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Life-long consequences of postnatal normoxia exposure in rats raised at high altitude

Delphine Lumbroso,[1] Alexandra Lemoine,[1] Marcelino Gonzales,[2] Gabriela Villalpando,[2] Tommy Seaborn,[1] and Vincent Joseph[1]

[1]*Department of Pediatrics, Laval University, Centre de Recherche Centre Hospitalier Universitaire de Québec, Hôpital St-François d'Assise, Quebec, Canada; and* [2]*Instituto Boliviano de Biología de Altura, Universidad Mayor de San Andrés, La Paz, Bolivia*

Lumbroso D, Lemoine A, Gonzales M, Villalpando G, Seaborn T, Joseph V. Life-long consequences of postnatal normoxia exposure in rats raised at high altitude. *J Appl Physiol* 112: 33–41, 2012. First published October 13, 2011; doi:10.1152/japplphysiol.01043.2011.—We tested the hypothesis that exposure of high-altitude (HA) rats to a period of postnatal normoxia has long-term consequences on the ventilatory and hematological acclimatization in adults. Male and female HA rats (3,600 m, $Po_2 \simeq 100$ Torr; La Paz, Bolivia) were exposed to normal room air [HA control (HACont)] or enriched oxygen (32% O_2; $Po_2 \simeq 160$ Torr) from 1 day before to 15 days after birth [HA postnatal normoxia (HApNorm)]. Hematocrit and hemoglobin values were assessed at 2, 12, and 32 wk of age. Cardiac and lung morphology were assessed at 12 wk by measuring right ventricular hypertrophy (pulmonary hypertension index) and lung air space-to-tissue ratio (indicative of alveolarization). Respiratory parameters under baseline conditions and in response to 32% O_2 for 10 min (relieving the ambient hypoxic stimulus) were measured by whole body plethysmography at 12 wk. Finally, we performed a survival analysis up to 600 days of age. Compared with HACont, HApNorm rats had reduced hematocrit and hemoglobin levels at all ages (both sexes); reduced right ventricular hypertrophy (both sexes); lower air space-to-tissue ratio in the lungs (males only); reduced CO_2 production rate, but higher oxygen uptake (males only); and similar respiratory frequency, tidal volume, and minute ventilation. When breathing 32% O_2, HApNorm male rats had a stronger decrease of minute ventilation than HACont. HApNorm rats had a marked tendency toward longer survival throughout the study. We conclude that exposure to ambient hypoxia during postnatal development in HA rats has deleterious consequences on acclimatization to hypoxia as adults.

development; lungs; breathing

POSTNATAL DEVELOPMENT IN MAMMALS is a key period during which environmental stimuli induce immediate physiological responses, but also modulate development, leading to long-lasting physiological alterations in adulthood. Along with other nutrients, oxygen is crucial for adequate development. It is well documented that postnatal hypoxia blunts growth (16) and induces several alterations of the respiratory system in adults. In rats and mice, postnatal hypoxia results in a lung tissue with fewer but larger alveoli, reduced surface-exchange area, limitations of respiratory gas exchange, and signs of pulmonary hypertension (6, 31, 50). Similarly, adequate development of the respiratory control system is also dependent on postnatal oxygen level. Exposure to hypoxia during early life delays the postnatal maturation of ventilatory response to hypoxia (17, 21), reduces respiratory chemoreflex in

adult rats (3, 39), and reduces carotid body responses to hypoxia and hypercapnia (25). Typically, the period during which neonatal hypoxia induces such long-term changes includes the first 2 wk of postnatal development (3, 31, 39, 50). These experimental studies linking postnatal hypoxia with blunted chemoreflex responses help to explain results from human studies, showing that minute ventilation ($\dot{V}_E$), but also hypoxic ventilatory response are decreased in high-altitude residents compared with sea-level natives acclimatized to altitude (13, 23, 45, 46).

Yet and despite the fact that several high-altitude regions over the world are densely populated with fast-growing populations, we have little information related to the potential long-lasting consequences of exposure to ambient hypoxia during early development in the specific context of life-long hypoxic exposure. One of the important health issues for residents at high altitude is the development of chronic mountain sickness, a form of "mal-acclimatization" syndrome to hypoxia, characterized by high erythrocytosis, hypoventilation, and pulmonary hypertension (26). So far, the origin of this syndrome remains unknown, but recent studies have provided interesting insights linking perinatal hypoxia to chronic mountain sickness (35).

