Exposure to tributyltin (TBT) via food during developmental stages after weaning, or the exposure via the placenta and their dams' milk, inhibits body weight gain and behaviors in rats

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Objectives: Neurotoxicity is one of the major toxic effects of tributyltin (TBT). In our previous study, the mean values of total locomotor distance and instance of wall rearing among others in open field tests of F1 rats exposed to TBT via the placenta and their dams' milk were significantly lower than those in the control. The problem remained to determine the neurotoxic effects of exposure to TBT during the developmental stages after weaning. We, therefore, evaluated the effects of TBT on behavior by continuing exposure from fetus to 9 weeks of age for the comparison with the results among rats exposed to TBT via the placenta and their dams' milk only and rats exposed by their food only.

Methods: Male F1 rats were exposed to TBT in utero and postnatally via their dams' milk by the dams' chow containing TBT at 0 and 125 ppm. After weaning, they were either fed chow containing TBT at 0 or 125 ppm until 9 weeks of age. The pups at 9 weeks were composed of the control (control-control: CC), the group exposed by their food only (control-TBT: CT), that group exposed by the placenta and their dams' milk (TBT-control: TC), and the continuous exposure group (TBT-TBT: TT). Open field tests and prepulse inhibition (PPI) tests were performed at 9 weeks of age in F1 rats.

Results: In the open field tests, the mean values of total locomotor distance in the CT and TT groups were significantly lower than that in the CC group. For the TC group, the mean value of locomotor distance between 15 to 20 minutes was significantly lower than that of the control. The mean values of instances of wall rearing in the CT, TC, and TT groups were significantly lower than that in the CC group. The mean values of center rearing in the CT and TT groups were significantly lower than that in the CC group. The mean values of face washing in the CT and TT groups were significantly lower than that in the CC group. There were no significant differences in the PPI tests among the groups.

Conclusion: These results suggest that TBT exposure during the developmental stages after weaning, as well as the exposure via the placenta and their dams' milk, inhibits the body weight gain and behavior of F1 rats.

Key words: tributyltin, neurotoxicity, developmental stages, anti-fouling agents, rats

Introduction

Tributyltin (TBT) compounds have been widely used as biocides and anti-fouling agents.1-3 The restriction on the application of TBT as vessel anti-fouling agents have been introduced in Japan in 1990 as well as in developed countries.4 However, TBT pollution in fish and shellfish has been reported worldwide.5,6,7 Humans are exposed to TBT via eating TBT-contaminated fish and shellfish. Even though the fish is cooked well, the organ tin compounds, including TBT, are not destroyed.8 Thus, human beings are very likely exposed to these toxic compounds. The tentative ADI (acceptable daily intake) for TBT oxide compiled by the Ministry of Health and Welfare in Japan is 1.6 μg/kg bw/day as TBT oxide.9

Neurotoxicity, as well as immunotoxicity, is one of
the major toxic effects of TBT.\textsuperscript{1,2} Especially, in mice and rats, it is suggested that the neurotoxic effects from exposure to TBT via the placenta and their dams' milk are stronger than those in adult mice from exposure via their food as well as immunotoxicity.\textsuperscript{10-13} Among these studies, the study by Asakawa et al. focused on the behavioral changes of F1 rats exposed to TBT.\textsuperscript{12} In the study, the female F1 rats were exposed to TBT chloride via the placenta and the dams' milk. The exposure from the weaning to 9 weeks was stopped to distinguish the effects by the exposure via the placenta and their dams' milk and by the exposure via their foods. After the cessation, the F1 rats were randomly assigned to the groups which were not exposed to TBT or the TBT groups. The TBT groups were exposed to TBT again via their food from 9 to 15 weeks. The behavioral tests, an open field test and the prepulse inhibition (PPI) test, were used to evaluate neurotoxicity in the animals. The inhibition of F1 rats' development and locomotor activity in the open field test, induced by exposure to TBT via the placenta and dams' milk and/or food, was suggested at 15 weeks of age.

There are several important problems remaining. It is of interest whether or not the exposure to TBT at the early stages from weaning induces neurotoxicity.

