THE EFFECTS OF DEVELOPMENTAL NICOTINE EXPOSURE ON THE VENTILATORY RESPONSE TO HYPERTHERMIA OF NEONATAL RATS

By:

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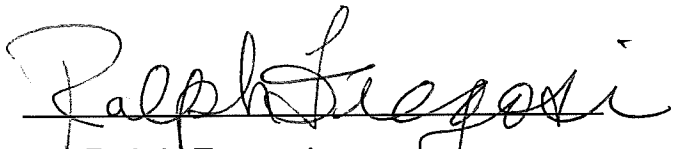

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Abstract

To determine the influence of developmental nicotine exposure on the ventilatory response to hyperthermia, respiratory airflow of head-out body plethysmography in awake neonates on postnatal days 1-3 was recorded. Data from 9 DNE neonatal rats were compared with data from 8 saline-exposed neonatal rats. The dams were exposed to nicotine (6 mg/kg of nicotine tartrate per day) or saline through the use of osmotic minipumps, transplanted subdermally on the fifth day of gestation. Each rat was tested at 33° C and 37° C. Treatment effects were found at 33° C for both ventilation and breathing frequency; however, this same trend was not found at 37° C. Tidal volume also did not see a trend at 33° C; however, there was a downward trend at 37° C, though it was just under statistical significance. There were no trends found in apnea number/10 minutes nor in apnea duration.

1. Introduction

Sudden Infant Death Syndrome (SIDS) is the sudden death of a seemingly healthy infant aged less than 1 year old due to unexplainable causes, even after a thorough investigation of the death is conducted (including autopsy, examination of death scene, and review of clinical history). SIDS is a subcategory of Sudden and Unexpected Infant Death (SUID), which is a term used to describe all such deaths, regardless of cause. It is after an investigation and autopsy of an infant death fails to yield any specific cause that the death is classified as SIDS (Centers for Control and Prevention 2014).

Previously regarded as one of the most confounding disorders in medicine, recent advances have helped to better understand SIDS. The most important of these was the discovery that the prone sleeping position in infants triples the risk of SIDS, leading to campaigns advocating the supine sleeping position. The result of simply changing sleeping position was a decrease in the rate of SIDS by over 50%, though it still remains the third leading cause of infant mortality. Being the leading cause of death in infants 1-12 months and the third leading cause of infant mortality in the United States, SIDS is a pressing concern in public health and further research of this matter is extremely important (Centers for Control and Prevention 2014).

There has also been evidence of developmental nicotine exposure (DNE) increasing the risk of SIDS. Apneas, delayed arousal responses, and suppressed chemoreceptor reflexes are a few of the respiratory control abnormalities that arise as a result of prenatal nicotine exposure. These abnormalities increase the risk of SIDS. For these reasons, it is important to study the cardiorespiratory system of nicotine-exposed neonates. A study (Huang et al., 2004) has shown evidence that the altered eupneic breathing pattern causes increased apnea incidence in pre-natal nicotine exposed rats (Huang, et al, 2004).

Many SIDS cases have also been attributed to hyperthermia. A study (Guntheroth, et al., 2000) shows a strong association between ventilatory control and thermal regulation, centering on prolonged apneas. The odds ratios of SIDS seemed to increase with increased body temperature. In the United States specifically, little attention has been paid to the dangers of thermal stress in

relation to SIDS.

Thermoregulation is an inherently essential function of the body, as vital organs must be kept at specific temperatures in order to survive and function optimally. Thermoregulation in neonates is relatively impaired as a result of differences in physiologic function and small body size. Thus, the neonates are prone to under heating and overheating. Neonates, with large surface area and high surface-to-mass ratio, lose body heat rapidly and temperature imbalance is a serious complication. The large surface area, limited insulation, and limited sweating ability of the neonate (It should be noted that rats cannot sweat at all, but rather constrict or expand blood vessels in their tail to thermoregulate) also makes overheating an issue. In a cool environment, heat can be produced rapidly via brown adipose tissue (BAT) in neonates. Brown adipose tissue (found in mammals) can produce heat in response to a cold environment, a fall in core body temperature, or the presence of pyrogens. This non-shivering or adaptive thermogenesis occurs when uncoupling proteins uncouple the mitochondria production of ATP (whereas normally chemiosmosis takes the energy of electrons and converts this energy to ATP). Thus, the energy that would have gone into the phosphate bond is released instead as heat. BAT has the uncoupling proteins activated by the sympathetic nervous system activity. BAT thermogenesis is usually found near critical organs in order to keep them warm (Morrison 11, 12). However, once this heat is produced, it cannot be reduced, and neither can basal metabolic rate. This leads to excessive fluid loss, an increase in breathing frequency, and metabolic rate can arrive at a potentially fatal level. Sweating is the only way to reduce body heat in extreme conditions (Thomas 1994).

