A Re-Examination of Physostigmine-Induced Cerebral Protection in the Hypoxic Mouse
A Critical Assessment of the Model

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SUMMARY Using the hypoxic (FiO₂ = 0.05) mouse model as originally described, the survival time following pretreatment with physostigmine was examined. The maximum increase in survival time was 87% following a physostigmine dose of 0.4 mg/kg. This increase was considerably less than that previously reported for this drug in a hypoxic mouse study wherein the standard method for exposing mice to hypoxia was altered. We speculate that this alteration in methodology resulted in small variations in FiO₂ sufficient to account for the differences between these studies.

PHYSOSTIGMINE has recently been reported to provide cerebral protection in the hypoxic (FiO₂ = 0.05) mouse. At the maximally protective dose (0.3 mg/kg) survival was increased by 542% (survival time in controls was 4.3 min) and 4 of 14 animals survived more than one hour. These increases in survival are strikingly greater than any previously observed with this model for a variety of cerebral protective agents (barbiturates, anticonvulsants, anesthetics) or conditions (hypothermia, hypercarbia) either singly or in combination.

Though the hypoxic mouse model is based on a simple concept it is known to be quite sensitive to a number of variables, most importantly FiO₂. Accordingly, it is desirable for experimental animals to be tested simultaneously with control animals in the same hypoxic environment. By this means any unintended variation in FiO₂ will be recognized by abnormal survival times in the controls. In the aforementioned physostigmine study individual control or experimental mice were sequentially introduced into a single test chamber. This modification may have introduced unrecognized variations in FiO₂ resulting in altered survival times. The present study was designed to re-examine the increase in survival afforded by physostigmine using standard conditions for this model. We also examined the effect of both temperature and elapsed time from injection of the drug, two other conditions that may alter survival times.

Method

Subjects were 170 white male ARS HA/ICR albino mice (Sprague Dawley, Madison, WI) (weights 24-32 g) given free access to food pellets and tap water. Groups of 5 mice were weighed individually then injected intraperitoneally with either physostigmine (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, or 0.7 mg/kg), an equal volume of 0.9% saline (controls), or pentobarbital (PTB) 50 mg/kg (controls). The injected volume ranged from 0.24 cc to 0.64 cc. Ten minutes later one animal was placed in each of 5 inter-connected airtight compartments breathing room air supplied at 4 L/min for an additional 10 min of temperature equilibration. The compartments were kept in a versa-range test chamber (Blue-M Engineering Co., Division of Blue-M Electric Co., Blue Island, IL) which, at an ambient temperature of 35°C, maintained mouse intraperitoneal temperature at 37.0 ± 0.2°C. At the onset of each test period the room air gas supply was replaced by a mixture of N₂, 88% and 5.22% O₂ (by Haldane analysis) in N₂, 15 L/min. After 60 seconds the N₂ flow was reduced to 0.25 L/min and after 120 seconds the O₂/N₂ flow reduced to 3.5-4.0 L/min to produce a rapid fall of O₂ concentration in all chambers to 5-5.25% (in-line Beckman O₂

Physostigmine-Induced Cerebral Protection

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analyzer) with maintenance of that concentration thereafter. Values from any chamber where the O₂ concentration exceeded the specified range were discarded. Survival time for each animal was defined as the time from initiation of hypoxic gas flow delivery to cessation of respiration.

At least 10 animals were studied at each physostigmine dose simultaneously with saline injected controls. At 0.3 mg/kg physostigmine, the experimental animals were studied simultaneously with both saline and PTB 50 mg/kg injected controls. For each group of 5 animals, 1–2 control animals were sequentially rotated among compartments. For each group behavioral observations were also made. Prior to hypoxic exposure (but after intraperitoneal injections) exploratory behavior, response to stimulus, righting reflex and respiratory rate were assessed. The intensity of terminal convulsive activity was also assessed.

To test the effect of drug exposure time, additional groups treated with physostigmine 0.3 mg/kg were studied at both 10 and 40 min after injection. To test the effect of temperature, an additional group treated with physostigmine 0.3 mg/kg was studied at a chamber temperature of 25°C, previously shown to result in mouse intraperitoneal temperature of 35°C. Survival data were subjected to analysis of variance. Where variance was confirmed further comparisons were made with Student's t-test for unpaired samples.