Although the importance of the exposure in utero and via dams’ milk was suggested in the previous study, the data for toxicity of TBT by continuing exposure to developmental rats have not yet been evaluated.\textsuperscript{12} In addition, Aou et al. pointed out that TBT neurotoxicity may be different between the sexes.\textsuperscript{14} It was also of interest whether or not TBT neurotoxicity was induced in male F1 rats by the exposure via the placenta and their dams' milk.

We, therefore, evaluated the effects of TBT on behavior by continuing exposure from fetus to 9 weeks of age for the comparison with the results from rats exposed to TBT via the placenta and their dams' milk only and rats exposed by their food only, and rats exposed via both ways. The objective of this study was to elucidate the neurotoxic effects of the male F1 rats' exposure to TBT via the placenta, their dams' milk, and/or oral exposure via food after weaning without the cessation.

**Materials and Methods**

**Experimental animals and the treatment**

Wistar rats, 9 weeks of age and pregnant for the first time (Oriental BioService, Tokyo), were administered 0 and 125 ppm of TBT chloride in their food (n = 5 for the

![Figure 1](image.png)

**Figure 1.** The protocol for the TBT exposure for the male F1 rats

For TBT exposure via placenta and their dams' milk, the pregnant rats were exposed to TBT chloride via food which contained 125 ppm of TBT chloride. Their offspring were exposed to TBT chloride via the placenta and their dams' milk.

For TBT exposure by oral exposure via food, the male F1 rats were exposed to TBT chloride via food which contained 125 ppm of TBT chloride (n = 10 per group).

CC, control-control (No exposure)

CT, control-TBT (Exposure to TBT by oral exposure via food at 3-9 weeks of age)

TC, TBT-control (Exposure to TBT via the placenta and their dams' milk)

TT, TBT-TBT (Exposure to TBT via the placenta, their dams' milk and oral exposure via food at 3-9 weeks of age.)
control, n = 7 for TBT-exposed groups) in polycarbonate shoebox-style cages at 22°C and 45% humidity, with a 14/10-hour light/dark cycle. Rodent chow containing TBT was prepared by Oriental BioService. The rats were given free access to food and tap water. The body weight and the intake of food and water were recorded daily up to the time of delivery.

The F1 rats were exposed to TBT in utero and postnatally via their dams' milk. After weaning, male F1 rats were randomly assigned either chow containing TBT at 0 or 125 ppm until 9 weeks of age. They were divided into 4 groups: the control (control-control, CC); the group exposed to TBT via their food (control-TBT, CT); the group exposed to TBT via the placenta and their dams' milk (TBT-control, TC); and the continuous exposure group (TBT-TBT, TT) (n = 10/group). Figure 1 shows the protocol for the treatments in the present study.

The F1 rats were also given free access to food and water after weaning. From 3 to 9 weeks of age, the body weight and the intake of food and water were recorded daily. At 9 weeks of age, behavioral tests (open field test and PPI test) were performed. Care and treatment of rats were in accordance with the guidelines established by the Animal Experimentation and Ethics Committee of Kitasato University School of Medicine and were approved by the committee.

Open field test
At 9 weeks of age, an open field test was performed using the method described in previous studies.12,15,16 Every rat was placed in the center of a white square box (width, 1.0 m; height, 0.4 m) one at a time. The locomotor behavior was recorded with a video camera for 30 minutes. The record was analyzed using an Image Open Field 2.15r (Obara Medical, Tokyo). Total locomotor distance and locomotor distance every 5 minutes were calculated. During a 30-minute observation period, the following behaviors were recorded: the time of the first grooming, the number of times of wall rearing (WR), center rearing (CR), face washing (FW), body washing (BW), defecation, or urination.

PPI test
In the morning after the open field test, the PPI test was performed using the method described in previous studies.12,15,16 A Startle Response System SR-LAB ABS System (San Diego Instruments, San Diego, CA, USA) was used, which was composed of a startle chamber equipped with an electric sensor and a speaker mounted 24 cm above the floor to create an acoustic noise burst. Each rat was placed in a cylindrical holder in the chamber and allowed to acclimate for 5 minutes before the test session. In the PPI test session, there were three types of prepulses: a burst of 70 dB (PP70 dB), that of 75 dB (PP75 dB), and that of 80 dB (PP80 dB). Four types of acoustic stimulation were given to each rat in a pseudo-random order: a startle pulse of a burst of 120 dB without prepulse (P alone), combined trials of PP 70 dB followed by a pulse of 120 dB (PP70&P), PP 75 dB followed by the pulse of 120 dB (PP75&P), and PP80 dB followed by

Figure 2. Daily change in mean body weight of male F1 rats exposed to TBT chloride via the placenta and their dams' milk and/or oral exposure via food.