Accordingly, in the present study, we tested the hypothesis that, in rats living at high altitude, postnatal hypoxia elicits a long-lasting influence on the acclimatization process to chronic hypoxia. To test this hypothesis, rats from the Bolivian Institute for Altitude Biology (IBBA, La Paz, Bolivia, 3,600 m above sea level), implanted there more than 15 yr ago, were born and raised for 2 wk after birth in a room filled with a gas mixture containing 32% O_2, recreating the sea-level Po_2. Our results show that postnatal exposure to sea-level Po_2 reduces hematocrit (Hct) and hemoglobin (Hb) levels, attenuates right ventricular (RV) hypertrophy, and improves lung architecture, resulting in higher oxygen uptake ($\dot{V}o_2$). These results are associated with a marked tendency toward a reduced mortality in high-altitude rats. We conclude that exposure to ambient postnatal hypoxia is a key factor that might contribute to the development of a form of mal-acclimatization to hypoxia in high-altitude rats.

MATERIALS AND METHODS

Animals

All experiments have been performed on rats from a colony bred since 1992 in the IBBA, La Paz, Bolivia (3,600 m, mean atmospheric pressure = 490 mmHg, corresponding to a $Po_2 = 100$ Torr). We used a total of 81 male and 79 female Sprague-Dawley rats from 14 primiparous females. The animals have access to water and food ad libitum and are maintained under a 12:12-h light-dark cycle, with controlled humidity and temperature. The protocol design has been reviewed and approved by

Table 1. *Body weight at the age of 2, 12, and 32 wk in male and female rats raised under control conditions at high altitude, or exposed to normoxia*

	2 wk	12 wk	32 wk
HACont male	23.6 ± 0.5	299 ± 11	379 ± 4
HACont female	24.0 ± 0.4	185 ± 5*	219 ± 11*
HApNorm male	24.1 ± 0.5	297 ± 3	376 ± 8
HApNorm female	23.5 ± 0.5	184 ± 2*	240 ± 6*

Values are means ± SE in g; *n* for each age, sex, and group are as follows for male HACont, male HApNorm, female HACont, and female HApNorm, respectively: 28, 20, 20, 17 (2 wk); 17, 17, 16, 16 (12 wk); 12, 14, 8, 16 (32 wk). HACont, high-altitude control; HApNorm, high-altitude postnatal normoxia. *$P < 0.05$, males vs. females for corresponding group.

the scientific committee of IBBA and was in accordance with the guidelines of the Canadian Council on Animal Care.

General Experimental Design

The control group [high-altitude control (HACont)] are rats continuously exposed to ambient room air (Po_2 = 100 Torr), while another group comprised rats born and raised for the first 2 postnatal wk in a sealed room flushed with 32% O_2 (postnatal normoxia group or HApNorm; Po_2 = 160 Torr, i.e., the sea-level partial pressure of O_2). This postnatal period corresponds to functional development of the respiratory control system and lungs (11, 37) and also includes the critical time window during which hypoxic exposure induces long-lasting effects on these systems (3, 31, 39, 50). After this period of postnatal exposure, rats were returned to standard conditions. All experiments were conducted in parallel: pregnant female rats were alternatively assigned to the HACont or HApNorm group. Some animals were used at the age of 2, 12, and 32 wk of age (for body weight, Hct, and Hb levels). Adult rats at the age of 12 wk were either killed to perform cardiac dissections and lung tissues sampling, or used to determine respiratory parameters, $\dot{V}o_2$, and CO_2 production ($\dot{V}co_2$) rate using whole body plethysmography in an open-flow system. Finally, a subsample of rats was followed up to 600 days of age to report occurrence of spontaneous death.

Specific Experimental Details

Normoxia exposure in newborn rats at high altitude. We used standard reproduction guidelines to obtain time-pregnant female rats. One day before delivery, pregnant females were placed in a 50-liter Plexiglas chamber. HApNorm pups were raised under hypobaric normoxia (32% O_2, corresponding to a Po_2 of 160 Torr) until postnatal *day 15*. O_2 level inside the chamber was maintained by a continuous flow of calibrated gas permanently flushed through the chamber. CO_2 level was checked twice daily with a dedicated CO_2 sensor and never exceeded 0.3%. The air inside the chamber was continuously mixed with a small fan. The chamber was opened every 3–5 days for 15 min to clean the cages. HACont pups were raised under ambient hypobaric hypoxia (21% O_2, corresponding to a Po_2 of 100 Torr) in the same room (outside the Plexiglas chamber). After 2 wk of exposure, the animals were returned to room air.

Hematological parameters. Blood samples were drawn in nonanesthetized rats by a tail puncture. Hct was measured by microcentrifugation, and the Hb concentration was determined using the Hemocue field spectrophotometer (Agelholm, Sweden). All samples were processed in duplicate.