These experiments were designed to examine the influence of developmental nicotine exposure on thermoregulation and the control of breathing. To perform such a study, rats were used as models at the systems and cellular level for three main reasons: ease of exposing the fetus to nicotine, short gestation period, and large litter size. It should also be noted that rats cannot sweat at all, but rather constrict or expand blood vessels in their tails to thermoregulate (Lentini 2007). We tested the hypothesis on the influence of developmental nicotine exposure on the ventilatory response to hyperthermia.

2. Methods

2.1 Animals

For ventilation measurements, the neonates came from three saline-exposed and three nicotine-exposed Dams; 2-4 neonates were taken per litter. Ventilation recordings were taken from eight saline-exposed and nine DNE neonates on either postnatal day 2 or 4.

2.2 Prenatal nicotine exposure

The six mother rats used for the experiments were implanted with 28-day osmotic mini pumps on the 5^{th} day of gestation. Implantation on this day assured nicotine exposure began early in embryonic development. Three of the rats had nicotine pumps (6 mg/kg per day at a rate of 2.5 uL/h) and three had physiologic saline pumps (also at 2.5 uL/h). The rats were anesthetized with a sub-cutaneous injection of a mixture of xylazine (8.0 mg/kg), ketamine (25 mg/kg) and acepromazine (1.0 mg/kg). Buprenorphine (0.5 mg/kg) was also injected to mitigate post-surgical pain. After surgical anesthesia was achieved, a small incision was made between the scapulae and an osmotic minipump containing either nicotine or saline. Penicillin was given to treat infection and the animals were taken back to their cages. Nicotine was delivered at a rate of 6 mg/kg/day for 28 days; physiological saline was released at the same overall rate. Since gestation in the rat is 21 days, the neonates were nicotine or saline exposed from gestational day 5-21 via the placenta, and throughout the postnatal period via breast milk.

2.3 Measurements

To measure ventilation, head out plethysmography was used in awake neonates, between the times of 10:00 am and 5:00 p.m. The homemade chamber that held all pups had dimensions of 6 cm and was made from plastic syringe bottles. A seal was made for the animal's head by cutting out a circle (that could easily fit over the top of the chamber) from a latex glove and then cutting a small hole into the center of the glove. This hole was large enough to fit over the neonate's head, though it also had to be airtight when around its neck. The glove was secured to the

chamber using a rubber band.

In order to measure the rate of respiratory airflow, the chamber had three ports: one for thermocouple probe insertion (in order to regulate temperature), one as a means to inject volume for calibration, and one connected to a pneumotachometer to measure changes in chamber airflow. The Spike II software system was used to digitize and display analog voltage outputs from the pneumotachometer and an analog integrator. Volume calibration was done by injecting 0.1, 0.2, and 0.3 ml into the chamber, using a graduated 1ml syringe. During these injections, the chamber was closed by securing a circular patch of latex glove (no hole in the center of this one) to the open side. The pneumotachometer was connected to the third port via Tygon tubing. When the neonate inhaled, air was pushed across the pneumotachometer and exhalation resulted in opposite airflow changes. The two ports of the pneumotachometer were attached to the positive and negative ports of a pressure transducer with a range of ±2 cmH_2O (Validyne DP45-16, Northridge, CA). The pneumotachometer contains a fine mesh screen positioned between the two pressure ports, and the differential pressure is measured as gas flows across the screen. The transducer is connected electronically to a demodulator, which then produces an analog voltage signal proportional to the differential pressure. The voltage output of the pressure transducer was sent in parallel to an analog integrator (Grass model 7, Quincy, MA) and the A/D board. The differential pressure, which is proportional to gas flow into and out of the chamber, as well as the integral of flow with respect to time were recorded and displayed on a computer screen using Spike II software. The neonates' weight in grams was taken before the experiment began.