Day 0: 3 weeks of age; Day 37: 9 weeks of age. Mean values are indicated. The mean values of the body weight in the TC or TT group were significantly lower than those in the CC group from day 1 to day 37. The mean values in the CT group were significantly lower than those in the CC group from day 4 to day 37 (n = 10 per group).
a pulse of 120 dB (PP80&P). For each rat, there were 11 acoustic stimulations in each test session for P alone, PP70&P, PP75&P, and for PP80&P, plus 10 no acoustic stimulation sessions randomly inserted. The startle response was measured by an electric sensor. The mean value of the responses for respective stimulations in a session was calculated. The percentage PPI of a startle response was calculated by the following formulae.

\[
\%\text{PPI at PP70} = \left[ 1 - \frac{\text{PP70&P}}{\text{P alone}} \right] \times 100
\]

\[
\%\text{PPI at PP75} = \left[ 1 - \frac{\text{PP75&P}}{\text{P alone}} \right] \times 100
\]

\[
\%\text{PPI at PP80} = \left[ 1 - \frac{\text{PP80&P}}{\text{P alone}} \right] \times 100
\]

Statistical analyses
The mean values of body weight, the daily intake of food and water per body weight, and the indexes of the behavioral tests of the groups were calculated. Data were analyzed by one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD) test as a post hoc test, using StatView J-5.0 software (SAS Institute, Cary, NC, USA). The level of significance was P < 0.05.

Results
Figure 2 illustrates the mean body weight of male F1 rats exposed to TBT chloride via the placenta, their dams' milk, and/or their food from 3 weeks of age (observation day 0) to 9 weeks of age (observation day 37). On day 0, the mean body weight ± SE was 56.3 ± 1.7 g for the CC group, 56.3 ± 1.7 g for the CT group, 37.4 ± 1.1 g for the TC group, and 52.6 ± 2.3 g for the TT group. The mean body weight of the CC group was significantly higher than that of the CT group at 9 weeks of age. The mean body weight of the CC group was significantly higher than that of the TT group at 9 weeks of age. The mean food intake of the CC group was significantly higher than that of the CT group at 9 weeks of age. The mean water intake of the CC group was significantly higher than that of the CT group at 9 weeks of age.
for the TC group, and $35.6 \pm 1.3$ g for the TT group. There were significant lower mean values of body weight in the TC and TT groups compared with that in the CC group during the observation period. The mean value of body weight in the CT group was significantly lower than that in the CC group from day 4 to day 37. Mean values of body weight in the TT group were significantly lower than those in the TC group from day 4 to day 37. On day 37, the mean body weight $\pm$ SE was $320.8 \pm 6.9$ g for the CC group, $277.9 \pm 2.9$ g for the CT group, $257.3 \pm 7.4$ g for the TC group, and $209.8 \pm 5.5$ g for the TT group.

Figure 3 illustrates the mean food consumption/body weight of the male F1 rats from 3 to 9 weeks of age. The amounts of food intake per body weight of the rats in the CT and TT groups were lower than those of the CC and TC groups for the first week. After the first week, the amounts of food intake among the groups were similar. From days 4 to 7, the food intake in each group was transiently higher compared with that in other weeks. The amount of food intake of the rats in each group gradually stabilized after day 7. The mean food consumption/body weight $\pm$ SE during the observation period was $108.5 \pm 4.6$ g/kg for the CC group, $115.7 \pm 4.6$ g/kg for the CT group, $98.7 \pm 3.3$ g/kg for the TC group, and $103.8 \pm 3.0$ g/kg for the TT group. The mean dose of TBT chloride exposure via food, estimated at each daily body weight and food intake measurement, was $14.46 \pm 0.57$ mg/kg body weight in the CT group, and $12.97 \pm 0.38$ mg/kg body weight in the TT group.
Figure 4 illustrates the mean water consumption/body weight of female F1 rats from 3 to 9 weeks of age. The amounts of water intake of the rats in the CT and TT groups were lower during the period of observation. The transient increase in water intake was also observed from days 4 to 7. The mean water consumption/body weight ± SE during the observation period was 185.2 ± 6.0 g/kg for the CC group, 166.0 ± 7.7 g/kg for the CT group, 196.5 ± 8.9 g/kg for the TC group, and 158.4 ± 7.7 g/kg for the TT group.

