0031-3998/02/5206-0886
PEDIATRIC RESEARCH
Vol. 52, No. 6, 2002
Printed in U.S.A.
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Effects of Hyperthermia and Muramyl Dipeptide on IL-1β, IL-6, and Mortality in a Neonatal Rat Model

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ABSTRACT

The mechanism of sudden infant death syndrome (SIDS) may be linked to an interaction between the SIDS risk factors of hyperthermia and infection, and between their effect on cytokine production and arousal. This study investigated the effects of hyperthermia and a surrogate of infection (muramyl dipeptide or MDP) on cytokine production and mortality in a neonatal rat model. Four temperature groups were studied: 34°C (baseline), 38°C, 39°C, and 40°C. Body temperatures of neonatal rat pups in the hyperthermic groups were raised and maintained at the desired temperature (38°C, 39°C, or 40°C) for 1 h and then returned to the baseline temperature (34°C) for a further hour. The heat source was a covered, heatable aluminum metal plate in a Perspex heating chamber. Intraperitoneal (IP) injection of 0.1 mL normal saline was given 30 min before the start to control for MDP (protocol A). Four equivalent treatment groups were pretreated with MDP (25 nmol/animal) instead of normal saline (protocol B). IP ketamine (55 mg/kg) was used for anesthesia during the experiments and for euthanasia. Blood was collected by direct cardiac puncture immediately after the 2-h experiments and assayed for the cytokines IL-6 and IL-1β by ELISA. Hyperthermia significantly increased the production of IL-6 (p = 0.049) but not IL-1β and significantly increased mortality. Administration of MDP significantly increased the IL-1β production (p = 0.006) but not IL-6. Cox regression analysis showed that MDP in combination with hyperthermia had a significant effect on mortality in the neonatal rat. The risk of experiencing mortality was two and half times higher in the MDP group than in the non-MDP group (p = 0.016) [hazard ratio (95% confidence interval) = 2.66 (1.20–5.92)]. We conclude that hyperthermia and a surrogate of infection (MDP) influence cytokine production and that the combination of heat stress and MDP increases mortality in the neonatal rat. **(*Pediatr Res* 52: 886–891, 2002)**

Abbreviations

SIDS, sudden infant death syndrome
TNF, tumor necrosis factor
LPS, lipopolysaccharide
MDP, muramyl dipeptide or *N*-acetyl-muramyl-L-alanyl-D-isoglutamine
WBH, whole-body hyperthermia
HPA, hypothalamic-pituitary-adrenal
SHRP, stress-hyporesponsive period
CRF, corticotrophin-releasing factor

Despite impressive recent decreases in incidence, SIDS remains a leading cause of postneonatal mortality in Western countries. These decreases in incidence have been attributed to campaigns advising parents and healthcare providers about modifiable SIDS risk factors. These factors, identified in numerous case-controlled studies, include prone (front) sleep position, smoking by mother or father, bed sharing (especially if the mother smokes), sleeping under bedclothes, not breastfeeding, and not using a pacifier (dummy) (1–4). Other factors such as young maternal age, low socioeconomic status, high parity, low birth weight, male infant, winter months, and cold climates have long been known to be associated with SIDS (5), but are to a large extent considered to be immutable or nonmodifiable. The prone sleep position is the most important SIDS risk factor and is likely to be causal (6). However, the mechanism whereby prone sleep position causes SIDS remains unknown. Suggested mechanisms include reduction of heat loss, which increases the risk of relative or absolute hyperthermia (7), rebreathing of expired gases (8, 9), and airway obstruction (10).

Prone sleep position will reduce heat loss (11), as will covering the head with bedding. Galland and colleagues (12, 13) induced hyperthermia in neonatal piglets by covering the head with bedding and showed that this could lead to death in both sedated (with chloral hydrate) and nonsedated animals.

Received January 29, 2001; accepted April 30, 2002.
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W.Y. was supported by a research studentship from the Research Grants Council, Hong Kong.

DOI: 10.1203/01.PDR.0000036625.39038.D0

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Postmortem examination of these animals showed findings similar to those seen in heatstroke and some SIDS victims, *e.g.* intrathoracic petechial hemorrhages, thymic congestion, pulmonary hemorrhage, edema, and congestion (12). It has also been suggested that the possible effects of temperature in SIDS may be influenced by co-existing infection, and several species of toxigenic bacteria or their toxins have been identified in SIDS infants (14). Both hyperthermia and infection influence the production of cytokines.

