Observation of the Development of Tolerance to and Physical Dependence on Barbital by Cortical Evoked Potential in Rats

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ABSTRACT—To observe the dispositional and functional tolerance to and physical dependence on barbital, the influence of repeated administration of the drug on serum barbital levels, coordinative motion, body weight, and cortical evoked potential was assessed. Rats administered the first dose of barbital showed marked impairment of gross behavior and then loss of the righting reflex. While they were repeatedly treated with barbital for a 4-week period, the CNS depression became weaker and weaker, and loss of the righting reflex was no longer observed. Serum barbital levels after administration of barbital tended to decrease by the 28th day of repeated drug administration. Coordinative motion was markedly impaired after administration of the first dose, but gradually recovered during the repeated administration period. Barbital at 100 mg/kg, i.p., prolonged the latent time of the evoked potential in normal untreated rats but not in tolerant rats. During the withdrawal period, no particular change was observed in the animals' gross behavior. However, body weight loss and shortening of the latent time of the evoked potential were observed at 60 to 72 hours of withdrawal. These results suggest that cortical evoked potential can serve as a useful method for observing tolerance to and physical dependence on barbital.

It is well-known that tolerance to barbiturates consists of both dispositional and functional components (1–5). However, some studies indicate disinvolvement of the dispositional tolerance: Ebert et al. (6) reported that the distribution and metabolism of barbital-14C in tolerant rats were similar to those in non-tolerant rats, and several studies reported no development of dispositional tolerance to barbital (7–11). On the other hand, biochemical analysis of hepatic microsomal enzymes indicates that dispositional tolerance to barbital as well as other barbiturates may be developed due to the increase of drug metabolizing enzyme activity by repeated administration of the barbiturates (12, 13).

In physical dependence studies in rats, withdrawal manifestation is usually observed in terms of the body weight change. Recently, use of the cortical evoked potential of the rat was reported as a sensitive method for observing the development of tolerance to and physical dependence on opioid analgesics (14). However, since no attempt was made to use the evoked potential for observation of tolerance to and physical dependence on barbital, the present study was conducted.
MATERIALS AND METHODS

Twenty-six male Sprague-Dawley rats (Clea Japan Inc., Tokyo) weighing between 149 to 375 g were used in the present experiments.

The barbital (Yoshida & Co., Ltd., Tokyo) used in the experiments was suspended with 0.5% w/v sodium carboxymethyl cellulose (CMC, Daiichi Pure Chemicals Co., Ltd., Tokyo) and administered to the abdominal cavity of rats at a fixed volume of 1 ml/kg. The dosing schedule was: 75 mg/kg twice daily at 9:00 a.m. and 5:00 p.m. for the first two weeks, then 100 mg/kg twice daily at the same times for the other two weeks, except for 4 holidays when only the morning dose was given at 1.5 times the normal dose. Then, the rats were withdrawn by discontinuation of the drug for either 12 to 14 hours, 60 to 72 hours, or 10 to 14 days.

**Experiment 1: Observation of gross behavior, coordinative motion, and body weight**

Gross behavior was observed in all rats. Each group of the rats was accommodated in a transparent acrylic cage, 26 cm in length, 42 cm in width, and 16 cm in height. The observation was performed immediately before and at one hour after every morning dose, roughly following the observation method of Irwin (15). Namely, the appearance of spontaneous motor activity, response to touch, abnormal gait, and righting reflex were observed in each rat. Observation of the righting reflex was performed by placing each animal in a dorsal position on the palm of the observer's hand and then noting whether the animal could return to a normal posture. Appearance of tremor, diarrhea, and convolution was also observed for every morning after discontinuation of the drug for 12 to 14 hours, 60 to 72 hours, or 10 to 14 days.

**Experiment 2: Determination of serum barbital levels**

The same 6 rats from the rotarod test were also used in this experiment. The serum barbital levels were determined on the 1st, 3rd, 7th, 14th, 21st, and 28th days of treatment from the blood samples obtained just before and 60 minutes after administration in the morning. The levels were also determined at 12 to 14 hours, 60 to 72 hours, or 10 to 14 days, after discontinuation of the drug. Blood samples, 400 μl in volume, were collected by a syringe from the tail vein of each rat. Each blood sample was centrifuged at 3000 r.p.m. for 15 minutes, and then a serum sample was collected. Barbital was extracted from the serum with ethylacetate. Namely, to each 100 μl serum sample, 50 μl of 4.0 M NaH₂PO₄ and 5 ml of ethylacetate were added, after which the mixture was shaken for 10 minutes and then centrifuged at 2000 r.p.m. for 5 minutes. A 4-ml sample of the ethylacetate layer was removed and evaporated to dryness with nitrogen gas at 45°C. The barbital level was determined by gas chromatography. A glass column (4 mm in internal diameter, 1.5 m in length) packed with 1.5% SE30, 60 to 80 mesh, was used for chromatographic separation of barbiturates. The nitrogen carrier gas flow rate was 75 ml/min, and oxygen and hydrogen gas pressures were 0.6 and 1.2 g/cm², respectively. The temperatures of the column oven, detector, and injection ports were maintained at 140°C, 253°C, and 265°C, respectively. Serum barbital levels were calculated from the ratio of the area under the curve (AUC) of the barbital peak to that of the internal

