Carbon Monoxide (CO)-Induced Hypoxia in Mice: Evaluation as an Experimental Model of Cerebral Ischemia for Drug Screening

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Abstract—An injection of 12.5 ml of carbon monoxide (CO) gas into an air-filled chamber (780 ml in volume) caused the death of the ICR or ddY mouse (6–8 weeks old) inside. The average survival time was 2.5 min for either sex of animals treated with nothing or saline and never exceeded 8 min. Pretreatment with pentobarbital Na (30 mg/kg, i.p.), hopantenate Ca (100 mg/kg, i.p.), vinpocetine (5 mg/kg, i.p. or 50 mg/kg, p.o.), flunarizine HCl (5 mg/kg, i.p.), glucose (6 g/kg, i.p.), phenobarbital (30 mg/kg, i.p.), phentoin (20 mg/kg, i.p.), arginine HCl (100 mg/kg, i.p. or 1 g/kg, p.o.) and alanine (100 mg/kg, i.p. or 1 g/kg, p.o.) prolonged the survival time of male mice. Insofar as tested, female mice responded rather poorly to these pretreatments. Survival for longer than 8 min occurred in some of the drug-pretreated animals of either sex. To be noted is the finding that most of the animals which survived 8 min once were able to survive the second 8 min on the following day without any drug-treatment. Monitoring of the time course of carboxyhemoglobin formation revealed that the carboxyhemoglobin level reached a plateau of 70% saturation within 2 min and then gradually increased. The lethal level was about 72%. Pentobarbital decreased the formation rate but did not elevate the lethal level. The results indicate that the CO-induced hypoxia model of mice is usable for screening of drug candidates which may be effective for treatment of human ischemic diseases.

A variety of cerebral ischemic models of mice have been developed for anti-ischemic drug screening: ligation of bilateral carotid arteries (1), inhalation of hypobaric air (2, 3), or normobaric hypoxic air (2, 4–7), intravenous injection of KCN (2, 3) and so on. The method of choice would be the ligation method by which one can limit the ischemia to the head, but the reproducibility of the results appears to be low, possibly due to the severe surgical damage. The normobaric hypoxic air method is the most widely used, and it has better reproducibility. Inhalation of hypobaric air or injection of KCN may induce other effects in addition to hypoxia.

Carbon monoxide, a classical asphyxiant, is one of the common causes of world-wide chemical poisoning. The use of gas as the asphyxiant in animal experiments, however, is very limited, though the mechanism of its asphyxic action is principally the same as that of hypoxic hypoxia (8, 9) and it may be possible to monitor the hypoxic level of the blood by measuring the degree of saturation of hemoglobin with the gas.

In this experiment, we attempted to evaluate the usability of the CO-inhalation method in screening drug candidates for cerebral ischemic diseases. The experiment consisted of 1) comparison of the “apothanasic” effect of various drugs on CO-induced asphyxia including pentobarbital, and 2) monitoring...
of the time course of the hypoxic level during the gas inhalation and effect of pentobarbital pretreatment. The obtained results indicate the usability of the CO-inhalation method for drug screening.

Materials and Methods

Six to eight weeks old mice of the ICR and ddY strains (Shimizu Experimental Animals, Kyoto) were used. Chemicals for injection were dissolved in saline except for vinpocetine which was dissolved in 10% ascorbic acid solution.

CO-inhalation (10): The mouse was placed on the top of a multi-perforated plastic lid of a petri dish (Sogorikagaku, Kyoto, 87 mm in diameter and 19 mm in height) on a glass plate, over which a glass bell jar (Sogorikagaku, Kyoto, 780 ml in volume) was put. The jar was made air-tight using silicone grease, a silicone stopper for the top opening and a gum tubing for tabulation. CO-gas (99.9% Japan Oxygen Co., Tokyo) was pressed into a 20 ml syringe; and after adjusting the pressure inside the syringe by taking the needle away for seconds, the gas was injected into the bell jar via a needle through the silicone stopper over a period of 10 sec. The air in the jar was mixed by a stirrer bar rotating under the plastic lid. The time from the end of gas injection to respiratory arrest was recorded up to 8 min as the survival time. Any animal that survived for 8 min was freed from the jar, and in some cases, challenged again on another day. The drug pretreatment was performed 15 min before CO-inhalation for the peritoneal route and 30 min for the oral route. The room temperature was between 22 and 26°C.