Cytokines are protein or glycoprotein factors secreted by cells of both monocytoid and lymphoid lineages. These proteins function as up- and down-regulators of immunologic, inflammatory, and reparative host response to injury (15). Externally induced WBH induce production of the cytokines IL-1 and IL-6 in rats (41.5°C for 60 min) (16) and IL-1β and IL-6 in humans (41.8°C for 60 min) (17). Infection in normal-weight and low-birth weight neonates may be predicted by increased levels of IL-6, and to a lesser extent IL-1β (18, 19). Tobacco smoke, another important SIDS risk factor, may also influence cytokine production. Bronchoalveolar lavage supernatant of adult smokers has been shown to contain higher levels of IL-1β and IL-6 than does that of nonsmokers (20). Cytokines are involved in physiologic sleep regulation, *e.g.* IL-1 increases slow wave sleep (21), and it has been proposed that IL-1 may be the link between infection and SIDS by its adverse effect on respiratory control and arousal mechanisms (22). IL-1 may also increase the severity of apneas (23).

Muramyl peptides are derived from bacterial cell wall peptidoglycan, which constitutes as much as 90% of the cell wall of Gram-positive bacteria. In Gram-negative bacteria, only 5–20% of the cell wall is peptidoglycan, with the remainder being composed predominantly of LPS (21). MDP is a synthetic muramyl peptide. Both synthetic MDP and naturally occurring muramyl peptides exert their biologic effects, in part by inducing IL-1 (24).

An infant at a vulnerable developmental age may enter a state of positive thermal balance by virtue of the prone sleep position, soft under-bedding, heavy clothing, and/or room heating (5, 25). This could influence the production of cytokines (IL-1β, IL-6), as could other risk factors such as recent upper respiratory infection or possibly parental smoking. In turn, an increased level of IL-1 may increase slow wave sleep, blunt arousal, and increase the severity of apnea in the presence of moderate hyperthermia. This study aimed to explore the hypothesis that a mechanism of death in SIDS is linked to an interaction between hyperthermia, cytokine production, and sleep state by looking at the combined effects of hyperthermia and a surrogate for infection (MDP) on cytokine production and mortality in a small animal model (neonatal rat).

METHODS

Female Sprague Dawley rats (weights: 17.3–24.5 g) aged 10 d were used in this study. The original stock of rats was obtained from Animal Resource Centre, Perth, Australia, and mated at the Laboratory Animal Science Centre of the Chinese University of Hong Kong. The neonatal rats were kept with the mother until the start of each experiment. Three sets of experiments were conducted: protocol A to assess the effects of hyperthermia; protocol B to assess the effects of hyperthermia and MDP; and control experiments to assess possible effects of anesthesia and direct cardiac puncture on cytokine production. Animals from multiple litters were randomly assigned to the study groups.

Control of WBH. Animals in protocols A and B were removed from the mothers and placed in a Perspex heating chamber (12.5 × 22.5 × 19.5 cm) for the 2-h duration of the experiment. The heat source in the heating chamber consisted of a covered, heatable aluminum metal plate onto which the animal was placed. The plate was connected to a temperature controller, which maintained the animal at a desired temperature by using a feedback temperature recorded from a sensor placed in the animal's rectum. The experiments were conducted in an environmental temperature of 23°C with humidity of 50–70%.

Protocol A: Animal model to examine the effects of hyperthermia. Ten animals were studied at each of four temperatures (34°C, 38°C, 39°C, 40°C). The 34°C group (baseline temperature) was the sham heat-treatment group. This baseline temperature was determined by measuring rectal temperatures of neonatal rats aged 10–14 d ($n = 12$) immediately after removal from the mother using the temperature sensor connected to the temperature controller in an environmental temperature of 23°C, humidity 50–70% (the mean temperature was 33.75°C, SD 0.86). Each study animal was given 0.1 mL i.p. normal saline to control for injection of MDP (protocol B). After injection of normal saline, the pups were marked on the back and returned to the mother for 30 min. The animal was then taken from the mother again and anesthetized with ketamine hydrochloride (55 mg/kg i.p.). The pup was placed in the heating chamber and the body temperature was kept at the desired temperature (34°C, 38°C, 39°C, or 40°C) for 1 h and then returned to baseline temperature (34°C) for 1 h. The time to reach the desired temperature was 26.67 min (SD 15.15) for 38°C, 25.22 min (SD 14.03) for 39°C, and 20.29 min (SD 9.27) for 40°C. A second dose of ketamine (55 mg/kg i.p.) was given before euthanasia by direct cardiac puncture using a 27-gauge needle.