RV hypertrophy. The animals were anesthetized by an intraperitoneal injection (0.1 ml/100 g body wt) of ketamine (87.5 mg/ml) and xylazine (12.5 mg/ml) and then perfused through the heart with iced-cold PBS. The heart was dissected out, quickly rinsed in saline, and weighted. The atria were separated from the ventricles; the RV was cut off from the left ventricle (LV) with the septum (S) left as a part of LV. The RV and the (LV + S) were immediately weighed and used to report the ratio of RV/(LV + S), an index of RV hypertrophy, which is directly related to pulmonary hypertension (41).

Lung morphology. To evaluate lung morphology and histology, another group of rats was perfused through the heart with iced-cold PBS. After perfusion, small fragments ($\approx 4 \times 4 \times 4$ mm) sampled from the distal lateral region of the left lobe were cut and fixed in 4% paraformaldehyde solution overnight at 4°C, cryoprotected in 30% sucrose solution during 1–2 days, stored at −80°C, and then shipped to Quebec on dry-ice. Sections 10 μm thick were cut with a cryostat (Leica, CM 1900), mounted on glass slides, countercolored with Harris hematoxylin solution (VWR), rinsed in water for 1 min, exposed in acid-alcohol solution (5 successive immersions in 1% HCl, 70% ethanol), washed with water, and dipped in Bluing Reactif RTU (VWR) and then in water for 1 min. The slides were then mounted in SHUR/MOUNT Liquid Mounting Medium. Images were captured using a Nikon digital imaging system at ×100 magnification. We randomly selected 16 non-overlapping fields from each slide, using 2 slices per animal and 5 animals/group. The air space-to-tissue ratio was determined by the point-counting method (33) by applying a 10×10 grid on each image and counting the ratio of intersections falling on air space to intersections on tissue.

Survival analysis. Rats from the HACont and HApNorm groups were followed to report spontaneous death up to 600 days of life. Death was described as spontaneous when an animal was found dead in its cage without any previous signs of suffering (lordosis or
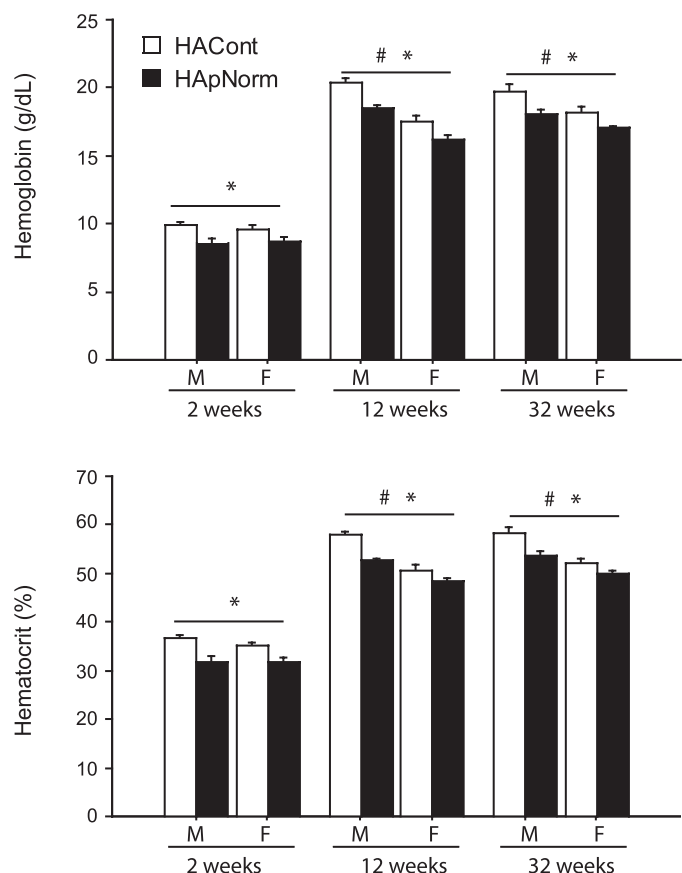

Fig. 1. Hemoglobin (g/dl) and hematocrit (%) at the age of 2, 12, and 32 wk in male (M) and female (F) rats raised under control conditions at high altitude (HACont), or exposed to postnatal normoxia (HApNorm). Values are means ± SE; *n* for each age, sex, and group are as given in Table 1 legend. *$P < 0.05$, HACont vs. HApNorm for corresponding sex. #$P < 0.05$, males vs. females for corresponding group. * or # above a bar indicates a group or sex effect without interaction.

piloerection) or weight loss. A total of 16 HACont males, 8 HACont females, 10 HApNorm males, and 11 HApNorm females were included in this survey. For each group, we reported the percentage of surviving animals throughout the duration of the study.

