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Effects of Pentobarbital and Isoflurane on Conditioned Learning after Transient Global Cerebral Ischemia in Rabbits

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Background: **The acquisition of a conditioned eyeblink response has been used extensively to study the neurologic substrates of learning and memory. We examined the effects of the anesthetics isoflurane and pentobarbital, or hypothermia (30°C), on the ability of rabbits to acquire an eyeblink conditioned response after 6.5 min of cerebral ischemia.**

Methods: **New Zealand white rabbits (n = 48) were randomly assigned to sham, normothermic, hypothermic, isoflurane, or pentobarbital groups. In the normothermic, hypothermic, isoflurane, and pentobarbital groups, 6.5 min of global cerebral ischemia was produced. In animals randomized to the isoflurane and pentobarbital groups, a pattern of burst suppression was achieved on the electroencephalogram before the start of the ischemic episode. Animals in the hypothermia group were cooled to 30°C before ischemia. Seven days after ischemia, eyeblink training was started using an audible tone presented for 100 ms as the conditioned stimulus. The unconditioned stimulus was an air puff directed at the cornea. The delay between the end of conditioned stimulus and the start of the unconditioned stimulus (the trace interval) was 300 ms in duration. A conditioned response was defined as an eyeblink that was initiated during the trace interval. Eighty trials per day and 15 days of training were delivered.**

Results: **Neurologic deficits were greatest in the normothermia group, and these animals also had fewer conditioned responses than those in the sham, hypothermia, or pentobarbital groups. Animals in the isoflurane group had an intermediate number of conditioned responses that was not significantly different from the normothermia group.**

Conclusions: **This study demonstrates that a brief episode of cerebral ischemia results in the impairment of associative learning. Hypothermia and burst-suppressive doses of pentobarbital were able to improve neurobehavioral outcome as measured by ability to acquire a trace conditioned response. (Key words: Eyeblink; neuroprotection; reflex.)**

CLASSICAL conditioning paradigms have been used extensively to analyze the neurobehavioral substrates of associative learning.[1] Studies of the eyeblink conditioned response (CR) have been particularly popular because of the ease in eliciting the response and the lack of painful or injurious stimulation to subjects. Acquisition of a CR is mediated, in part, by the hippocampus[2] and cerebellum.[3]

Transient global cerebral ischemia, such as that which occurs during cardiac arrest and resuscitation, is a major cause of serious neurologic morbidity. The pyramidal cell layer of the hippocampus and the Purkinje-cell layer of the cerebellum are selectively vulnerable to ischemia.[4] The selective vulnerability of these neurons may be related to the high concentration of excitatory neurotransmitters (*i.e.*, glutamate) in these regions.[5] Excessive interstitial glutamate in the hippocampus has been found during transient global cerebral ischemia, and it may worsen the ischemic injury by facilitating calcium entry into the neuron.[6]

Many attempts have been made to prevent or attenuate neuronal injury after global cerebral ischemia. The neuroprotective effect of hypothermia is potent and widely acknowledged. Hypothermia may provide neuroprotec-

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tion through a combination of metabolic suppression and an attenuated release of excitatory neurotransmitters.[7] Similar decreases in cerebral metabolic rate can be achieved pharmacologically with pentobarbital and isoflurane. A burst-suppressed pattern on electroencephalogram (EEG) can easily be achieved with these drugs and is associated with approximately 40% reduction in the cerebral metabolic rate for oxygen. This is comparable to the reduction in cerebral metabolic rate for oxygen produced by moderate hypothermia (30°C).[8] Because of their ability to depress cerebral metabolic rate, it has been hypothesized that barbiturates and isoflurane would possess neuroprotective properties. However, studies have yielded conflicting data on this issue.[9–11] In the present experiment, we examined the effect of isoflurane, pentobarbital, or hypothermia (30°C) on the ability of rabbits to acquire a trace conditioned eyeblink response after 6.5 min of transient global cerebral ischemia. We opted to use a trace-conditioning rather than a delay-conditioning paradigm because there is evidence that some trace conditioning may be hippocampally dependent.

Materials and Methods

Animals

After the study was approved by the institutional Animal Care and Use Committee, 48 male New Zealand white rabbits (age, 3 months; weight, 3.0–4.0 kg) were randomly assigned to one of five groups: sham-operation group (n = 8), normothermic group (n = 13), hypothermic group (n = 11), pentobarbital group (n = 9), and isoflurane group (n = 10). The animals were housed one per cage at the institutional Animal Resource Center, where they received routine veterinary care.

