates; therefore, neonates can be more sensitive to caffeine than older infants. There may be a biological reason why cyp genes are expressed at very low levels in human neonates.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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