The locomotor distance every 5 minutes for 30 minutes in the F1 rats in the open field test is illustrated in Figure 5. For the locomotor distance every 5 minutes compared with the CC group, the mean values in the CT group were significantly lower during the 10-15- and 15-20-minute periods. The mean value of the locomotor distance in the TC group during the 15-20-minute period was significantly lower than that in the CC group. The mean value of the locomotor distance in the TT group during the 15-20-minute period was significantly lower than that in the CC group. And the mean value of the locomotor distance in the TT group was significantly lower during the 10-15- and 25-30-minute periods.

Figure 6 illustrates the total locomotor distance in F1 rats in the open field test. The mean value of the total locomotor distance in the CT and TT groups was significantly lower than that in the CC group, and the mean value of the total locomotor distance in the TT group was also significantly lower than that in the TC group.

The mean instances of typical behaviors in the groups are illustrated in Figure 7. The mean instances of WR in the CT, TC, and TT groups were significantly lower than that in the CC group. The mean instance of WR in the TT group was also significantly lower than that in the TC group. The mean instances of CR in the CT and TT groups were significantly lower than those in the CC and TC groups. The mean instances of FW in the CT and TT groups were significantly lower than that in the CC group. The mean instance in the CT group was also significantly lower than that in the TC group. And the mean instance of BW in the CT group was significantly lower than those in the CC, TC, and TT groups. There were no significant differences among the groups for the mean instances of defecation, urination, or the time of the first grooming.

Results of the PPI test are shown in Table 1. There were no significant differences among the groups in %PPI at any prepulses, 70 dB, 75 dB, or 80 dB.

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**Figure 7.** Times of typical observed behaviors of male F1 rats exposed to TBT chloride via the placenta and their dams' milk and/or oral exposure via food in the open field test.

WR, wall rearing; CR, center rearing; FW, face washing; BW, body washing

Note) Each bar represents mean value, and each error bar represents standard error.

P < 0.0001 for WR, P = 0.004 for CR, P = 0.013 for FW, P = 0.003 for BW, P = 0.798 for defecation, and P = 0.109 for urination by ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001 compared with the CC group, #P < 0.05, ##P < 0.01, ###P < 0.001 compared with the CT group, †P < 0.05, ††P < 0.01 compared with the TC group (n = 10 per group).
There were no significant differences in any %PPI by ANOVA.

prepulse was 80 dB. %PPI when prepulse was 75 dB. %PPI at PP80: %PPI when prepulse was 70 dB. %PPI at PP75:

Each number represents mean value ± standard error. n = 10 per group.

Discuss the development of F1 animals exposed to TBT via the placenta and their dams’ milk and/or oral exposure via food.

TBT inhibits body weight gain and behaviors in rats

It has been suggested that the neurotoxic effects of TBT are different between the sexes. It is of interest whether or not the inhibitory effects induced by TBT via the placenta and their dams’ milk and/or their food are also observed in male F1 rats as well as female F1 rats in Asakawa et al.’s study. To test the hypotheses and interest, we set the 4 groups of male F1 rats: the control (the CC group); the group exposed to TBT via their food after weaning (the CT group); the group exposed to TBT via the placenta and their dams’ milk (the TC group); and the continuous exposure group (the TT group) (n = 10/ group).

