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Review Article

Temporary forced oral breathing affects neonates oxygen consumption, carbon dioxide elimination, diaphragm muscles structure and physiological parameters

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ABSTRACT

Objectives: We studied adaptation of diaphragm, oxygen consumption and carbon dioxide elimination to forced oral breathing (lasting for only 4 days) following reversible bilateral nasal obstruction performed on day 8 post-natal male rats.
Methods: Diaphragm myosin heavy chain (MHC) composition, oxygen consumption, carbon dioxide elimination and hormones level were analysed during nasal obstruction period.
Results: Diaphragm muscle showed significant increases in adult isoforms (MHC 1, 2a) in oral breathing group versus control. Reversible nasal obstruction was associated with a decrease of oxygen consumption and carbon dioxide elimination. Nasal obstruction period was associated with reduced growth of the olfactory bulbs and an initial decrease in lung growth. One day after implementing nasal obstruction, basal corticosterone levels had increased (by over 1000). Oral breathing was also associated with a lower level of thyroid hormone.
Conclusions: We conclude that a 4 day nasal obstruction period in young rats leads to hormonal changes and to Diaphragm myosin heavy chain structural adaptation.

Contents

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1. Introduction

Nasal obstruction is considered a risk factor in sleep-disordered breathing [1–3], which has a very negative impact on quality of life in children and adults with increased daytime sleepiness [4]. This symptom resembles that of obstructive sleep apnea (OSA) caused by episodes of upper airway obstruction leading to episodic hypercapnic hypoxia which alters upper airway muscle structure and fibre type expression [5]. The most common clinical manifestations of OSA are nocturnal snoring, respiratory pauses, restless sleep and oral breathing [6]. This disturbed breathing is known to produce lethargy, cognitive and sleep impairment, especially in children [7,8]. Chronic nasal obstruction is a non-specific condition observed in many pathophysiological conditions e.g. allergic rhinitis, rhinosinusitis, adenoid hypertrophy and nasal polyps. Impaired nasal breathing results in obligatory oral breathing, which can be divided into two components: chronic absence of active nasal respiration that results in an olfactory deprivation [9] and chronic mouth opening [10]. Furthermore, in contrast to oral breathing, nasal breathing allows the optimal conditioning of inhaled air, clearing, moistening and warming the air before the gas exchange in the lungs [11,12]. Obligatory oral breathing has been observed in human babies and has been associated with a number of conditions that could have both short and long term effects on the physiology and thus behaviour of these infants later on in adolescence.

Stressful situations correspond to particular changes in environmental conditions that induce modifications in different physiological parameters like plasma hormonal levels. For example, stressful situations produce an adrenal hypertrophy and an increase of plasma glucocorticoid levels [13,14], which are known to induce alterations in MHC isoforms expression [15]. Plasma levels of thyroid hormones can be reduced in stressful situations and these hormones are affecting by severity airway obstruction [16,17].

To our knowledge no work has been published on oxygen consumption and carbon dioxide elimination changes during total closure of the nostril in neonates animals before any normal ageing processes could intervene.

Thus, our hypothesis was that early oral breathing (from postnatal day 9 to day 11) would be associated thus having a negative impact on oxygen consumption and carbon dioxide elimination associated diaphragm structure and physiological parameters.

2. Materials and methods

2.1. Animal care

Twenty eight males Wistar rats (IFFA-CREDO, France) were used for this experiment. The animals were born in the laboratory from 20 l, culled to 7 pups per litter to ensure normal body growth. The animals were housed in standard cages under controlled temperature conditions (22 ± 1° C). Food and water were available ad libitum throughout the experiment. From birth, the rats were kept on a reversed 12:12 light–dark cycle (dark period 08:00–20:00).

2.2. Nasal obstruction procedure

All experiments conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (no. 85-23, revised 1996), the recommendations of the European Community Council for the Ethical Treatment of Animals (no. 86/609/EEC)—and the regulations of the University of Lorraine. All efforts were made to minimise animal suffering. At the age of 8 days (D8), the litters were first anesthetised. Animals were weighed and they were then divided randomly into one control group and one experimental group (oral breathing). Bilateral nasal obstruction, resulting in forced oral breathing, was performed in experimental animals (7 per age) as described previously by [18]. The selected method consisted in the cauterisation of the external nostrils, which is the most common and simple procedure allowing spontaneous reopening of nostrils after 4 days. The tissue surrounding the external nostrils was burned by placing a surgical cauterising instrument (1 mm in diameter) on the nostrils, consequently occluding the orifice of the nostrils without mechanical or chemical damage to the olfactory mucosa. This procedure induced complete nasal obstruction between D8 and day 11 (D11) with 100% of the nostrils spontaneously reopened by day 15 (D15). The sampling experiments were conducted during complete nasal obstruction day 9 (D9) (Fig. 1).