Recording of Respiratory Parameters, $\dot{V}_{O_2}$, and $\dot{V}_{CO_2}$

Setup details. Ventilatory parameters were measured in awake and unrestrained rats using a whole body plethysmograph chambers designed by Emka Technologies for adult rats, as previously described (14, 31). The respiratory flow trace was recorded using a differential pressure transducer (ML141, ADInstruments, Colorado Springs, CO). The flow of air through the chamber was set and continuously monitored at 1,000 ml/min using a pump and gas flow restrictor/monitor (G265, Qubit systems, Kingston, ON, Canada). Inlet and outlet gases were alternatively subsampled, directed toward a water pressure analyzer (RH-300, Sable System, Las Vegas, NV), and then the air was dried and directed to an oxygen/carbon dioxide analyzer (ML206 Gas analyzer, AD Instruments) for respiratory gases analysis. All signals (plethysmograph, gas analyzers, and flowmeter) were directed toward a PowerLab acquisition interface for analog-to-digital conversion and storage on a computer running the Chart software (AD Instruments).

Recording routine. After calibration of the chamber with a known volume of air (5 ml), the animal was placed in the chamber for a period of tranquilization (30–45 min). Rectal temperature was measured once the animal laid quiet for at least 5–10 min. Next, basal recordings with ambient air were initiated for 10 min when the animal was quiet after temperature measurements (usually after 5–10 min). The chamber was then flushed with a gas mixture ensuring exposure to 32% O_2 (P_{O_2} = 160 Torr, or sea-level value) within 2–3 min. This O_2 level was maintained for 10 min, then the chamber was immediately opened to measure rectal temperature.

Analysis of respiratory parameters, $\dot{V}_{O_2}$, and $\dot{V}_{CO_2}$. Respiratory frequency (fR) and tidal volume (V_T) were recorded from the plethys-mograph signal. V_T was calculated based on barometric pressure, room and body temperature, and humidity using the Bartlett and Tenney equation (2). $\dot{V}_E$ was calculated as $\dot{V}_E = fR \times V_T$. According to our setup (29), $\dot{V}_{O_2}$ was calculated as $\dot{V}_{O_2} = $ flow $\times [(O_2 \text{ in} - O_2 \text{ out}) - O_2$ out $\times (CO_2 \text{ out} - CO_2 \text{ in})]/(1 - O_2 \text{ out})$, and $\dot{V}_{CO_2}$ as $\dot{V}_{CO_2} = $ flow $\times [(CO_2 \text{ out} - CO_2 \text{ in}) - CO_2 \text{ out} \times (O_2 \text{ in} - O_2 \text{ out})]/(1 - CO_2 \text{ out})$. All metabolic volumes are expressed in conditions of ambient atmospheric temperature and pressure (ATP) and expressed per 100 grams of body weight. These values were used for calculation of the ventilatory equivalents for carbon dioxide ($\dot{V}_E/\dot{V}_{CO_2}$), oxygen ($\dot{V}_E/\dot{V}_{O_2}$), and the respiratory exchange ratio ($\dot{V}_{CO_2}/\dot{V}_{O_2}$). Ventilatory and metabolic values were averaged over the 10 min of recordings in room air and then over the last 5 min of exposure to 32% O_2.

Statistical Analysis

We performed two-way ANOVA (Statview 5.0, SAS Institute, Cary, NC) for each parameter using sex and group (HACont or HApNorm) as independent variables. When values are obtained at different ages, they were analyzed separately for each age (i.e., the effect of age was not tested). If a significant effect or interaction between sex and group appeared, the data were split to perform relevant ANOVA using group or sex as the independent variable. Significance of P value was set at 0.05. Data are reported as means ± SE.

Survival curves were constructed using the product limit method of Kaplan and Meier and were compared using the log-rank test with GraphPad Prism version 4.0c (GraphPad Software).