In the present study, the exposure to TBT after the weaning caused significant decrease in body weight which was clearly apparent in the comparisons between CC vs. CT and TC vs. TT. Carthew et al. reported a 16% reduction compared with the control for the body weight of female F344 rats exposed to TBT oxide in the diet from weaning to 6 weeks of age. For adult mice exposed to TBT, the final mean body weight was not significantly different between the control and the group exposed to TBT at 125 ppm in their food. In Asakawa et al.’s study, no significant differences in body weight were observed between the control and the group exposed to TBT via food after 9 weeks of age. For the effects of TBT on body weight, the timing of exposure is important. The exposure to TBT via food from weaning caused decrease in the rats’ body weight. In Asakawa et al.’s study, the body weight in the TT group exposed via the placenta and their dams’ milk and food after 9 weeks of age were not significant different from that in the TC group. In contrast, the TT group in the present study which was exposed to TBT continuously had significant lower body weight compared with that in the TC group. The additive inhibitory effects on the body weight would likely be observed if the exposure to TBT via food starts just after the weaning.

The inhibition of the development for male F1 rats was observed by the TBT exposure via the placenta and their dams’ milk as well as the female F1 rats in the previous study. In other previous studies concerning the development of F1 animals exposed to TBT via the placenta and their dams’ milk, Konno et al. demonstrated that the mean body weight of male mice exposed to TBT chloride at 50 ppm via drinking water was significantly lower than that in the controls at 3 weeks of age, which is the time of weaning. It is noteworthy that the inhibition...
of development also continued in the TC group after the stopping of TBT exposure at the time of weaning in this study as well as female F1 rats in the previous study. The exposure to TBT caused permanent decrease in body weight for both male and female F1 rats. The cumulative effect evidenced by these results suggested that the exposure of TBT during the early stage (3-9 weeks of age) caused the decrease in body weight as did the exposure via the placenta and their dams' milk.

The lower amounts of food intake in the CT and TT groups compared with those in the CC and TC groups in the first week could have been due to the taste of the food containing TBT. In the previous study, lower amounts of food intake were also observed in the CT and TT groups.\(^{12}\) After the first week, the rats in the CT and TT groups could have become used to the taste of the food. The transient higher amounts of food intake in each group from days 4 to 7 could have been due to the process after the animals' adaptation to their new environment. The mean food consumption per body weight among the groups ranged from 98.7 mg/kg to 115.7 mg/kg. The mean food consumption of the CT group, which showed the significant decrease in body weight, was the highest. The decrease in body weight may not be due to the decrease in the consumption of food.

The mean water consumption per body weight was the lowest in the TT group followed by the CT group. It may be related to the significant decreases in body weight in the groups. In Asakawa et al.'s study, the amounts of mean water intake were also lower.\(^{12}\) Another possibility was that the amount of water in the food containing TBT was different from the commercial rodent chow due to the preparation process of the food containing TBT. The transient higher water intake in each group from days 4 to 7 may also be due to the process after the adaptation to the new environment.

The total locomotor distance in the open field test was significantly lower than those in the CT and TT groups compared with the CC group. The lowest mean total locomotor distance was observed in the TT group, which suggests the additive inhibitory effects of activities by the exposure via food. The mean total locomotor distance in the TC group was lower than that in the CC group but not significantly different. In the Asakawa et al. study, the total locomotor distance in the TC and TT groups were significantly lower than that in the CC group.\(^{12}\) The difference between studies in the CT group was likely the difference of the timing of exposure via food. The exposure from weaning may also have stronger inhibitory effects on activities compared with that after 9 weeks of age. The difference for the TC group between studies may be the sexes. Female F1 rats exposed to TBT via the placenta and their dams' milk might be more sensitive to TBT in behavioral activities.

The mean values of locomotor distance in every 5-minute period were significantly lower in the CT group in the 10-15- and 15-20-minute periods compared with that in the CC group. In Asakawa et al.'s study, the mean value of locomotor distance in the 15-20-minute period in the CT group was significantly lower than that in the CC group.\(^{12}\) From the analysis of locomotor distance during every 5-minute period, the exposure to TBT after weaning may have enhanced inhibitory effects compared with the exposure to TBT after 9 weeks of age. The mean value of locomotor distance during every 5-minute period was significantly lower in the TC group in the 15-20-minute period compared with that in the CC group. In Asakawa et al.'s study, those in the 5-10-, 15-20-, and 20-25-minute periods were significantly lower than that in the control. This analysis also suggested stronger inhibitory effects on activities in female F1 rats than those in male F1 rats. The locomotor distances in every 5-minute period for the TT group were the lowest of those among the groups. This was in accordance with that of Asakawa et al.'s study.\(^{12}\) It is noteworthy that significant difference was observed in the observation period after 10 minutes. The later observation period may be important for the evaluation of TBT toxicity.