The animals started breathing through their mouths immediately after nasal occlusion, as has been reported in infants [19]. Nostril cauterisation earlier in life resulted in rapid death of the pups. In the control group (7 per age), the nostrils were not sealed but the cauterising instrument was placed about 1–2 mm above each nostril to burn the skin. After cauterisation, the nostrils were washed with chlortetracycline (Aureomycine Evans 3%) to prevent infection. The pups were warmed (37 °C) for 30 min and returned to their mothers.

2.3. Olfactory bulbs and lungs

Seven pups male rats per group (control and oral breathing) and per age (D9, D11), were removed, immediately humanely killed and weighed. Olfactory bulbs and lungs were removed and weighed.

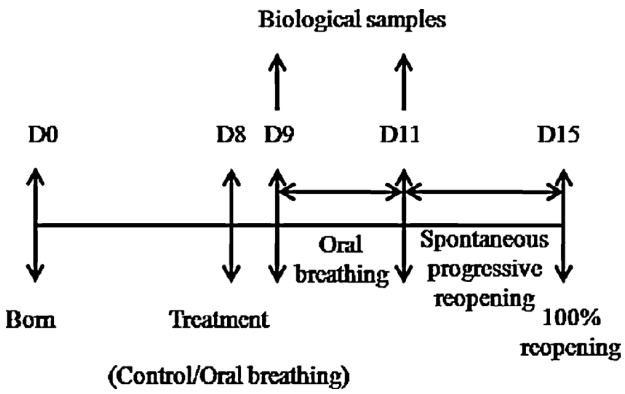

Fig. 1. Time line of the experimental protocol.

2.4. Muscle sampling and myosin extraction

After sample collection, the entire Diaphragm (Dia, respiratory muscle) was dissected. After dissection, muscles were weighed and myosin was isolated in a high ionic strength buffer, as described by [20].

2.5. Electrophoretic analysis of myosin heavy chain isoforms

Electrophoresis was performed according to the method of Talmadge and Roy [21], with little modification. This allowed the separation of the developmental MHC as described by [22,23]. Mini-gels were used in the Bio-Rad Mini-protean II Dual Slab Cell. Electrophoresis took place in a cold room (temperature of 6° C) for the whole run. To separate all the heavy chains, the duration of the run was 32 h (70 V). Three separate loads were made per sample (2.5 mg of protein/well). The MHC isoforms were identified according to migration rates compared with an adult diaphragm containing only adult isoforms 2a, 2x, 2b and 1 [24]. The gels were stained with Coomassie blue R-250.

The relative amounts of the different myosin heavy chains were measured using an integration densitometer Bio-Rad GS-800 and analysed with the Molecular Analyst Program (except "program" In Computers) (Quantity One 4.2.1)

2.6. Oxygen consumption and carbon dioxide elimination

The measurement of gas exchange was made using a pulse Oxymeter 0, 00-20,00MG/L (Fischer, France). The rats are placed in a respiratory chamber. Subsequently the animal is introduced into the chamber for a period of 5 min. At the end of the experiment, the oxygen consumption (O_2) and carbon dioxide (CO_2) elimination were measured.

2.7. Hormone assays

Corticosterone concentration was measured without an extraction procedure, using a commercially available EIA kit and performed according to the manufacturer's guidelines (Assay Designs Inc., USA). The concentration of corticosterone in plasma samples was calculated from a standard curve and expressed as ng/ml. The intra- and inter-assay coefficients of variation were under 8.4% and 13.1%, respectively, for corticosterone, 10.8% and 14.6%, respectively.

Thyroxine (T4) and triiodothyronine (T3) were assayed using commercial RIA kits and performed according to the manufacturer's guidelines (Immunotech SA, Marseille,France). The concentrations of T4 and T3 in plasma samples were calculated from standard curves and expressed as pg/ml. The intra- and inter-assay coefficients of variation were, respectively, under 6.7 and 6.5% for T4 and under 6.4 and 5.5% for T3.

Table 1
Effects of temporary forced oral breathing on body weight (g), olfactory bulbs and lungs specific weights (mg/g).