Surgical Preparation

All rabbits (except animals in the isoflurane group) were anesthetized with 4% halothane in oxygen in a plexiglass box. Animals in the isoflurane group were anesthetized with 5% isoflurane in oxygen. After loss of consciousness, they were removed from the Plexiglas box and allowed to breathe the selected anesthetic from a face mask for 3–5 min before tracheal intubation was performed. Mechanical ventilation was started with a respiratory rate of 15–20 breaths/min and a tidal volume of 40–60 ml. End-tidal carbon dioxide partial pressure (35–40 mmHg) and anesthetic concentration (0.8–1.0% halothane or 1.0–1.2% isoflurane) were monitored (Capnomac Ultima; Datex, Helsinki, Finland). A 20-gauge ear vein catheter was inserted, and 0.9% saline was infused as maintenance fluid at 6 ml $\cdot$ kg^{-1} $\cdot$ h^{-1}. The ear artery was cannulated with a 20-gauge catheter for continuous monitoring of mean arterial blood pressure (MAP). Body temperature was measured with an esophageal probe and was automatically maintained at 38°C with a heating lamp and a warming mattress in the sham, normothermia, pentobarbital, and isoflurane groups. In the hypothermia group, esophageal temperature was reduced to 30°C before the onset of ischemia and maintained throughout the ischemic period by placing ice packs on the dorsal region of the rabbit. The scalp was infiltrated with bupivacaine (0.25%), incised in the midline, and reflected laterally to expose the skull using an aseptic technique. Four burr holes (1.2 mm) were drilled into the skull, and stainless metal screws were inserted. Dental acrylic was used to attach a stainless-steel machine bolt to the skull. This bolt served as a fixation point to attach the conditioned stimulus (CS) apparatus (see *Trace Conditioning*). Needle electrodes were placed in the scalp for continuous recording of the frontoparietal EEG (D.P-304 Differential Amplifier; Warner Instrument Corp., Hamden, CT).

Induction of Ischemia

An inflatable neck tourniquet (6 cm width) was placed loosely around the neck of the rabbit. In the pentobarbital group, pentobarbital was administered at a dose of 2 mg $\cdot$ kg^{-1} $\cdot$ min^{-1} until burst suppression of the EEG was achieved. Inspired halothane concentration was unchanged during the administration of pentobarbital. In the isoflurane group, the inspired concentration of isoflurane was increased to 3 vol% to achieve burst suppression of EEG. To induce ischemia, MAP was lowered to 25–30 mmHg with an intravenous bolus dose of 5 mg trimethaphan. The neck tourniquet was then inflated to a pressure of 700 mmHg within 0.5 s using a regulated tank source of compressed air. Ischemia was considered to be effective if EEG isoelectricity was achieved within 30 s after inflation of the tourniquet. After 6.5 min of ischemia, the neck tourniquet was deflated, and MAP was restored with an intravenous bolus dose of 10 μg phenylephrine. The EEG was examined for evidence of spontaneous activity in the postischemic period. After ischemia, the halothane, pentobarbital, or isoflurane administration was discontinued, and the animals were extubated as soon as spontaneous ventilation was adequate. Before extubation, body temperature

Table 1. Rabbit Neurologic Deficit Grading Scale

Assessment	Score	Maximum Score
Level of consciousness		
Normal	0	
Clouded	5	
Stuporous	10	
Comatous	25	25
Respirations		
Normal	0	
Abnormal	5	5
Cranial nerves		
Normal	0	
Vision	1	
Light reflex	1	
Oculocephalic	1	
Corneal	1	
Facial sensation	1	
Auditory	1	
Gag reflex	1	7
Motor and sensory function		
Normal	0	
No flexor response to pain, front	2	
No flexor response to pain, rear	2	
No righting reflex	10	14
Gait		
Normal	0	
Minimal ataxia	5	
Moderate ataxia	10	
Able to stand	15	
Unable to stand	20	
No purposeful movement	25	25
Behavior		
Normal	0	
No grooming	4	
No eating/drinking	10	
No exploratory movement	10	24
Total		100

was restored to 38°C in the hypothermia group. Thereafter, the ear vein catheter was removed, the animals were placed in the plexiglass box, and oxygen was insufflated into the box until the animals were awake. Free access to food and water was then allowed.