RESULTS

Postnatal Normoxia Reduces Hct and Hb Level

HApNorm rats had similar body weight to HACont rats at 2, 12, and 32 wk of age for both males and females (Table 1). On
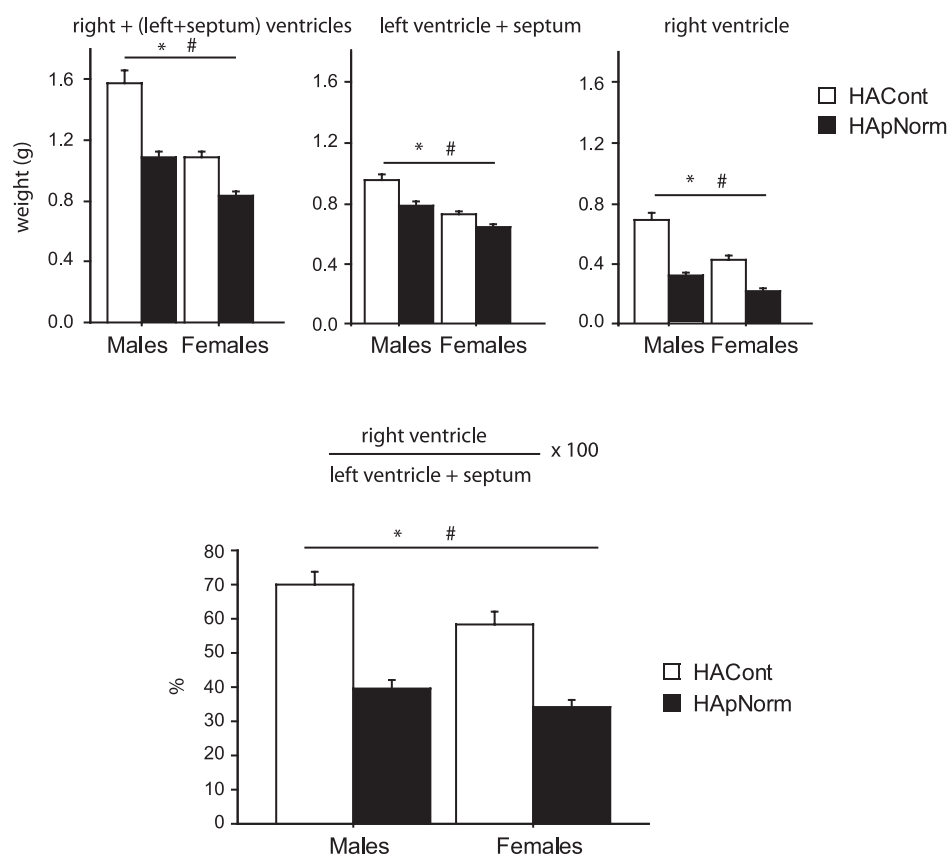
Fig. 2. Weight (g) of the right + (left + septum) ventricles, left ventricle + septum, right ventricle, and %right-to-left ventricle weight ratio in 12 wk-old male and female HACont and HApNorm rats. Values are means ± SE; *n* for each age, sex, and group are as follows: male HACont = 17 and HApNorm = 20, female HACont = 16 and HApNorm = 19. *$P < 0.05$, HACont vs. HApNorm for corresponding sex. #$P < 0.05$, males vs. females for corresponding group. * or # above a bar indicates a group or sex effect. Interaction terms are noted in the text.

the other hand, in both juvenile and adult HApNorm rats, Hct and Hb levels were lower compared with HACont (Fig. 1, P values for group effects at 2, 12, and 32 wk are <0.0001, <0.0001, and 0.0006 for Hct; and 0.0007, <0.0001, and 0.0009 for Hb, respectively). This effect of treatment was not sex specific (P value for sex $\times$ group = nonsignificant). In 12- and 32-wk-old females (of both groups), the mean Hb and Hct values were lower than in corresponding males (P value for sex effect <0.0001 at 12 and 32 wk for Hct, $P < 0.0001$ and $P = 0.0015$ at 12 and 32 wk, respectively, for Hb). The reduced Hct and Hb level in 2-wk-old rats indicates that normoxia exposure effectively relieved hypoxic stress during postnatal development, whereas the fact that these differences are maintained up to 32 wk of age (i.e., 7.5 mo after the end of the normoxic exposure) shows the profound, long-term effect of the postnatal period on hypoxic acclimatization later in life.