Group	D9	D11
Control group ($N=7$)		
Body weight (g)	18.60 ± 0.49	23.08 ± 0.59
Olfactory bulb (mg/g)	1.62 ± 0.13	1.21 ± 0.03
Lungs (mg/g)	20.60 ± 0.52	18.83 ± 0.54
Oral breathing ($N=7$)		
Body weight (g)	15.51 ± 0.48	19.75 ± 0.79*
Olfactory bulb (mg/g)	1.12 ± 0.12*	0.88 ± 0.04*,#
Lungs (mg/g)	20.61 ± 1.12	16.20 ± 0.02*,#

Note: Total of rat per group and per age.
Significant difference between D9 and D11 $P<0.05$.
* Significant differences from control vs oral breathing group $P<0.05$.

2.8. Statistical analysis

The results were expressed as group means ± SEM. Student's *t*-test was used to establish the comparison between control and oral breathing animals since all data were normally distributed. Body weight group differences were determined using a two-way ANOVA (factor treatment × factor age). Specific mean comparisons were then made using *t*-test with the Bonferroni correction. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Morphometric characteristics

Before treatment, at 8 days of age, the body weights of control and oral breathing pups were not significantly different: 17.78 ± 0.52 g and 17.95 ± 0.28 g, respectively, ($P > 0.14$). Table 1 shows that there was a significant difference in body weight already at D9 ($P < 0.05$) which continued on D11 ($P < 0.05$) between control and oral breathing rats. Body weight of animals decreases by 14% at D9 in the oral breathing group compared to weights at D8 (respectively, 15.51 g vs 17.95 g) and also by 14% compared to control at D11 (respectively, 19.75 g vs 23.08 g).

Relative organ weights are presented in Table 1. Olfactory bulb weights: a significant reduction in olfactory bulb weight was found for the two ages in the oral breathing group compared to control animals ($P < 0.05$). The reduction was 30% during nasal obstruction in oral breathing males compared to control animals. Lung

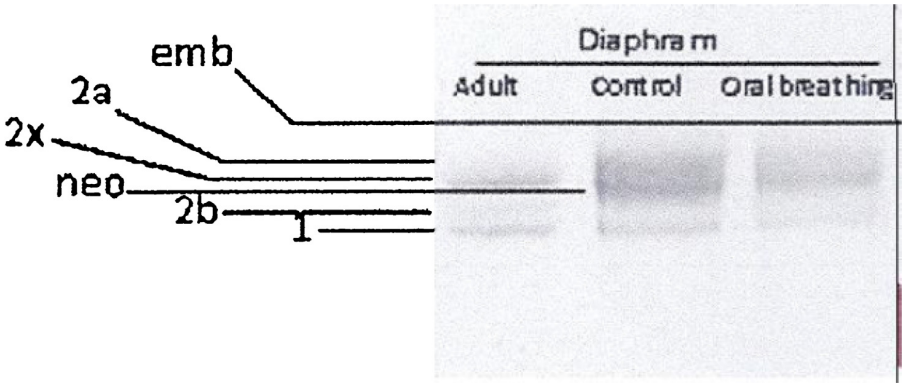

Fig. 2. Example of effects of temporary forced oral breathing on myosin heavy chain expression in four skeletal muscles: embryonic (emb), neonatal (neo), adult fast 2a, adult fast 2x, adult fast 2b, and slow adult 1 type.

Table 2
Myosin heavy chain distribution in diaphragm in control and rats exposed to temporary forced oral breathing.

Days	Groups ($N=7$)	emb	neo	1	2a	2x	2b
D9	Control		54 ± 0.01	9 ± 0.07	37 ± 0.02		
	Oral breathing		55 ± 0.01	$13 \pm 0.01^{*}$	$32 \pm 0.02^{*,\#}$		
D11	Control		$78 \pm 0.08^{\#}$	10 ± 0.01	$12 \pm 0.01^{\#}$		
	Oral breathing		$41 \pm 0.01^{*,\#}$	$18 \pm 0.04^{*,\#}$	$41 \pm 0.09^{*,\#}$		

Note: Total of rat per group and per age. emb: embryonary myosin isoform; neo: neonatal myosin isoform.
$^{\#}$ Significant difference between D9 and D11 $P < 0.05$.
* Significant differences from control vs oral breathing group $P < 0.05$.

weights: A significant reduction of lung weight was observed only on D11 in the oral breathing group compared to the control group ($P < 0.05$). The reduction was 14% after three days of nasal obstruction.