Neurologic Examination

A daily neurologic assessment was made by an observer unaware of the treatment group using the grading scale shown in table 1. The neurologic deficit score could range from 0 (normal) to 100 (severely impaired).

Trace Conditioning

Trace conditioning was begun on the seventh postischemia day. The animals were habituated to restraint in a padded, sound-attenuated, and ventilated chamber (Model ac-3; Industrial Acoustics Company, Bronx, NY) for 1 h on the day before the start of training. Body movements were minimized by placing the rabbit in a canvas bag that was secured around the neck and the hind legs. A panel set 35 cm behind the animal contained a speaker that delivered an 85-dB, 6-kHz tone for 100 ms. This 6-kHz tone represented the CS in this study. The unconditioned stimulus (UCS) was a 150-ms air puff generated by opening a valve separating a compressed (3×10^4 Pa) air tank from a length of tubing. The tube ended at a 2-mm nozzle positioned 2 mm from the cornea. An optoelectronic sensor (Optec OPB704; TRW Electronics, Carrollton, TX) was used to detect closure of the eyelid or nictitating membrane. This sensor, along with the compressed air tubing, was attached to the bolt cemented to the skull and positioned 4 mm from the cornea. Any movement of the nictitating membrane or eyelids was detected by a light-emitting diode/phototransistor combination. A CR was defined as an eyeblink that occurred between the end of the CS and the onset of the UCS (the trace interval) with an amplitude exceeding 4 SDs of baseline fluctuations as measured for 500 ms before the start of the CS. One conditioning trial consisted of a 100-ms CS, a 300-ms trace interval, and a 150-ms UCS. Eighty trials per day were administered. The interval between each trial was randomized between 30 and 60 s, and the average interval was 45 s. The entire training period lasted for 15 days. All behavioral trial presentations, data collection, and data reduction were controlled by a personal computer (Macintosh Quadra 950; Apple Computer Inc., Cupertino, CA) with custom-written software.

Learning Curves

Learning curves were produced by calculating the cumulative CR count on each day for each of the 15 days of training for each animal. The mean values ± SEM of the cumulative CR counts were plotted against the number of days of training.

Statistics

Neurologic deficit scores were dichotomized as 0 (for a zero score) or 1 (for all positive scores). The five groups were then compared for the proportion of positive scores using a chi-square test for days 1, 3, 5, and 7. Cumulative CR counts were analyzed for day 15 using one-way classification analysis of variance with Dunnett's one-tailed *t* test for multiple comparisons of the normothermia group *versus* the sham and treatment

Table 2. Neurologic Deficit Scores on Postischemia Days 1, 3, 5, and 7

		Treatment Groups				
	Deficit Score	Normothermia (n = 13)	Isoflurane (n = 10)	Pentobarbital (n = 9)	Hypothermia (n = 11)	Sham (n = 8)
Day 1	19+	5	0	0	0	0
	15	3	1	0	0	0
	10	1	2	2	0	0
	5	2	3	2	3	0
	0	2	4	5	8	8
Day 3	19	1	0	0	0	0
	10	2	1	0	0	0
	5	6	2	2	2	0
	0	4	7	7	9	8
Day 5	10	1	0	0	0	0
	5	7	2	1	1	0
	0	5	8	8	10	8
Day 7	5	6	1	1	1	0
	0	7	9	8	10	8

Distribution of deficit scores by groups and postischemia day. Note that neurologic deficit scores improved over time and that by day 7, nearly all animals in the isoflurane, pentobarbital, and hypothermia groups had normal neurologic examinations (deficit score = 0). Approximately half of the animals in the normothermic group had very minimal deficits on day 7, as evidenced by mild ataxia, yielding scores of 0 and 5. All groups were significantly different from the normothermic group by chi-square analysis.

groups. In all cases, a P value < 0.05 was regarded as statistically significant.