The temperature on the thermocouple was calibrated between 32-34° Celsius, corresponding to the normal nesting temperature for neonatal rats which is between 30-34° C (Mortola, 1984), using a control unit (TCAT-1A Temperature Controller, Physitemp, Clifton, NJ). The control unit was connected to a lamp that was set to turn on at the set point temperature, and then turn back off when chamber temperature exceeded the set point. After the recording, the rat was taken out of the chamber and core temperature was measured by inserting a separate thermocouple into the rectum. Table 1 shows the similarities between the rectal temperature in saline and DNE rats.

2.4 Experimental protocol

The recording began after the rat was secured in the chamber. The rat was kept in the chamber for 660 seconds. During this time, copious notes were taken on the level of movement made by the rat. 10 of the most stable and continuous 20 second breathing periods were used to compute breathing frequency and tidal volume. Apneas were measured as an absence of breath for at least double a normal breath cycle. All apneas were counted within the 660-second experiment and the durations were taken note of. After the rat had been recorded at 33° C, he was taken out of the chamber and given approximately 10 minutes to rest. During this rest time, the temperature of the chamber was changed to 37° C by recalibration. The rat was not placed back inside the chamber until the control unit read approximately 37° C.

2.5 Statistics

All data studied (tidal volume, ventilation, breathing frequency, apnea number, and apnea duration) was compared using ANOVA analysis. Temperature and treatment (nicotine or saline) were the main factors. Student–Neuman–Keuls post hoc test was used for post-hoc analysis of all comparisons. Statistical significance was defined as having a p-value of 0.05 or less.

3. Results

Plethysmograph recordings of a nicotine-exposed and a saline-exposed neonate (P3) at both 33°C and 37ºC are shown in figure 1.

The graph of average apnea number in figure 2 shows the saline-exposed rats with a larger average number of apneas at 33°C and the nicotine-exposed rats with a larger average number of apneas at 37°C. However, the data from one of the nicotine-exposed rats was removed as a result of its outlier-like qualities (having respectively 49 and 87 apneas at 33°C and 37°C). The graph with the outliers included is shown in figure 3. Overall, even with some trends, no statistical significance was found. The average duration of apneas in nicotine-exposed rats (shown in figure 4) was larger at 33°C than saline-exposed and about equal for saline and DNE at 37°C. The average duration of apneas increased slightly in saline-exposed rats from 33-37°C and remained roughly the same in nicotine-exposed rats. No statistical significance was found.

As seen in figure 5, at 33° C, there was a downward trend in ventilation with saline-exposed rats having a consistently higher ventilation rate than DNE. This treatment effect had statistical significance with p-value=0.008. At 37° C, however, treatment did not seem to have an effect on the ventilation rate, with no statistical significance and no clear trend. Although, it was found that DNE rats tend to breathe more in response to an increase in temperature, while this did not occur in saline-exposed rats. Breathing frequency results (figure 6) were very similar to ventilation. At 33° C a treatment effect could be seen between the saline-exposed and DNE rats, with a downward trend and p-value=0.001. At 37° C, no effect was found. Tidal volume results (shown in figure 7) were different than ventilation and breathing frequency. At 33° C, there was no treatment effect seen between saline-exposed and DNE and no significance was found. At 37° C, tidal volume trended downwards for saline vs. DNE, but just missed significance with p-value=0.12.

4. Discussion

The results of the experiments led to several trends. Though there were trends in the data analysis, most statistics were under significance. It is reasonable to suggest that the temperature change was not drastic enough to produce significant results. The temperature of 37°C was initially chosen as the high temperature because research showed 39°C to be the upper limit as to what hyperthermic conditions neonatal rats can tolerate; 37°C seemed to be appropriate. To increase the chance of significant results, further experiments should be performed at 33C and 38C, as 37C did not result in significant findings, yet 39°C is too stressful to be of practical use in experimentation.