3.2. MHC isoform expression in neonatal during oral breathing (D9, D11).

Based on densitometric analysis of the SDS–PAGE, the relative MHC isoform compositions of diaphragm muscles were determined. Results are shown in Fig. 2. The order of increasing electrophoretic mobility of developmental and adult MHC isoforms is as follows: embryonic myosin isoform (emb), adult fast 2a, adult fast 2x, neonatal myosin isoform (neo), adult fast 2b, and slow adult 1 type.

In the neonatal diaphragm muscle at D9 and D11, MHC neonatal (neo) and adult isoforms were observed in both control and oral breathing animals. In the diaphragm we found a significant difference in the relative electrophoresis distributions of MHC isoforms between control and oral breathing animals (Table 2). Oral breathing was associated with an increase of MHC 1 in the diaphragm at D9 and D11 ($P < 0.05$).

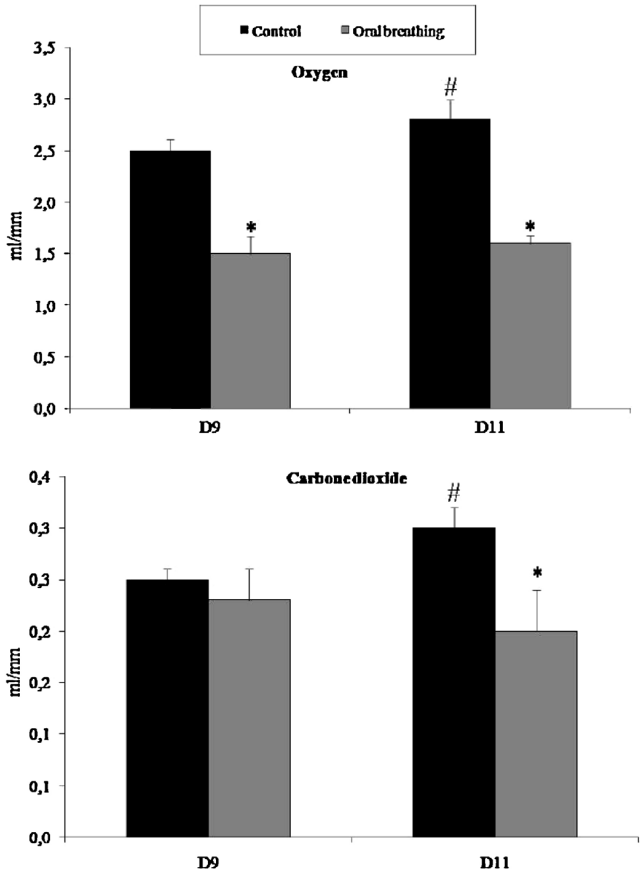

Fig. 3. Oxygen consumption and carbon dioxide elimination in control and rats exposed to temporary forced oral breathing. $^{\#}$ Significant difference between D9 and D11 $P < 0.05$ *Significant differences from control vs oral breathing group $P < 0.05$.

3.3. Oxygen consumption and carbon dioxide elimination

Before treatment, at 8 days of age, the average oxygen consumption and carbon dioxide elimination of control (O2: 2 ml/mm; CO2: 0.2 ml/mm) and oral breathing pups (O2: 2.2 ml/mm; CO2: 0.25 ml/mm) during 5 min were not significantly different. Fig. 3 shows that There was a significant decrease in the O2 consumption at D9 ($P < 0.05$) and D11 ($P < 0.05$) during 5 min.

The oxygen consumption decrease, respectively, by 40% at D9 and 46% at D11 in the oral breathing group compared to control group. At D11 we observe a decrease by 33% in carbon dioxide elimination between oral breathing and control group ($P < 0.05$).

3.4. Hormone assays

As shown in Table 3 impact of oral breathing in corticosterone and thyroid homones plasma level. Plasma corticosterone levels were significantly different between the experimental groups at D9 and D11.

Twenty-four hours after treatment, nasal obstruction was associated with a significant augmentation in corticosterone ($P < 0.05$). At D11 plasma corticosterone level were significantly increased in oral breathing rats ($P < 0.05$). Twenty-four hours after the treatment (D9), thyroxin (T4) concentration was significantly reduced in nasal obstruction compared to control animals ($P < 0.05$): At D9, plasma T4 levels were significantly reduced by nasal obstruction (24% $P < 0.05$). This difference in plasma T4 levels was maintained at D11 (15%; $P < 0.05$). Plasma triiodothyronine (T3) levels were significantly different between the experimental groups at all ages tested ($P < 0.05$). Indeed animals with nasal obstruction had lower levels of T4 throughout the period studied. The reduction was 49% at D9, 23% at D11.