Results

Physiologic Variables

There were no statistically significant differences in body weight, *p*H, arterial carbon dioxide partial pressure, hemoglobin concentration, or base excess between groups. In the hypothermia group, arterial oxygen partial pressure (mean ± SD) during reperfusion (645 ± 51 mmHg) was higher than the normothermia group (555 ± 55 mmHg), isoflurane group (509 ± 49 mmHg), or pentobarbital group (515 ± 80 mmHg) because of the increased oxygen-carrying capacity of blood at 30°C. There were no statistically significant differences in preischemic and postischemic MAP. The hypotensive time (MAP < 50 mmHg) before tourniquet inflation was 16 ± 3 s in the normothermia group, 18 ± 5 s in the hypothermia group, 17 ± 2 in the isoflurane group, and 18 ± 3 s in the pentobarbital group. The hypotensive time after tourniquet deflation was 25 ± 6 s in the normothermia group, 40 ± 13 s in the hypothermia group ($P < 0.05$ *vs.* other groups), 20 ± 5 s in the isoflurane group, and 22 ± 3 s in the pentobarbital group. The time (mean ± SD) required to obtain an isoelectric EEG was 11 ± 3 s, 15 ± 6 s, 9 ± 3 s, and 12 ± 2 s in the normothermia, hypothermia, isoflurane, and pentobarbital groups, respectively (not significant). In all animals, the EEG became isoelectric within 30 s.

Neurologic Deficit Score

The 50th percentiles for the neurologic deficit scores on days 1, 3, 5, and 7, respectively, were as follows: normothermia group, 14, 5, 5, and 2.5; sham group, 0, 0, 0, and 0; hypothermia group, 0, 0, 0, and 0; pentobarbital group, 0, 0, 0, and 0; and isoflurane group, 5, 0, 0, and 0. The neurologic deficit score in the normothermia group was significantly greater than any of the other groups on postoperative days 1, 3, 5, and 7 (table 2). Corneal reflex and hearing were intact in all animals.

Trace Conditioning

On the 15th training day, the cumulative CR counts (mean ± SD) were 536 ± 247 in sham group, 247 ± 241 in normothermia group, 565 ± 274 in hypothermia group, 511 ± 190 in pentobarbital group, and 450 ± 278 in isoflurane group (fig. 1). The cumulative CR counts for the normothermia group were significantly less than those of the sham, hypothermia, and pentobarbital groups ($P < 0.05$). There were no statistically significant differences in cumulative CR counts between the normothermia and isoflurane groups.

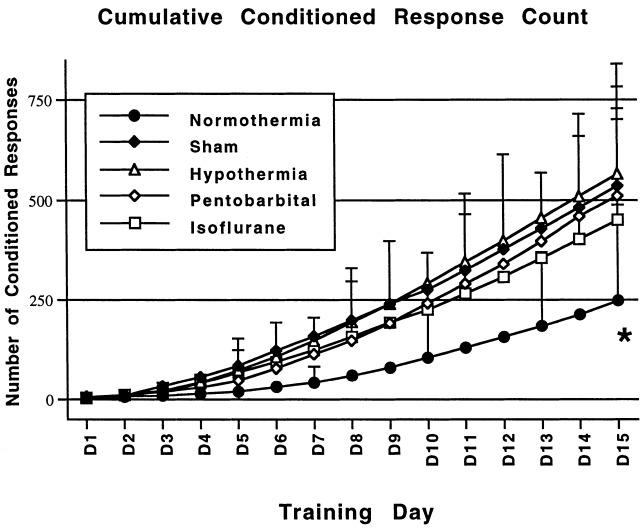

Fig. 1. Cumulative conditioned response counts (mean ± SD) for each of the experimental groups over the course of the study. *$P < 0.05$, normothermic *versus* sham, hypothermia, and pentobarbital groups.

Discussion

This study demonstrates that a brief episode of cerebral ischemia results in an impairment of the acquisition of a trace conditioned eyeblink response despite the nearly normal neurologic appearance of the test animals at the time the conditioning was conducted. Both mild hypothermia and a dose of pentobarbital sufficient to produce burst suppression on the EEG were neuroprotective for this behavioral test.

Classic conditioning using the eyeblink response has been widely used to investigate associative learning. The advantages of eyeblink conditioning for the study of learning are the ease of eliciting responses, lack of painful or injurious stimulus, and absence of gross movement during training.[12] It has been shown that a region of the cerebellum ipsilateral to the trained eye (lateral interpositus nucleus) is essential for learning and memory of the eyeblink CR but not for the unconditioned response.[13,14] The UCS (corneal air puff) pathway appears to consist of somatosensory projections to the dorsal accessory portion of the inferior olive and its climbing fiber projections to the cerebellum. The CS (tone) pathway consists of projections to pontine nuclei and their mossy fiber projections to the cerebellum. The efferent (eyelid closure) CR pathway projects from the interpositus nucleus of the cerebellum to the red nucleus and then *via* a descending rubral pathway to act ultimately on the motor neurons of the facial nerve. The red nucleus may also exert inhibitory control over the transmission of somatic sensory information about the UCS to the inferior olive, so that when a CR occurs (eyelid closures), the red nucleus dampens UCS activation of climbing fibers. Current evidence is most consistent with storage of the memory traces in localized regions of cerebellar cortex and interpositus nucleus.[1]