There might be some doubt about the validity of our experiments due to the fact that smokers self-regulate their nicotine intake, while the neonatal rats in this experiment were exposed to a fixed dosage of nicotine. The question of whether or not this model can be used to show the effects of pre-natal nicotine exposure on the mammalian respiratory control system arises. There are, however, three reasons why this specific dosage and method of administration were chosen. Firstly, the method of infusion does not replicate the ischemic effects of nicotine injection.

Second, infusion can be used to deliver a precise dose of nicotine equal to that of the blood levels recorded in an average smoker. Third, it can be seen in table 2 that the dose of nicotine used here does not stunt neonatal growth, as the weight of both saline-exposed and nicotine-exposed rats differ very slightly (Huang, et al, 2004). In fact, these reasons bring up the question of whether or not the use of nicotine patches and e-cigarettes is harmful during pregnancy. If it can be validated that the specific dosage and method of administration used in these experiments is viable, then it is reasonable to question the harmful effects of nicotine patch and e-cigarette use during pregnancy, as our results show that ventilatory output is altered, particularly at 33 degrees C. Although we did not show that DNE altered the ventilatory response to hyperthermia, some of the measured variables were close to being statistically significant, suggesting that studying a larger number of animals would reveal significant effects.

When compared to literature, the tidal volume, ventilation, and breathing frequency values were similar to values reported by others, validating the accuracy of the results (Bamford et al., 1996; Saetta and Mortola, 1985; St-John and Leiter, 1999). For example, our values for breathing frequency, 123 breaths/minute, and tidal volume, 0.08 ml, at P3 differ very slightly from Bamford et al., 1996 at 123 breaths/minute and 0.09 ml also at P3.

While the majority of our results lacked an appropriate level of significance, some trends can be seen within the data that could guide future research. First, tidal volume in nicotine-exposed neonates was consistently lower than that of saline-exposed neonates. This trend agrees with the findings of several other studies in neonatal rats (Huang, et al, 2004) and in neonatal lambs (Hafstrom, et al., 2002). The trend for breathing frequency, however, was opposite of the trends found in Huang, et al., 2004 and Hafstrom, et al., 2002, with saline-exposed rats having a higher breathing frequency than nicotine-exposed. Several aforementioned factors such as the hyperthermic conditions not being high enough and the neonates not being old enough could have led to these results.

Treatment effects were seen (with statistical significance) in ventilation and breathing frequency at 33°C. The DNE rats had consistently lower ventilation rates in relation to the saline-exposed

rats. This is viable data as similar results and trends have been found in several other studies including Hafstrom et al., 2002; Ueda et al., 1999; and Robinson et al., 2002.

One possible explanation for the lack of significant results could be the timeframe from which we were examining our neonates. Huang, et al (2004) found that nicotine-exposed neonates did not yield significant changes in tidal volume and breathing frequency until P10 was reached. All of our experiments were performed on day 2-4 of the neonates' life. It is possible that the changes did not have adequate time to develop yet. This is reasonable, as on P12 there is an important alteration in neurotransmitter receptor expression on the brain stem respiratory neurons. Future direction might include performing similar experiments on older neonates.

In conclusion, this experiment raises interesting trends that bear further examination. Future direction might include older neonatal rats tested at 33°C and 38°C, as well as a larger sample size for both saline-exposed and DNE rats. There is much to be learned about the influence of developmental nicotine exposure on the ventilatory response to hyperthermia.