result obtained was expressed in seconds. For 7 days prior to starting the treatment, the rats were trained to stay on the rotating bar. The test was carried out before and one hour after the morning dose throughout the repeated administration and withdrawal periods. In these 6 rats, body weights were also measured just prior to the morning dose throughout the repeated administration and withdrawal periods.
standard, allobarbital (Ono Pharmaceutical Co., Ltd., Osaka). AUCs were analyzed automatically by a Chromatopac R-A1 analyzer (Shimadzu, Tokyo).

Experiment 3: Observation of cortical evoked potential

Fifteen rats weighing between 149 and 254 g, divided into three groups of five rats each, were treated with barbital according to the previously mentioned dosing schedule for 4 weeks. These three groups of rats were designated to be withdrawn for 12 to 14 hours, 60 to 72 hours, and 10 to 14 days after the last administration of the drug. In these rats weighing between 277 and 375 and in 5 normal rats weighing between 320 and 375 g, the influence of a test dose of barbital (100 mg/kg, i.p.) on the latent time of evoked potentials was observed and recorded by the previously reported method (14). Namely, the rat's head was held immobile by a stereotaxic instrument under ether anesthesia. A polyurethane-coated monopolar silver ball electrode, 0.5 mm in diameter, was placed through hole in the skull over the dura mater of the sensory cortex, while a reference electrode was placed at the frontal sinus. Each rat was intramuscularly administered 0.3 mg/kg of d-tubocurarine chloride (Amerizol*, Yoshitomi Pharmaceutical Co., Tokyo). Immediately thereafter, artificial respiration was initiated and maintained at a tidal volume of 1 ml/breath and a rate of 90 breaths/min. The sciatic nerve, cut off at the lower end of the thigh region, was electrically stimulated by 20 single square waves, 0.1 msec in duration and 2 sec apart. Then, the evoked potentials were obtained in the contralateral cerebral cortex. An average noise-analyzed response obtained every twenty electrical stimuli was registered on an XY recorder. Then, the latent time of the second negative phase of the evoked potential wave was measured and expressed in milliseconds. The rats used in this experiment were sacrificed by intravenous administration of sodium pentobarbital (Nembutal*, Dainippon Pharmaceutical Co., Ltd., Osaka) after the experiment was completed.

Statistical analysis was performed using the two-tailed Student's t-test for experiments 1, 2, and 3, but the Fisher's exact probability test was used for body weight change in the experiment 1. When the value of \( P \) was less than 0.05, the difference was considered significant.

RESULTS

Experiment 1: Influence on gross behavior, coordinative motion, and body weight

Rats administered the initial dose of barbital first showed decreased spontaneous motor activity, abnormal gait, suppression of response to touch, and disappearance of the righting reflex within 60 minutes after administration. As the administration continued during 4 weeks, these manifestations became very slight and with no loss of the righting reflex. During the withdrawal period, no withdrawal signs were observable.

In the rotarod test, the staying time on the rod in normal untreated rats was 114.0 ± 3.8 (mean ± S.E.) seconds. Following the first dose of barbital (75 mg/kg, i.p.), coordinative motion was markedly impaired, and the staying time was shortened dramatically to 5.0 ± 2.2 sec. The staying time gradually recovered during the first 2 weeks of the treatment. By the 14th day of administration, the time was 48.0 ± 16.4 sec. As the dose increased to 100 mg/kg on the 15th day, coordinative motion was again impaired markedly, but this also recovered gradually during the last 2-week period. Thus, the staying time on the 28th day was 77.8 ± 18.8 sec. When barbital at 100 mg/kg was readministered to these rats after 12 to 14 and 60 to 72 hours of withdrawal, the results were almost the same as those observed on the 28th day of treatment. On the other hand, the test on days 10 to 14 of withdrawal showed a marked shortening of the staying time (Table 1).

Body weight increased during the repeated administration period, but by discontinuing the drug administration, it decreased in 2 out of 6 rats withdrawn for 12 to 14 hours and in
4 out of 6 rats withdrawn for 60 to 72 hours. However, the average body weights of these groups did not decrease significantly (Table 2).