4. Discussion

We have shown for the first time that a few days of forced oral breathing during reversible nasal obstruction-induced decrease of oxygen consumption and carbon dioxide release. This finding can be explained by the effect of nasal obstruction: it induces an increase in lung resistance and a decrease in lung compliance, affecting chest and leading to inadequate alveolar ventilation [25].

Our results show that nasal obstruction causes early changes in structural development of the respiratory muscle, which begins within 24 h after nasal obstruction. During the nasal obstruction period, our results showed a decrease in neo (the predominant neonatal isoform) to the benefit of MHC 1, 2a (the mature isoform) in the diaphragm. These results showed that nasal obstruction induced accelerated structural development of the diaphragm. During development muscles usually change directly from

Table 3
Impact of temporary forced oral breathing on plasma corticosterone and thyroids hormones levels.

Days	Groups ($N=7$)	Thyroxine (pg/ml)	Triiodothyroxine (pg/ml)	Corticosterone (ng/ml)
D9	Control	10 ± 0.5	3 ± 0.6	2.66 ± 0.1
	Oral breathing	$5 \pm 0.8^{*,\#}$	$1.5 \pm 0.01^{*}$	$72.60 \pm 5^{*,\#}$
D11	Control	$18 \pm 1.2^{\#}$	$6 \pm 0.4^{\#}$	$15.45 \pm 1.3^{\#}$
	Oral breathing	$14 \pm 0.9^{*}$	$3 \pm 0.3^{*,\#}$	$164.84 \pm 8.4^{*}$

Note: Total of rat per group and per age.
$^{\#}$ Significant difference between D9 and D11 $P < 0.05$.
* Significant differences from control vs oral breathing group $P < 0.05$.

embryonic to neonatal to fast, or from embryonic to neonatal to slow isoforms [26,27] have shown that neo increases between D0 to D14 and thereafter decreases to disappear at the age of 28 days. Our results showed that between D9 and D11 neo increased in control and decreased in nasal obstruction animals, which is in accordance with the results [27] for control animals. This leads apparently to an accelerated maturation for the forced oral breathing animals because the decrease of neo was to the benefit of MHC adult isoforms. Thus, oral breathing rats presented a profile in MHC adapted to the transition from nasal to oral breathing, in other words a change facilitating respiration. This is in agreement with results in the literature [28] that show that environmental conditions (such as hypergravity) could induce structural changes in the development of the muscles. The first day of nasal obstruction appears to be a significant stress that stimulated a large release of corticosterone, especially in the oral breathing. A similar stress effect has been observed previously by [29] looking at facial muscle changes with oral breathing at weaning. Neonates exposed to nasal obstruction showed a decrease in plasma T4 and T3 levels. Different factors could be involved in these modifications amongst which are nutritional depletion and the associated secretion of glucocorticoids. A suppressive impact of nutritional deprivation on T3 and T4 levels has been shown [30], and these effects could be mediated by activation of the hypothalamo–pituitary–adrenal axis [31]. Hypothyroidism observed in animals exposed to nasal obstruction could produce several deleterious effects such as a maturation defect of the central nervous system [32], or a decrease of basic metabolism and thermogenesis [33]. The severity of airway obstruction is also know affects thyroid gland function [3,4].

Our model of temporary nasal obstruction could be an appropriate model for looking at potential changes in hormones and other physiological parameters of obstructive sleep- disordered breathing. In conclusion, the present study has shown that the respiratory activity and diaphragm muscle structure can be altered by temporary forced oral breathing. The observed changes began very early during the period of nasal obstruction. Indeed, in animals exposed to temporary forced oral breathing, muscle involved in oxygen consumption and dioxid elimination presented an increased relative expression of fatigable MHC isoforms. These modifications could contribute in some part to various human pathologies, and it would interesting to specify the factors that act to produce these changes in respiratory activity and muscular structures could be involved.

This could indicate that our model of temporary nasal obstruction could be an appropriate model for looking at potential changes physiological parameters of rhinitis or other temporary obstructive nasal breathing pathology.

Conflict of interest

The authors declare that they have no conflict of interest.

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