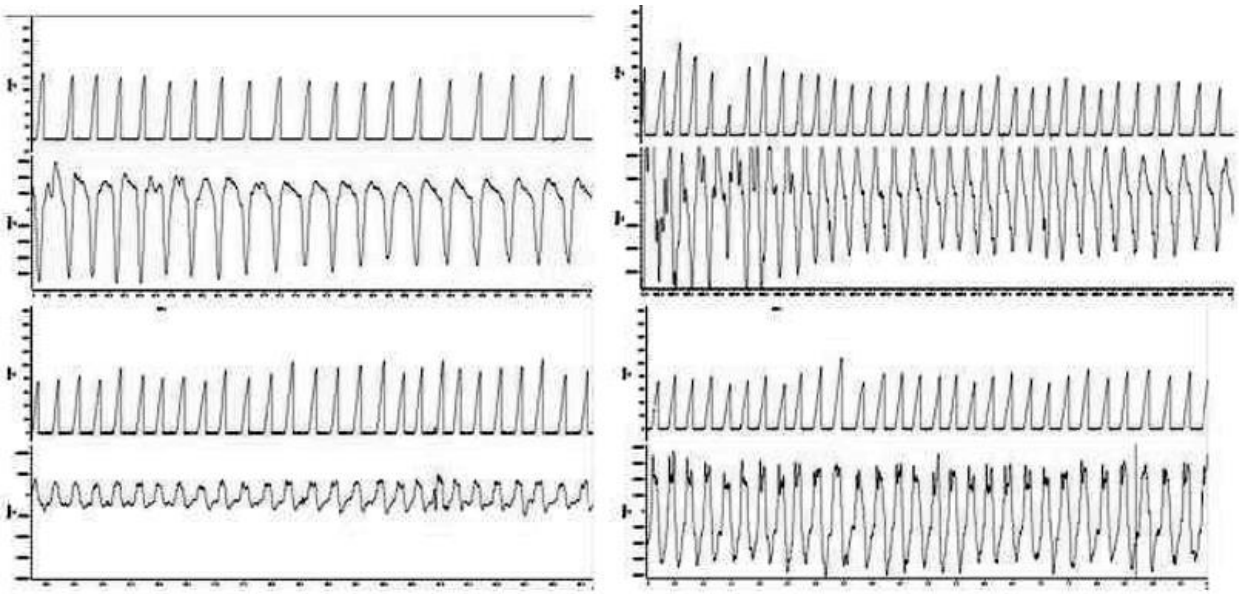

Fig. 1. Representative tidal volume recordings in two awake, P-3 neonatal rats that were DNE (top) or prenatally saline-exposed (bottom). The left side shows the recordings at 33°C and the right side show the recordings at 37°C.