The hippocampus is believed to be involved in associative learning, which involves temporal coding. Lesion studies using delay eyeblink conditioning suggested some form of modulation of expression of the CR, either in latency to onset or in CR amplitude.[15,16] The hippocampus has been shown to be required in more complex eyeblink paradigms, including trace procedures. When the trace interval is 500 ms, hippocampectomized rabbits do not acquire the eyeblink CR.[2,17,18] When the trace interval is 300 ms, rabbits acquire but do not extinguish the CR. Hippocampal multiple neuron activity shows firing patterns that correlate with the developing CR in both delay and trace conditioning. Single hippocampal CA1 neurons show large alterations in firing rate early in the acquisition process, before the trace eyeblink CR is well learned.[19] Both *in vitro* recording of CA1 cellular excitability changes[20] and bilateral hippocampal lesions[17] demonstrate that the hippocampus is required during the consolidation period of the developing trace CR. These studies suggest that the hippocampus is involved in the mechanism by which animals learn and consolidate the association between the CS and UCS.

In pilot studies, we examined the effect of various ischemia durations on brain temperature, neurologic injury, and histologic outcome. Brain temperature decreased by 1°C at the end of 6.5 min of ischemia. Despite this slight decrease in brain temperature, ischemia of 6.5 min duration was found to be sufficient to cause histologic evidence of neurologic injury. Neuronal damage was widely distributed throughout the brain, involving cortical neurons as well as hippocampal CA1 and cerebellar Purkinje cells. However, the neurologic injury after ischemia was not so severe to cause feeding difficulty or prolonged disability, and the neurologic deficit score improved to nearly normal values within a week. During eyeblink conditioning in this study, the neurologic deficit score of all rabbits was < 5.

Electroencephalogram suppression states can be achieved with a variety of anesthetic agents, including intravenous and inhaled agents. At the point at which complete EEG suppression is achieved, the cerebral metabolic rate for oxygen has been reduced to approximately 40% of the awake baseline value. The concept

that this decreased demand for oxygen might result in an increased tolerance for ischemic events arose several decades ago. Barbiturates were one of the first agents to be tested for neuroprotective properties. A series of investigations demonstrated the cerebral protective effects of barbiturates in a setting of standardized focal ischemic insults.[21,22] However, in models of global ischemia, a number of investigations have failed to demonstrate any neuroprotective benefit.[9–11] In the present study, we studied a model of mild global ischemic injury. The relatively short period of ischemia (6.5 min) differs from many previous studies[23] in which severe brain injury ensued. The normothermic ischemic rabbits showed minimal neurologic deficits and were almost completely recovered after a week.

Moderate hypothermia (30°C) was effective in this study in preventing an impairment of neurobehavioral function. Hypothermic neuroprotection is well recognized and is associated with metabolic suppression, decreased extracellular levels of excitatory amino acids, decreased free radical formation, and inhibition of cytokines. The cerebral protective effects of hypothermia after focal or global ischemia have been supported by many investigations.[24,25]

Burst suppression of the EEG was easily achieved by 3% isoflurane in the present study without a significant reduction in arterial blood pressure. There were no significant differences between the various groups in preischemic MAP. Although there have been numerous studies examining the putative neuroprotective properties of isoflurane,[26–28] few have been successful in demonstrating a beneficial effect.[28] However, more recently, Miura *et al.*[29] have shown that isoflurane is neuroprotective in a setting of near-complete global ischemia. This positive observation once again raises the possibility that anesthetic agents may improve outcome from global ischemic insults. In the present study, both hypothermia and pentobarbital resulted in statistically significant improvement in neurobehavioral (eyeblink) scores as compared with the normothermic controls. The CR count for the isoflurane group, although numerically superior to that of the normothermic group, did not quite reach statistical significance ($P = 0.06$).

We conclude that a brief episode of cerebral ischemia with minimal neurologic sequelae can result in the impairment of associative learning. Both hypothermia and pentobarbital may be neuroprotective in this setting of subtle neurobehavioral damage.

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