Protocol B: Animal model of MDP (infection surrogate) and hyperthermia. This protocol was identical to protocol A with the exception that the animals were given MDP (25 nmol/animal i.p.) instead of normal saline at the start of each experiment. MDP powder (Wako Pure Chemical, Osaka, Japan) was reconstituted and stored in aliquots at −70°C until use.

Control protocol. Three groups of experiments were conducted to assess the possible effects of anesthesia and direct cardiac puncture on cytokine production. In the first control group, five animals were studied. Each pup was given 0.1 mL i.p. normal saline and returned to the mother for 2.5 h without heating. Euthanasia and blood collection was by percutaneous cardiac puncture without anesthesia. In the second control group, 10 animals were studied. Each pup was given 0.1 mL i.p. normal saline and returned to the mother for 2.5 h without heating. Euthanasia and blood collection was by percutaneous cardiac puncture after administration of ketamine 55 mg/kg

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Table 1. *Summary of IL-1β and log (IL-6) (pg/mL) in three control groups (animal with mother) and sham heat-treatment group at 34°C (animal in Perspex heating chamber on a heatable aluminium plate)*

	No.	IL-1β Mean (SD)	Log(IL-6) Mean (SD)
Control 1	5	33.21 (20.14)	2.59 (0.78)
Control 2	10	41.92 (14.50)	2.67 (0.39)
Control 3	10	37.45 (17.07)	2.63 (0.43)
Sham heat-treatment group (34°C)*	10	34.23 (11.44)	2.45 (0.56)
p Value		0.651	0.799

* Non-MDP group (Protocol A).

i.p.. The third control group of 10 animals was identical to the second control group, with the exception that blood was taken by direct cardiac puncture. This third control group was compared with the 34°C (baseline temperature) sham heat-treatment group (protocol A).

Data collection. The PowerLab system (ADInstruments, Castle Hill, NSW, Australia) was used to record rectal temperature, skin temperature, power output of temperature controller, heat plate temperature, ambient temperature, concentration of ambient oxygen, and ambient carbon dioxide. All data were recorded for the duration of the experiment, with a sampling rate of two per second. Body temperature was recorded continuously from thermistor probes placed under the thigh (skin temperature) and in the rectum (inserted about 3 mm into the rectum with lubrication). Visual monitoring of movement, respiratory rate (by observing the movement of abdominal wall), and skin color was made every 15 min and recorded manually on a chart.

Blood collection and storage, and cytokine assay. After collection by cardiac puncture at the end of each experiment, the blood sample was placed into a blank tube (Bio Plas, San Francisco, CA, U.S.A.) and centrifuged at 3000 rpm for 10 min. The serum was stored in two or three aliquots (120 μL) at −70°C until cytokine assay. IL-6 and IL-1β assay were undertaken in batches using commercially available ELISA kits (BioSource, Camarillo, CA, U.S.A.). Measurements were performed according to manufacturer's instructions and standard curves were generated for each measurement. The minimum detectable doses for IL-1β and IL-6 were noted to be <3 pg/mL and <8 pg/mL, respectively. For the IL-1β immunoassay kits, the percentage coefficients of variation for the intra-assay precision were noted to be 6.7–8.2 and for the interassay precision 8.7–9.7 (BioSource). Corresponding data for IL-6 immunoassay kits were 3.7–4.9 and 5.9–9.9.

Statistical analysis. All values were expressed as mean (SD). Level of IL-6 was log transformed to make the data more normally distributed and homogenous of variance for further analysis. One-way ANOVA was used to evaluate the effects of anesthesia and direct cardiac puncture on cytokine production in the control group. Comparison of IL-1β and log (IL-6) measures in the non-MDP and MDP groups were analyzed by means of two-way ANOVA. Mortality in non-MDP and MDP groups was examined using the Cox proportional hazard model. All analyses were adjusted for any weight difference.