Spectrophotometric estimation of carboxyhemoglobin level (11): Blood was taken by cardiac puncture using a heparinized needle at various time intervals after CO-gas injection. A 50-μl blood sample was diluted with 0.1% Na carbonate solution (saturated with N2 gas) to 10 ml. Ten milligrams of Na hydrosulfite was dissolved in the blood solution; and 15 min later, the absorptions at 538 nm (E538) and at 555 nm (E555) were measured. When the ratio of E538/E555 was \( A_2 \); the ratio for the blood of the no CO-inhalated animal, \( A_0 \); and the ratio for the blood saturated with CO, \( A_{100} \); the carboxyhemoglobin level, CO-Hb (%), was calculated according to the following equation:

\[
\text{CO-Hb} (\%) = \frac{(A_2 - A_0)}{(A_{100} - A_0)} \times 100
\]

Drugs: Hopantenate Ca was kindly provided by Tanabe Pharmaceutical Co., Ltd.; Vinpocetine by Takeda Pharmaceutical Co., Ltd.; and Flunarizine HCl by Kyowa Hakko Co., Ltd.

Statistics: The numerical results were expressed as mean±S.E.M. Statistical analyses were performed using Student's \( t \)-test, and differences were considered significant when \( P<0.05 \).

Results

1) CO-induced asphyxic death and protection by drugs: In the standard procedure employed herein, 12.5 ml of CO gas was injected into the jar which was later found to saturate almost 70% of the hemoglobin within 60 sec (see the saturation curve of the saline-treated control in Fig. 1), and the time from the injection to complete arrest of respiration was recorded up to 8 min. This procedure was set based on the following preliminary observations: i) The average survival time of the naive or saline-treated ICR mice was around 160 sec; and saline treatment

Fig. 1. Time course of carboxyhemoglobin formation during CO inhalation and effect of pentobarbital. ⭕: saline-treated control mice. ○: pentobarbital Na (30 mg/kg, i.p.)-treated mice. Pretreatment was made 15 min before time 0 when CO inhalation was started. **: significantly different from the control (\( P<0.01 \)).
CO-induced hypoxia model of mice tended to elongate the survival time, but never significantly; ii) the increase of gas volume to 15 ml did not change the survival time (166±6 sec, N=9 to 155±17 sec, N=5), but a decrease to 10 ml increased both the mean of the survival time and its variance (166±6 sec, N=9 to 270±34 sec, N=5); iii) ddY strain of mice also survived about 160 sec, on the average; iv) the survival time appeared not to differ between male and female ICR mice; and v) no mice were able to survive longer than 6 min without any drug pretreatment. For setting the maximal survival time as 8 min, the results reported by Von Krieglstein and Hauer (5) was also taken into consideration that NMRI mice survived normobaric hypoxia (3.5% O2 in N2) for an average time of 180 sec (control 338 mice), and the mean survival time for drug treated groups of mice that survived significantly longer than the control was 528 sec (221 mice), 3 times longer than the control average, though in their study no upper limit for the survival time was set. Thus, all the experiments below were made using ICR mice under the standard procedure.

As shown in Table 1, pentobarbital Na and hopantenate Ca, glucose, phenobarbital, phenytoin, vinpocetine and flunarizine HCl were found to elongate the survival time significantly. Of three amino acids tested similarly, arginine HCl and alanine also delayed the asphyxic death of animals. In addition, these two amino acids appeared to be active even when pretreated orally, but only in higher doses than the ones given intraperitoneally. Though the data are not shown, a lower dose (100 mg/kg, p.o.) of either amino acid was not effective. Throughout the experiments summarized in Fig. 1, out of 140 mice, 14 showed a survival for longer than 8 min. When each was challenged on the following day, 12 mice were able to survive the 2nd 8 min without any pretreatment.