Postnatal Normoxia Reduces Heart Weight and Ventricular Hypertrophy

HApNorm rats had a reduced RV + LV weight (Fig. 2, P value for group < 0.0001), this effect was more prominent in males compared with females (P value for group $\times$ sex = 0.017). The (LV + S) weight was only slightly reduced by postnatal normoxia (P value for group < 0.0001), with a similar effect in males and females (P value for group $\times$ sex = 0.12). The weight of the RV was drastically reduced by postnatal normoxia (P value for group < 0.0001), with a more prominent effect in males than females (P value for group $\times$ sex = 0.012). The ratio of RV to (LV + S) was also decreased by postnatal normoxia (P value for group = 0.009), but to a similar extend between males and females (P value for group $\times$ sex = 0.33). HACont females had a reduced RV
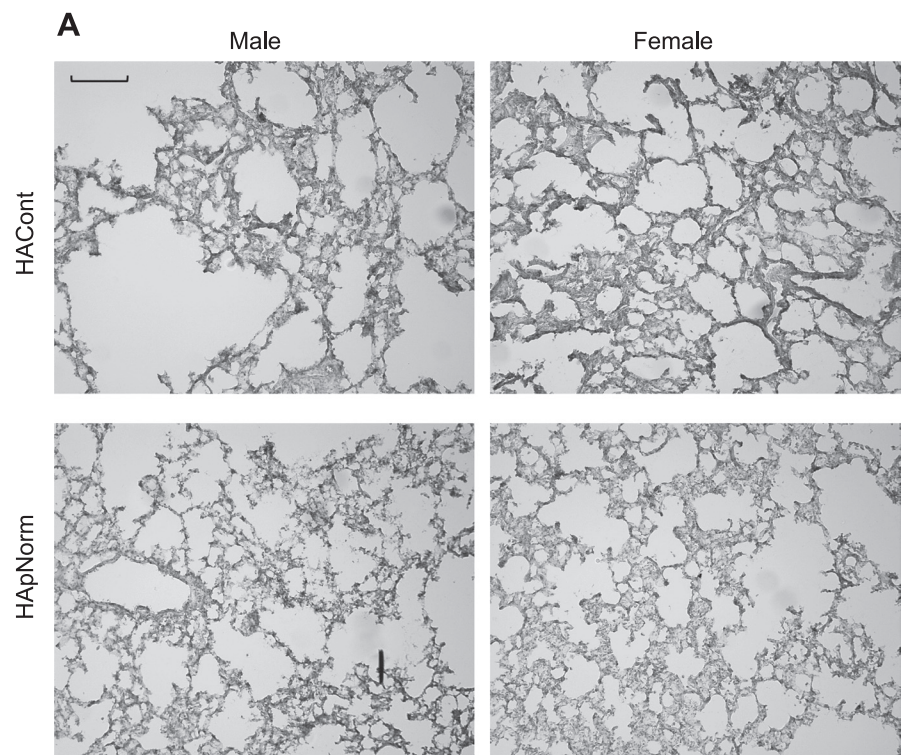

Fig. 3. Representative lung morphology picture and air space-to-tissue ratio at the age of 12 wk in male and female HACont and HApNorm rats. *A*: hematoxylin staining ($\times$100). *B*: air space-to-tissue ratio histogram for corresponding groups. Values are means $\pm$ SE; $n = 5$ animals in each group. *$P < 0.05$, HACont vs. HApNorm for corresponding sex. #$P < 0.05$, males vs. females for corresponding group.

hypertrophy [ratio of RV to (LV + S)] compared with males ($P < 0.0001$).

Because RV hypertrophy directly correlates with arterial pulmonary pressure, this suggests that postnatal normoxia reduces arterial pulmonary hypertension in both male and female high-altitude rats.

Postnatal Normoxia Reduces Lung Air Space-to-Tissue Ratio In Males

Compared with all other groups, HACont male rats have enlarged air spaces with an apparent reduction in the corresponding number of alveoli (Fig. 3). Therefore, the air space-to-tissue ratio was higher in HACont male rats compared with HApNorm males (+29.3%, $P = 0.02$ for group in males), but postnatal normoxia had no effects in females (P value for group × sex = 0.02, P value for group in females = 0.5). In the HACont group, females had a reduced air space-to-tissue ratio compared with males ($P = 0.003$ for sex in this group).

Postnatal Normoxia Reduces Mortality Rate

Drastic pulmonary hypertension, enlarged air spaces, and excessive erythrocytosis may threaten survival of the rats, especially under chronic hypoxic stress. Accordingly, we performed a survival analysis (Fig. 4A). When data of males and females are pooled, mortality showed a clear tendency to be higher in HACont rats compared with HApNorm ($P = 0.07$) with a death ratio of 2.8 (95% confidence interval = 0.9 to 7.5) (Fig. 4B).

Effects of Postnatal Normoxia on Respiratory Parameters, $\dot{V}o_2$, and $\dot{V}co_2$

Baseline values. There was no group-specific effect on respiratory variables, but there was an important sex effect (and no group × sex interaction). Females of both groups had higher V_T (P value for sex = 0.0005) and lower f_R than males (P value for sex = 0.002; Fig. 5).

For $\dot{V}co_2$, there was a group ($P = 0.03$) and a sex effect ($P = 0.02$), without group × sex interaction, with HApNorm showing lower $\dot{V}co_2$ than HACont (mostly apparent in males), and females having higher $\dot{V}co_2$ values than males (mostly in HApNorm). For $\dot{V}o_2$ there was a group × sex interaction ($P = 0.04$): $\dot{V}o_2$ was higher in HApNorm male rats than HACont ($P = 0.007$), and in HApNorm females vs. males ($P = 0.004$). There was a group effect ($P = 0.02$) and group × sex interaction ($P = 0.03$) for $\dot{V}E/\dot{V}co_2$, with HApNorm males showing much higher $\dot{V}E/\dot{V}co_2$ compared with HACont males ($P = 0.01$) and HApNorm females ($P = 0.04$). No significant effect or interaction appeared for $\dot{V}E/\dot{V}o_2$ (Fig. 5).