Ethical approval. The study was approved by the Animal Research Ethics Committee of the Chinese University of Hong Kong, and a license to conduct animal experiments was obtained from the Hong Kong Department of Health.

RESULTS

There was no significant difference in the IL-1β and log (IL-6) levels between the three control groups and the sham heat-treatment group (Table 1), indicating that anesthesia (ketamine 55 mg/kg i.p.), direct cardiac puncture, and the experimental set-up did not influence the levels of these cytokines. Although all animals were studied at the age of 10 d and received the same standard diet, the body weights were significantly heavier in the non-MDP group (protocol A) as compared with the MDP group (protocol B) (mean [SD] = 21.67 [2.38] *versus* 20.59 [1.59] g, respectively, $p = 0.019$) (Table 2). No significant differences in animal weights were found between the different temperature levels (34°C, 38°C, 39°C, 40°C) in either the non-MDP or the MDP groups ($p = 0.39$).

Table 2. *Body weight and IL-1β and log (IL-6) [mean (SD)] in nonmuramyl dipeptide (MDP) (Protocol A) and MDP (Protocol B) groups*

	Temperature				
	34°C (baseline)	38°C	39°C	40°C	Overall
Body Weight (g)					
Non-MDP	21.75 (3.27)	21.30 (1.23)	21.13 (2.17)	22.51 (2.53)	21.67 (2.38)
MDP	21.15 (1.20)	20.40 (1.73)	20.12 (1.89)	20.70 (1.50)	20.59 (1.59)
Overall	21.45 (2.42)	20.85 (1.53)	20.62 (2.05)	21.6 (2.23)	21.13 (2.08)
IL-1β* (pg/mL)					
Non-MDP	34.23 (11.44)	36.71 (11.80)	31.36 (10.24)	35.92 (13.19)	34.56 (11.44)
MDP	34.83 (13.73)	38.05 (15.65)	54.06 (18.70)	45.52 (12.50)	43.12 (16.53)
Overall	34.50 (12.30)	37.38 (13.50)	42.71 (18.73)	40.72 (13.44)	38.84 (14.77)
Log [IL-6]† (pg/mL)					
Non-MDP	2.45 (0.56)	2.70 (0.55)	3.50 (0.63)	2.86 (0.16)	2.88 (0.63)
MDP	2.36 (1.02)	3.35 (0.99)	3.09 (2.10)	3.51 (1.31)	3.08 (1.44)
Overall	2.41 (0.80)	3.02 (0.84)	3.30 (1.52)	3.19 (0.97)	3.00 (1.11)

$n = 10$ for each of non-MDP/MDP group at each temperature level (34°C, 38°C, 39°C, 40°C).

* Non-MDP/MDP groups, $p = 0.006$; temperature levels (34°C, 38°C, 39°C, 40°C), $p = 0.248$; non-MDP/MDP × temperature levels (34°C, 38°C, 39°C, 40°C), $p = 0.044$.

† Non-MDP/MDP groups, $p = 0.419$; temperature levels (34°C, 38°C, 39°C, 40°C), $p = 0.049$.

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The mean values of the cytokines, IL-1β and log (IL-6), for the non-MDP and MDP groups at each of the four different temperature levels (34°C, 38°C, 39°C, 40°C) are shown in Table 2. IL-1β was significantly higher in the MDP group (43.12 pg/mL) than in the non-MDP group (34.56 pg/mL) (p = 0.006). However, IL-1β did not differ significantly between the four different temperature group (34°C, 38°C, 39°C, 40°C) (p = 0.278). A significant interaction between non-MDP/MDP and temperature (34°C, 38°C, 39°C, 40°C) was observed (p = 0.044), suggesting that the change in IL-1β level between non-MDP and MDP group differed across the four temperature groups. In contrast, the log (IL-6) value at 34°C was significantly less than the values at 38°C, 39°C, and 40°C (p = 0.049). The log (IL-6) values did not differ significantly between the non-MDP and MDP groups (p = 0.419). These results remained unchanged after adjusting for weight difference.