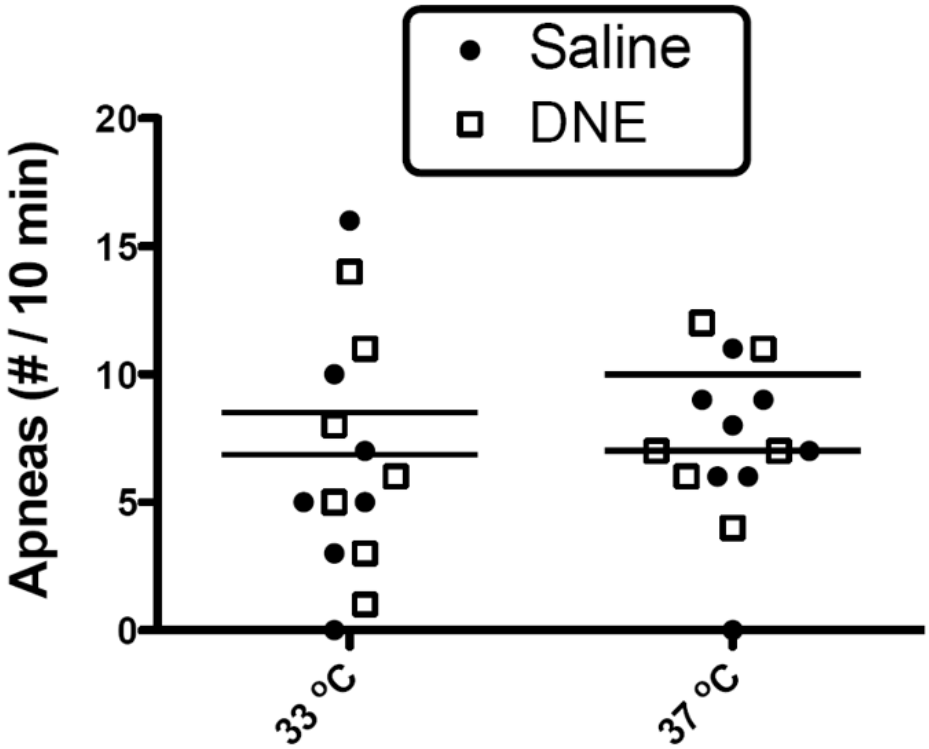

Fig. 2. The frequency of central apneic events during wakefulness at 33°C and 37°C in awake neonatal rats that were exposed to either saline (circles) or DNE (squares). The values are without outliers. No significance was found.

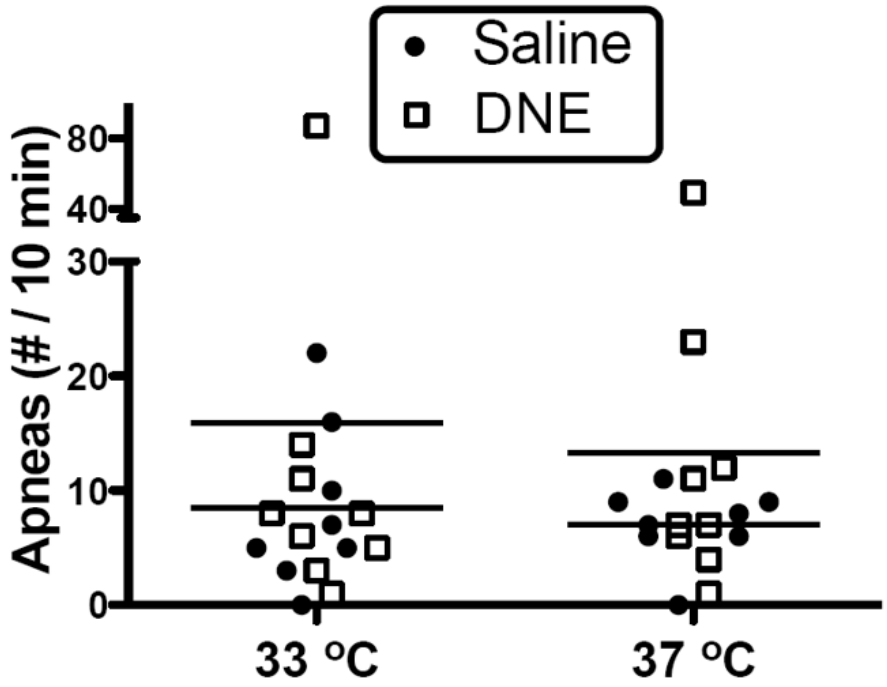

Fig. 3. The frequency of central apneic events during wakefulness at 33°C and 37°C in awake neonatal rats that were exposed to either saline (circles) or DNE (squares). The values are with outliers included. No significance was found.