For the respiratory exchange ratio ($\dot{V}co_2/\dot{V}o_2$), there was a group effect ($P = 0.0009$) and a group × sex interaction ($P = 0.002$), without sex effect; HApNorm males had lower values (0.59 ± 0.08) compared with HACont (1.03 ± 0.03; $P < 0.0001$ for group in males). In females, respiratory exchange ratio was at 0.92 ± 0.07 and 0.90 ± 0.07, respectively, for HACont and HApNorm. These differences in respiratory exchange ratio might indicate different utilization of metabolites (carbohydrate vs. fat) for oxidative reactions (see DISCUSSION).

Ventilatory responses to 32% O_2. Absolute values recorded under 32% O_2 are presented in Fig. 5. There were sex-specific effects (without group effect, or group × sex interaction) for
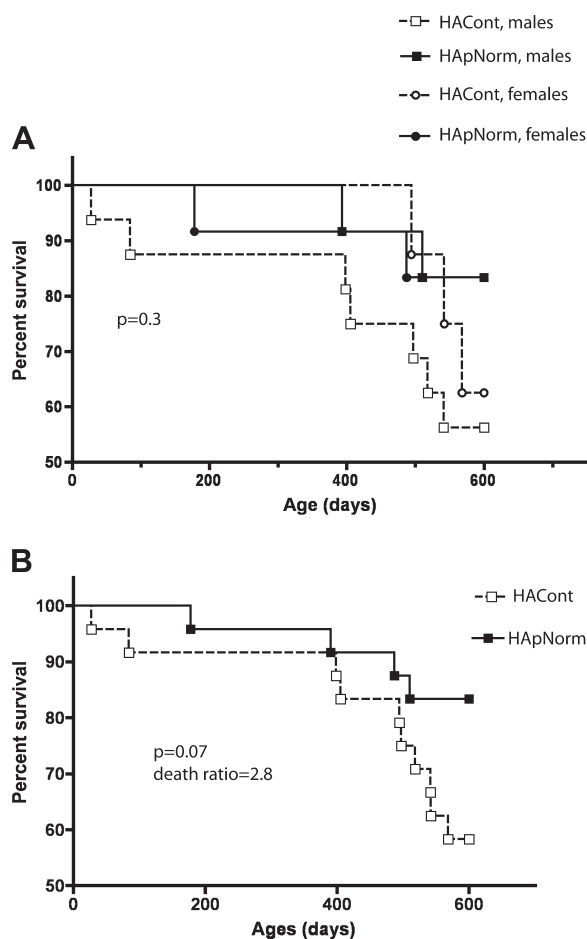

Fig. 4. Survival analysis showing percentage of male and female HACont and HApNorm rats living as a function of age. *A*: curves for each group and sex separately. *B*: curves when males and females are pooled. *P* values are the results of the log-rank test for comparing survival curves. *n* for each sex and group are as follows: male HACont = 16 and HApNorm = 12; female HACont = 8 and HApNorm = 12.

V_T ($P = 0.001$) and $\dot{V}E$ ($P = 0.02$). There were group ($P = 0.0005$) and sex ($P = 0.003$) effects for $\dot{V}co_2$, with HApNorm showing lower values vs. HACont and females vs. males (as observed at 21% O_2). For $\dot{V}o_2$, there was a sex effect ($P = 0.004$) and a group × sex interaction ($P = 0.01$), with HApNorm males showing higher values than HACont ($P = 0.02$), and HACont females vs. HACont males ($P < 0.0001$). For $\dot{V}E/\dot{V}co_2$, there was a group effect ($P = 0.02$), with HApNorm showing higher values than HACont. There was a group × sex interaction for $\dot{V}E/\dot{V}o_2$ ($P = 0.003$), with HACont males showing higher values than HApNorm ($P = 0.01$) and HACont females ($P = 0.01$).