**Experiment 2: Influence on the serum barbital level**

The serum barbital level at 60 minutes after the first dose of barbital at 75 mg/kg, i.p., was $121.5 \pm 6.3$ (mean $\pm$ S.E.) $\mu$g/ml. During the first 2 weeks of administration, no change was observed in the levels after each administration. The total serum level at 60 minutes after the morning dose tended to decrease by the 28th day of treatment, but no statistically significant difference was observed against the level on the 15th day (the first dose of 100 mg/kg), and it was still much higher than the level on the 1st day. The levels before each administration of the drug gradually increased throughout the repeated administration period (Table 3).
Table 3. Influence of repeated administration of barbital on serum concentration levels in rats

| Before/After administration | Serum barbital level (µg/ml) | Day of treatment | Time after the last dose |
|-----------------------------|-----------------------------|-----------------|-------------------------|
|                             | 75 mg/kg, i.p.            | 100 mg/kg, i.p. | 12-14 hr | 60-72 hr | 10-14 days |
| Before                      | 0.0 ± 13.7                | 46.3 ± 65.9     | 60.3 ± 13.5 | 2.0 |
|                             | ± ± ± ±                  | ± ± ± ±         | ± ± ± ±     | ± ± ± ± |
|                             | 0.0 ± 13.7                | 3.6 ± 7.0       | 7.7 ± 2.3   | 1.3 |

Each value represents the mean and standard error for 6 rats. Blood samples were collected immediately before and 60 minutes after drug administration. *: Significantly different from the value on the 1st day of treatment (P < 0.05, two-tailed Student’s t-test).

Table 4. Influence of repeated administration of barbital on the latent time of cortical evoked potential in rats

| Groups                     | Latent time of evoked potential (msec) |
|---------------------------|----------------------------------------|
|                           | Before | Time after barbital administration³ (min) | 15   | 30   | 60   |
| Untreated control         | 201.0 ± 13.2 | 219.5 ± 12.7 | 250.0 ± 15.1 | 269.0 ± 18.9 |
| Withdrawal for 12-14 hr   | 198.0 ± 8.7   | 196.0 ± 4.3 | 209.5 ± 5.7* | 211.0 ± 9.3* |
| Withdrawal for 60-72 hr   | 174.0 ± 18.5  | 189.0 ± 14.8 | 198.0 ± 12.1* | 192.0 ± 20.7* |
| Withdrawal for 10-14 days | 203.0 ± 9.8   | 210.0 ± 8.8 | 212.0 ± 8.9 | 209.0 ± 9.8* |

Each value represents the mean and standard error for 5 rats. ³: Rats were administered the test dose of barbital, 100 mg/kg, i.p. *: Significantly different from the values of the untreated control (P < 0.05, two-tailed Student’s t-test).

Experiment 3: Influence on cortical evoked potential

In normal rats, the average latent time of evoked potentials was 201.0 ± 13.2 (mean ± S.E.) msec. Prolongation of the latent time appeared from 15 minutes after administration and reached 269.0 ± 18.9 msec at 60 minutes after the administration. On the other hand, in groups of rats treated for 4 weeks and withdrawn, the latent times after administration of the test dose of barbital were significantly shorter than in the control group at 30 and 60 minutes in rats withdrawn for 12 to 14 hours and 60 to 72 hours and at 60 minutes in rats withdrawn for 10 to 14 days. In rats withdrawn for 60 to 72 hours, the latent time before administration of the test dose of barbital tended to become shorter than in the control group, but no statistically significant difference was observed (Table 4).

DISCUSSION

The first 2 experiments were carried out to make sure that the previously seen effects could be similarly obtained under the present
experimental conditions.

In gross behavioral observation, rats which received the first dose of barbital (75 mg/kg, i.p.) showed severe suppression of motor movement and loss of the righting reflex, while only slight suppression and no loss of the righting reflex were observed in the rats treated with barbital for 4 weeks. Thus, apparent development of functional tolerance was observed in these rats.

The serum barbital levels were almost constant during the first two weeks of treatment, but tended to decrease by the 28th day in spite of the dose increase and tendency of the drug to accumulate in the serum with repeated administration. In contrast to reports showing disinvolvement of dispositional tolerance to barbital (8, 11), there are reports showing possible development of the dispositional component of tolerance to the drug (12, 13, 16). The results in the present study may indicate involvement of dispositional tolerance to some extent in the overall tolerance to barbital. However, marked development of tolerance to barbital as observed in the gross behavior, and coordinative motion may be mainly attributable to the functional tolerance, since the serum level of the drug after the 28th day’s administration was much higher than the level on the 1st day.

The evoked potential latent time after administration of the drug in rats treated with barbital for 4 weeks then withdrawn became definitely shorter than that of the control group, which clearly indicates the development of functional tolerance to barbital. Although development of physical dependence was not clear in the behavioral observation, some amount of development was indicated by the weight decrease at withdrawal. Therefore, shortening of the latent time before administration of the drug may also reflect withdrawal. However, it is not clear whether the shortening of the latent time is attributable to tolerance development or physical dependence development. Nevertheless, since the latent time of normal rats differed significantly from that of the tolerant and physically dependent rats, observation of the evoked potential can be said to be a sensitive and useful measure for detecting the development of tolerance to and physical dependence on drugs of the barbiturate type.

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