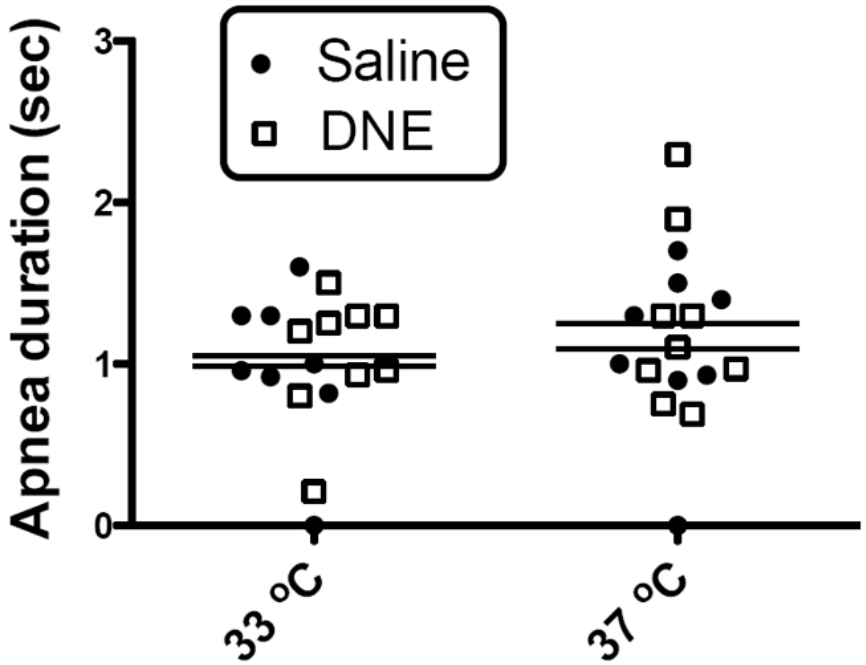

Fig. 4. The duration of central apneic events during wakefulness at 33°C and 37°C in awake neonatal rats that were exposed to either saline (circles) or DNE (squares). No significance was found.

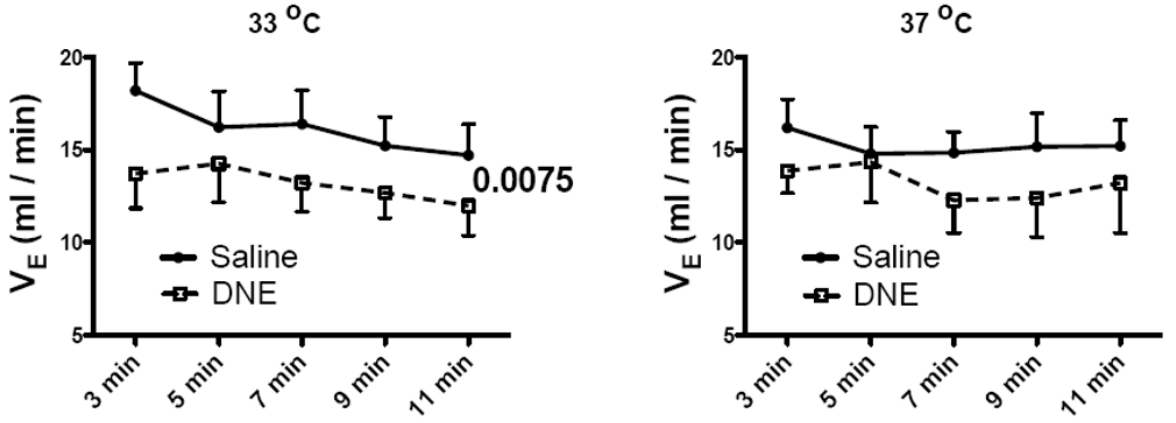

Fig. 5. Eupneic values for pulmonary ventilation rate in awake neonatal rats exposed to either saline (circles) or DNE (squares) at 33°C (left) and 37°C (right). At 33°C, a treatment effect can be seen with statistical significance (p=0.0075). There is a downward trend, with consistently higher saline-exposed values as opposed to DNE. Values at 37°C show no effect.