When compared with male mice, female mice appeared to respond poorly to the tested drugs. Pentobarbital Na, glucose or alanine increased the survival time significantly (Table 2), but, here again, 8 min survival was found to occur in most of the drug pretreated groups; and most of the survivors were able to tolerate the 2nd 8 min as observed with male survivors.

| Group No. | Treatment            | Dose   | Route | Survival time (sec) mean±S.E.M. | No. of 8 min survivors/No. of animals of group |
|-----------|----------------------|--------|-------|---------------------------------|-----------------------------------------------|
| 1         | Saline (control)     | 10 ml/kg | i.p.  | 154±17                          | 0/8                                           |
| 2         | Pentobarbital Na     | 30 mg/kg | i.p.  | 353±40*                         | 2/6                                           |
| 3         | Hopantenate Ca       | 100 mg/kg | i.p.  | 217±11*                         | 0/7                                           |
| 4         | Glucose              | 6 g/kg  | i.p.  | 283±26*                         | 0/7                                           |
| 5         | Phenobarbital        | 30 mg/kg | i.p.  | 241±20*                         | 0/11                                          |
| 6         | Phenytoin            | 20 mg/kg | i.p.  | 289±36*                         | 2/11                                          |
| 7         | 2% Ascorbic acid (control) | 10 ml/kg | i.p.  | 190±20                          | 0/7                                           |
| 8         | Vinpocetine          | 5 mg/kg  | i.p.  | 331±50**                        | 3/8                                           |
| 9         | Dist. water (control) | 10 ml/kg | p.o.  | 163±11                          | 0/6                                           |
| 10        | Vinpocetine          | 50 mg/kg | p.o.  | 313±38**                        | 1/6                                           |
| 11        | Flunarizine HCl      | 5 mg/kg  | p.o.  | 259±45*                         | 0/6                                           |
| 12        | Saline (control)     | 10 ml/kg | i.p.  | 152±14                          | 0/7                                           |
| 13        | Arginine HCl         | 100 mg/kg | i.p.  | 284±52*                         | 2/7                                           |
| 14        | Alanine              | 100 mg/kg | i.p.  | 267±53*                         | 2/8                                           |
| 15        | Glycine              | 100 mg/kg | i.p.  | 164±11                          | 0/7                                           |
| 16        | Saline (control)     | 10 ml/kg | p.o.  | 218±29                          | 0/7                                           |
| 17        | Arginine HCl         | 1 g/kg   | p.o.  | 330±47*                         | 2/7                                           |
| 18        | Saline (control)     | 10 ml/kg | p.o.  | 157±11                          | 0/7                                           |
| 19        | Alanine              | 1 g/kg   | p.o.  | 261±36*                         | 0/7                                           |

*: different from the control (P<0.05). **: different from the control (P<0.01).
2) Time course of carboxyhemoglobin formation and effect of pentobarbital: As illustrated in Fig. 1, the formation of carboxyhemoglobin proceeded rapidly; and in the saline-treated control mice, a 70% saturation was reached within 2 min. Pentobarbital pretreatment significantly delayed the formation rate: the mean±S.E.M. of the saturation levels estimated for the saline-treated control, which survived 178±12 sec (N=8) but died before the time set in Fig. 1, was 73±1.1%, which agreed well with 73±2.0% (N=9), the estimate for the drug-treated mice which survived 376±43 sec but died before the time set. The results show that pentobarbital Na pretreatment would not affect the lethal level of CO-saturation of hemoglobin.

Discussion

All the chemicals tested herein for their anti-asphyxic activity except amino acids have already been examined using other mouse models. Table 3 compares our results with some in the literature. Barbiturates including pentobarbital are known to first induce hypothermia, and the resulted hypothermia would prolong the survival time under the hypoxic condition (12). We did not measure the body temperature but the results in Fig. 1 clearly show that pentobarbital Na (30 mg/kg, i.p.) would exert its "apothanasic" effect by decreasing the rate of gas-exchange by respiration and not by elevating the lethal level of CO-induced asphyxia.