Results

Under standard conditions the mean survival times observed for each group pretreated with physostigmine as well as for the saline and PTB 50 mg/kg pretreated control groups are shown in table 1. The survival time of the saline treated controls (3.93 ± 0.07 min [mean ± SEM]) was not different from that observed in previous studies employing the same model. Likewise, the survival time of the group pretreated with PTB 50 mg/kg did not differ from that observed in a previous study employing this model. The maximally protective physostigmine dose was 0.4 mg/kg. At this dose survival time was 7.33 ± 0.46 minutes, an increase of 87% from saline treated controls. This increase in survival was significantly less than that afforded by pretreatment with PTB 50 mg/kg (13.99 ± 0.75 min).

The mean survival times of the additional groups pretreated with 0.3 mg/kg physostigmine are shown in table 2. Reducing the elapsed time to 10 min from intraperitoneal injection to the initiation of hypoxia did not change survival time compared to 20 min of drug exposure. Increasing the elapsed time to 40 min shortened survival time. In the group exposed to 25°C ambient temperature, the mean intraperitoneal temperature of the monitored mice was 35 ± 0.2°C during the 20–30 min interval after drug injection. The survival time of the untreated controls maintained at 35°C was 5.50 ± 0.35 min (mean ± SEM). The survival time of the group pretreated with 0.3 mg/kg physostigmine at 35°C was 8.01 ± 0.51 min (mean ± SEM). Lowering the ambient temperature significantly increased the mean survival time of both physostigmine treated and untreated animals compared to their normothermic counterparts. The greatest individual survival time observed for any physostigmine pretreated mouse was 11.5 min (0.3 mg/kg, 35°C).

Exploratory behavior was somewhat reduced after 0.1 mg/kg physostigmine and consistently reduced with doses of 0.2 mg/kg or greater. At doses of 0.5 mg/kg or greater terminal convulsive activity and response to stimulation were moderately reduced. The response to stimulation was characterized by prolonged repetitive rapid shaking movements. Respiratory rate was diminished at doses of 0.6 mg/kg or greater. Two of the 10 mice which received 0.7 mg/kg physostigmine became apneic and died prior to hypoxic exposure.

Discussion

It is likely that any protection afforded by physostigmine in this model is secondary to a drug induced increase in cerebral blood flow. The
neither a reduction in ambient temperature nor an alteration in drug exposure time sufficiently altered survival time to account for the differences between our study and that of Scremin and Scremin. We believe that the present study achieved standard conditions for this model since the survival times for both saline treated and PTB 50 mg/kg controls matched those of previous studies (see Results). Since the PTB-treated animals were tested simultaneously with the physostigmine-treated animals we are certain that the model as we used it was capable of demonstrating survival times greater than those observed in the physostigmine-treated groups.

The variable to which this model is most sensitive remains FiO₂, as reported by Secher and Wilhjelm. The figure is redrawn from their data with the inclusion of data from more recent reports from this laboratory. It shows the dramatic change in survival time that occurs with small changes in FiO₂. Since the curve for the protective agent (in this case thiopental) is shifted to the left of the control (saline treated) curve, an FiO₂ can be selected where small changes in FiO₂ (< 0.005) will only modestly alter control survival while dramatically altering survival in the protected group. It seems likely that this effect could account for the differences between our data and that of Scremin and Scremin. If reproducible results are to be achieved with this model, it is necessary that experimental animals always be observed simultaneously with controls exposed to the same environment, that the O₂ concentration in the gases supplying the compartments is precisely determined, that total gas flows to the chamber and equilibration times are exactly reproduced from one series of animal exposures to the next, and that the O₂ concentration achieved in each compartment is monitored. Attention should also be given to the other variables reported to alter survival time when using this model, namely variations in CO₂ concentration and temperature.

References
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FIGURE. Survival time for saline and barbiturate pretreated mice is plotted with respect to FiO₂. In the saline pretreated group small changes in FiO₂ when FiO₂ is near 0.05, produce only small changes in survival time. In barbiturate pretreated mice the survival curve is shifted upwards and to the left. When FiO₂ is near 0.05, small changes in FiO₂ now produce large changes in survival time.

magnitude of protection we observed was comparable to that previously reported for hypercarbia in this same model but considerably less than that reported by Scremin and Scremin in a modification of this model. Additionally, physostigmine-induced protection was less than that previously reported for other drugs (barbiturate™ and diphenylhydantoin®) and conditions (hypothermia®). At the 0.3 mg/kg dose level...
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