The mean numbers of instances of WR were significantly lower in the CT, TC, and TT groups compared with that in the CC group as well as to those in Asakawa et al.'s study.\(^{12}\) Those of CR were significantly lower in the CT and TT groups. While in Asakawa et al.'s study, the significantly lower mean value of instances of CR compared with that in the CC group was observed in the TC and TT groups.\(^{12}\) From these results for instances of rearing, the strong inhibitory effects of exposure via food from weaning was suggested. In addition, female F1 rats may be more sensitive to the exposure via the placenta and their dams' milk. In the study by Aou et al., the total instances of rearing were also lower in the TBT treated group.\(^{14}\)

The mean numbers of instances of FW were significantly lower in the CT and TT group compared with that in the CC group. The mean number of instance of BW was significantly lower to that in the CT group. In Asakawa et al.'s study, significantly lower instances of FW and BW were only observed in the TT group.\(^{12}\) The results of FW and BW also supported strong inhibitory effects of the exposure via food after weaning.

In contrast to locomotor activity and adaptive responses, there were no significant differences in any of
intraperitoneal injection of 2.0 mg/g TBT chloride. From elements in the hippocampus of rats that received a single injection in behavioral tests. The use of THA rats in the PPI tests is known for their very small individual variability of memory and learning, inhibited in the cerebrum of F1 mice exposed to TBT chloride via the placenta and their dams' milk. Arakawa showed alterations in trace elements in the hippocampus of rats that received a single intraperitoneal injection of 2.0 mg/g TBT chloride. From these studies, the impairment of memory and learning induced by TBT exposure may be indicated, and the PPI test may not be sensitive enough to detect the impairment of memory and learning induced by TBT exposure. Although the mean values of %PPI in the CT, TC, and TT groups were higher than those in the CC group, they were not statistically significant in the present study. In the previous study, the mean values of %PPI in the TBT-treated groups were not high. The standard errors in the PPI test in each group may be relatively high. The high value of standard errors in the PPI test may be partly due to the limited ability of the PPI test to detect differences among the groups. Tokai high-avoider (THA) rats are known for their very small individual variability in behavioral tests. The use of THA rats in the PPI tests may be useful to detect the difference among the groups. In addition, other tests for memory and learning such as the Sidman avoidance test may be worthwhile to test TBT toxicity.

The mechanisms of inhibition of development and behavior changes by the exposure of TBT chloride via the placenta and their dams' milk and/or oral exposure via food were not clear. Especially, for the rats exposed to TBT via food, the reason the exposure after weaning enhanced inhibitory effects remains unclear. In addition, the possible sexual difference in sensitivity to TBT exposure via the placenta and their dams' milk and/or oral exposure via food also requires further clarification. For the mechanism of development, examination of the alteration in metabolism related to body weight gain such as lipid metabolism may be useful. To elucidate the neurotoxic mechanism of TBT, and the difference due to timing of exposure via food or to that of the sexes, it may be useful to examine alterations in gene expressions. Recently, oligonucleotide microarrays have been developed and applied to the study of neurotoxic effects of environmental toxicants on the developing rat. It is of interest whether or not alterations in gene expression detected by the microarrays are observed under the protocol in the present study. The alteration in the gene expression may also be related to the decrease in water intake in the TT group. In addition, the toxic mechanisms of TBT and the no-observed-adverse-effect level (NOAEL) of TBT for F1 rats are not clear. The concentration of TBT in the food was set at 125 ppm in this study. To determine the NOAEL, lower concentrations of TBT should be tested.

In the present study, the exposure to TBT during developmental stages of rats after weaning induced the inhibition in body weight gain and behavior. It is difficult to compare the risks between the exposure to TBT during developmental stages and the exposure via the placenta and their dams' milk. From the results of body weight, the exposure via the placenta and their dams' milk has a stronger inhibitory effect than does that in the developmental stages. However, from the results of the open field test, the exposure during the developmental stages may have stronger effects than that the exposure via the placenta and their dams' milk. These results suggest that human exposure to TBT in the early developmental stages after weaning, as well as the exposure via the placenta and mothers' milk, should be avoided at all costs because of its high toxicity.

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