Baseline respiratory rates were around 150 breaths/min (93–150 breaths/min), and the respiratory rates in the heated groups (38°C, 39°C, 40°C) tended to be lower by 10–20 breaths/min. In the animals that died, the respiratory rate dropped below 90 breaths/min approximately 15 min before death (30–89 breaths/min). The color of the skin and mucosa of the animal turned pale and then dark before the breathing stopped. Both MDP and hyperthermia showed a significant association with mortality (Table 3). The risk of experiencing mortality was two and a half times higher in the MDP group than in the non-MDP group (p = 0.016) [hazard ratio (95% confidence interval) = 2.66 (1.20–5.92)]. Compared with temperature at 34°C, higher hazard ratios were found at higher temperature levels ($p < 0.0001$) (Table 3). Again, results were consistent after taking into account the weight effect.

Macroscopic findings at postmortem showed evidence of bleeding into the liver and kidney, but not into the lung, in the majority of the animals in the hyperthermia groups, with the proportion and degree of bleeding increasing with the level of temperature.

DISCUSSION

The study demonstrated that, in a 10-d-old neonatal rat model, hyperthermia and MDP influenced the production of the cytokines IL-1β and IL-6. An increase in body temperature in combination with a surrogate for infection (MDP) significantly increased the mortality rate in the neonatal rat. These findings are consistent with the hypothesis that hyperthermia and infection may interact with cytokine production to increase mortality.

Table 3. *Results from Cox proportional hazard model in non-MDP and MDP groups and temperature groups*

Variable	Hazard ratio	95% Confidence interval	p-Value
Group			0.016
Non-MDP	Ref	—	—
MDP	2.66	(1.20–5.92)	0.016
Temperature			< 0.0001
34°C	Ref	—	—
38°C	0.977	(0.06–15.63)	0.987
39°C	9.412	(1.17–75.43)	0.035
40°C	37.18	(4.85–285.15)	0.0005

Hyperthermia in both humans and animals has been associated with production of IL-1 and IL-6 (16, 17, 26). Although a few tissues express IL-1 constitutively, macrophages and most other cell types produce IL-1 only in response to external stimuli such as infections, bacterial LPS, and MDP (27). Two types of IL-1 (IL-1α and IL-1β) have been reported. IL-1β is the predominant form of IL-1 and the amount of IL-1β in activated cells is usually 50-fold greater than IL-1α. Both IL-1α and IL-1β have the same surface receptors and share similar biologic activities (27). Both forms of IL-1 can induce sleep, somnolence, and systemic acute phase response including fever, hepatic acute-phase protein synthesis, neutrophilia, hypozincemia, and increase level of hormones (28). Many of the biologic effects of IL-1 and TNF are similar to those observed during septic shock (29). IL-1 can also induce other cytokine synthesis, such as IL-6 and TNF (15). IL-6 can be produced by many cell types, including activated T and B lymphocytes, monocytes, endothelial cells, epithelial cells, and fibroblasts (27). It is stimulated by viral infection, bacterial products such as endotoxin and MDP, or other cytokines such as IL-1 or TNF (15, 30). IL-6 plays a central role in defense mechanisms, regulating the immune response, hematopoiesis, and acute phase reaction (31).

Our results showed that WBH (38°C, 39°C, 40°C) significantly increased the level of IL-6 compared with the sham heat-treated group (34°C), but that WBH did not significantly increase the level of IL-1β. A study of 28 heat-stroke patients showed that IL-6 was elevated in 100%, whereas IL-1β was only elevated in 39% (26). Haveman and co-workers (16) reported elevated cytokine levels in the serum of rats after both externally induced WBH (41.5°C 1 h) and local hyperthermia. Serum IL-1 and IL-6 were enhanced after WBH. The IL-1 started peaking during WBH about 15 min after reaching 41.5°C, and 2 h after WBH the IL-1 levels returned to baseline. In contrast, the IL-6 levels were not enhanced during WBH but peaked 1 h after WBH, with levels returning to baseline 4 h after WBH. For technical reasons in this neonatal rat model, it was only possible to collect a blood sample at one time point, which might therefore have missed an increase in IL-1β.