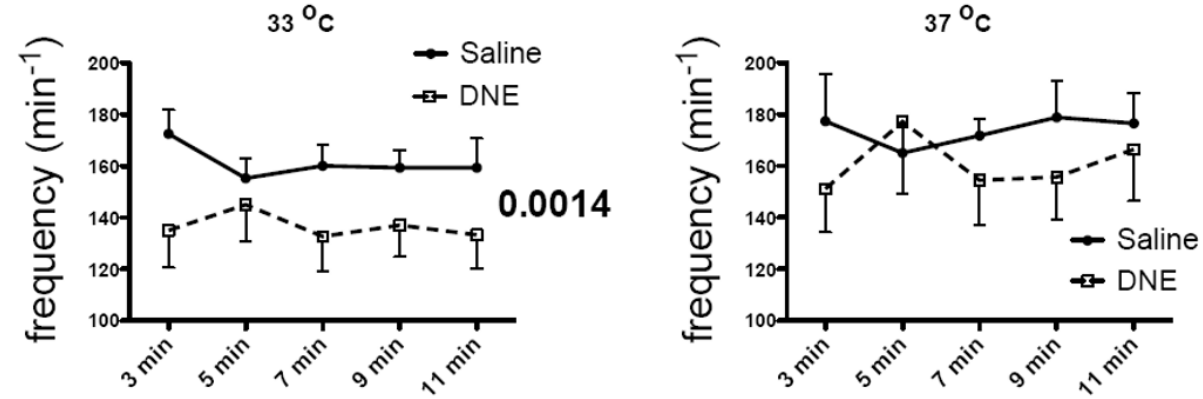

Fig. 6. Eupneic values for pulmonary breathing frequency rate in awake neonatal rats exposed to either saline (circles) or DNE (squares) at 33°C (left) and 37°C (right). At 33°C, a treatment effect can be seen with statistical significance (p=0.0014). There is a downward trend, with consistently higher saline-exposed values as opposed to DNE. Values at 37°C show no effect.

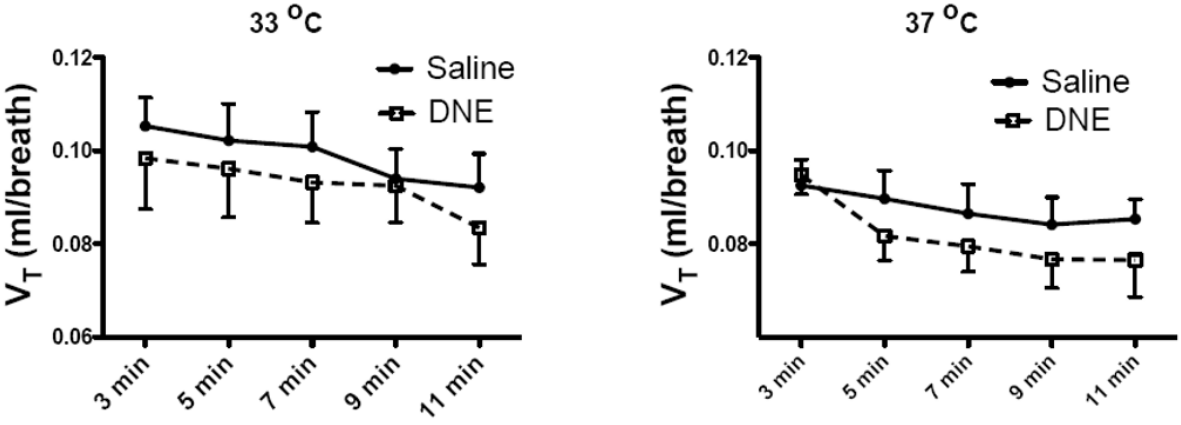

Fig. 7. Eupneic values for pulmonary tidal volume in awake neonatal rats exposed to either saline (circles) or DNE (squares) at 33°C (left) and 37°C (right). At 33°C, there is no effect. At 37°C, there is a downward trend, with consistently higher saline-exposed values as opposed to DNE; however, significance was just missed at p=0.12.

Temp. (° C)	**Saline**	**DNE**
33	**36.1**	**34.4**
37	**38.8**	**37.2**
33	**34.3**	**34.2**
37	**36.5**	**36.9**
33	**34.3**	**33.9**
37	**36.4**	**37.0**
33	**36.9**	**35.5**
37	**37.5**	**37.5**
33	**33.8**	**34.3**
37	**36.4**	**37.1**
33	**35.5**	**34.7**
37	**37.4**	**39.5**
33	**32.8**	**37**
37	**36.0**	**40.9**
33	**34.4**	**32.5**
37	**36.8**	**35.4**
33	**N/A**	**32.4**
37	**N/A**	**37.4**
Average @33:	**34.8+/- 1.3**	**34.3+/- 1.4**
Average @37:	**37.0+/- 0.90**	**37.3+/- 1.8**

Table 1 shows the core temp. of saline and DNE rats at 33° C and 37 ° C. It can benoted that the average temperatures do not differ much between saline and DNE rats.

	Saline	DNE
P2	8.87g	8.84g
P3	9.0g	9.2g
P4	13.8g	13.1g

Table 2 shows the average weight (in grams) of saline and DNE rats. It can be seen that the weight differs only slightly between the two groups.

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