Our results with hopantenate Ca, vinpocetine, flunarizine HCl, phenobarbital and phenytoin are also comparable with those reported by other investigators (Table 3). The protective effect of glucose noted by Von Krieglstein and Hauer (5) in the normobaric hypoxic model was confirmed in this experiment. The attempt to follow the time course of CO-hemoglobin formation failed in glucose treated mice due to a difficulty in blood sampling which was caused by a high increase in blood viscosity. On the other hand, a possibility that glucose-induced hyperglycemia increases secretion of various hormones including insulin which then could protect mice from asphyxia led us to test the effect of L-arginine, a strong stimulant of insulin and growth hormone secretion (13). The amino acid was found to be active both orally and intraperitoneally, but the effective dose by the former route was higher than the one by the latter route, indicating that the El axis (14) would not practically contribute to the action of the amino acid given orally. On the other hand, a later study on microsphere-embolized rats provided results suggesting that by post-embolism treatment, the amino acid may be effective in alleviating embolism-induced memory degradation (15).

When the results in Figs. 1 and 2 were compared, it may be noted that female mice tend to respond to various drug treatments rather

| Group No. | Treatment          | Dose   | Route | Survival time (sec) mean±S.E.M. | No. of 8 min survivors/No. of animals of group |
|-----------|--------------------|--------|-------|---------------------------------|-----------------------------------------------|
| 1         | Saline (control)   | 10 ml/kg| i.p.  | 188±14                          | 0/16                                          |
| 2         | Pentobarbital Na   | 30 mg/kg| i.p.  | 319±52*                         | 4/9                                          |
| 3         | Hopantenate Ca     | 100 mg/kg| i.p.  | 232±69                          | 1/6                                          |
| 4         | Glucose            | 6 g/kg  | i.p.  | 414±27***                      | 6/11                                         |
| 5         | Dist. water (control) | 10 ml/kg| p.o.  | 173±23                          | 0/6                                          |
| 6         | Vinpocetine        | 50 mg/kg| p.o.  | 284±63                          | 1/6                                          |
| 7         | Flunarizine HCl    | 5 mg/kg | p.o.  | 177±15                          | 0/6                                          |
| 8         | Saline (control)   | 10 ml/kg| i.p.  | 154±7                           | 0/7                                          |
| 9         | Arginine HCl       | 100 mg/kg| i.p.  | 240±83                          | 2/7                                          |
| 10        | Alanine            | 100 mg/kg| i.p.  | 266±57*                         | 3/8                                          |
| 11        | Saline (control)   | 10 ml/kg| p.o.  | 158±15                          | 0/6                                          |
| 12        | Arginine HCl       | 1 g/kg  | p.o.  | 219±46                          | 1/7                                          |

*: different from the control (P<0.05).  ***: different from the control (P<0.001).
poorly as compared to male mice, but insofar as concerned with the appearance rate of the 8 min survivor, there appears to be no sex difference. Another common observation for both sexes was that the animals that survived the first 8 min by any kind of drug pretreatment were in most cases able to survive at least the 2nd 8 min without any pretreatment in the consecutive daily exposure(s). It is known that chronic tolerance can develop to the toxic action of carbon monoxide in chronically exposed animals and humans, and this is possibly due to polycythemia (16-18). In addition, Winston and Roberts reported that there is a tolerance which could develop in as little as 24 hr (19). It is not clear what kind of tolerance developed in our experiment. Some experiments using the offspring from 8 min survivors of both sexes are in progress in order to answer this question.

In conclusion, a CO-induced asphyxic animal model tested herein would be usable for screening for a drug candidate with clinical effectiveness for preventing or ameliorating ischemia- or hypoxia-induced disturbances, with at least the advantage that the hypoxic level of the blood can be monitored.

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