MDP, given in a dose of 25 nmol/animal i.p. in the present study, led to a significant increase in the level of IL-1β (p = 0.006) but not IL-6 (p = 0.419). MDP is both pyrogenic and somnogenic (21). It has been shown to be somnogenic in rabbits, cats, rats, and monkeys. However, in rabbits, low somnogenic doses are pyrogenic only if given during the day but not at night (21). It is possible that muramyl peptides elicit their effects on slow wave sleep *via* newly induced IL-1 production (21). MDP has been noted to stimulate the production of IL-1, IL-6, TNF, and IL-1 receptor antagonist in human monocytes (30). In this latter *in vitro* study, the IL-1, TNF, and IL-1 receptor antagonist were induced after 4 h, whereas the IL-6 was produced at a later phase (30). Studies in dogs have shown that MDP *in vitro* can induce IL-6 after 2 h and that liposome-encapsulated muramyl tripeptide-phosphatidylethanolamine *in vivo* can induce IL-6 after 3–4 h (32) Our *in vivo* study showed a lack of effect of MDP on IL-6 production after

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a 2-h period, but whether any effect might be seen after a longer period is unknown.

As stressors, hyperthermia and infection may impact the HPA system in rats. In the adult rat, response to stress is characterized by increased levels of circulating ACTH and plasma corticosterone, whereas in the first 2 wk of life this response is greatly reduced (33). The period between 2 and 15 d of life in neonatal rats has been called the SHRP (33), and during this period basal levels of circulating ACTH and corticosterone are low and do not increase appreciably after stress (33). Reasons for this hyporesponsiveness are not clear but may relate to failure of the neonatal pituitary to respond to corticotrophin-releasing factor (CRF) or to an unusually strong negative feedback mechanism to circulating levels of corticosterone (33). However, other work has suggested that the HPA axis in neonatal rats is responsive to stress throughout development in a time-dependent and stressor-specific fashion (34). Results of recent studies suggest that although the adrenal undergoes SHRP, the rest of the HPA axis is entirely competent to respond during the neonatal period (35). In view of the stresses involved in our study, it would have been desirable to assay ACTH and corticosterone levels to evaluate the HPA response to the challenges of temperature and MDP. However, it was not possible to undertake these assays in view of the limited volume of blood obtained from each animal.

The study protocol required the use of anesthesia for ethical reasons and to enable data recordings to be made. This precluded any assessment of a possible role of hyperthermia, MDP, and cytokines on sleep state and arousal in this model. Neonatal rats of 10 d of age were used in this study for theoretical and practical reasons. It was not possible to conclude at what age a neonatal rat would be most equivalent to a 2- to 3-mo-old infant, but if animals much younger than 10 d of age were used, it would not be possible to collect sufficient blood for cytokine assay. Animals much older than this age start to develop significant covering of hair and were not considered suitable. Only female rat pups were used in the study so as to remove any possible effect that gender might have had on the results. However, cyclic changes in sex hormones would not be expected to occur at this age. The neonatal rat as a model to study the effects of hyperthermia and MDP on cytokines production and mortality has limitations but also potential advantages, including convenience and ready availability of commercial kits for cytokine assay. Previous studies have used a neonatal piglet model to look at the role of hyperthermia in sudden infant death (12, 36, 37). Our study was initially designed to try to mimic these previous experiments in a small animal model. However, instrumentation and monitoring in this small animal model proved difficult. Although described in adult rats, monitoring heart rate using a pulse transducer on the tail was not successful in the neonatal rat (38).

CONCLUSIONS

In conclusion, hyperthermia significantly increased the production of IL-6 but not IL-1β, and MDP, a surrogate for infection, significantly increased the IL-1β production but not IL-6. MDP in combination with hyperthermia had a significant effect on mortality in the neonatal rat. Previous study of sedated and nonsedated neonatal piglets subjected to head-covering has shown that a proportion of piglets did not arouse before dying during the development of hyperthermia (12, 36). These circumstances of head-covering are similar to those in which some cases of SIDS occur (1). To explore an animal model of SIDS further, it may be possible, after ethical consideration, to develop this neonatal rat model further. If the body temperature of the animal could be raised to a moderate level of 38°C to 39°C without instrumentation or anesthesia, then it might be possible to assess the effect of varying temperature, MDP dose, ambient oxygen, and carbon dioxide levels on sleep, arousal, and possible mortality.

Acknowledgment. The authors thank Dr. A.E. James, Laboratory Animal Sciences Centre of the Chinese University of Hong Kong, for providing practical advice and support.

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