When data are normalized as %changes from room air values (cf. Fig. 6), there was a group × sex interaction ($P = 0.05$) for %V_T, and a group effect for %$\dot{V}E$ ($P = 0.04$). Compared with HACont males, HApNorm males have a more pronounced decrease of V_T ($P = 0.008$ for group in males) and $\dot{V}E$ ($P = 0.002$), suggesting higher peripheral chemosensory drive. For $\dot{V}o_2$, there was a sex effect ($P = 0.02$), with females showing a higher increase when exposed to 32% O_2. For $\dot{V}E/\dot{V}co_2$, there was a sex × group interaction ($P = 0.03$), with HACont males showing a lower decline than HApNorm ($P = 0.05$) and HACont
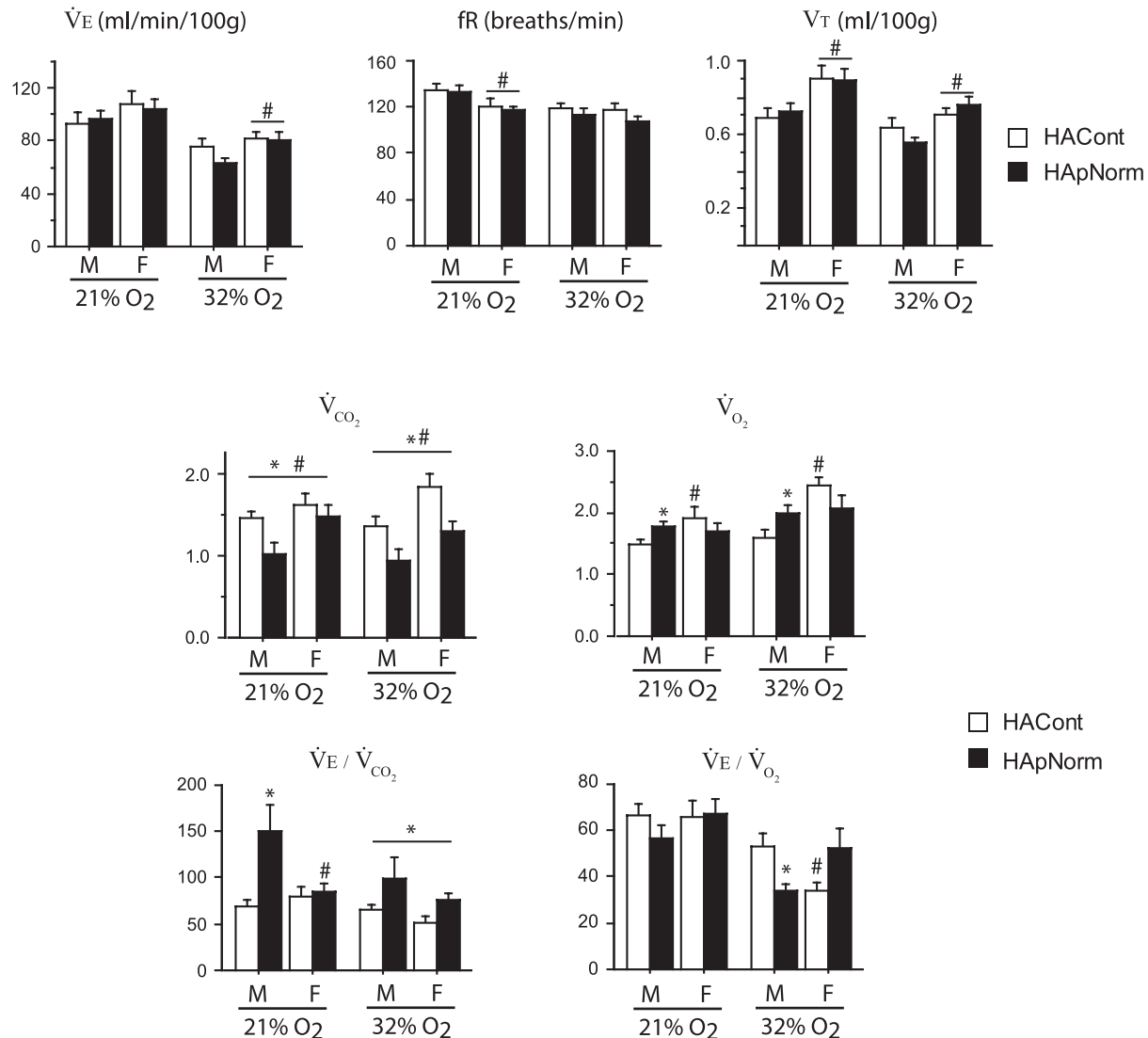
Fig. 5. Basal respiratory frequency (fR; breaths/min), tidal volume (V_T; ml/100 g), minute ventilation ($\dot{V}_E$; $ml \cdot min^{-1} \cdot 100 \ g^{-1}$), CO_2 production ($\dot{V}_{CO_2}$; $ml \cdot min^{-1} \cdot 100 \ g^{-1}$), O_2 uptake ($\dot{V}_{O_2}$; $ml \cdot min^{-1} \cdot 100 \ g^{-1}$), $\dot{V}_E/\dot{V}_{CO_2}$, and $\dot{V}_E/\dot{V}_{O_2}$ recorded in normal room air (21% O_2) and in response to 32% O_2, in male and female HACont and HApNorm rats. Values are means ± SE; n for each sex and group are as follows: male HACont = 20 and HApNorm = 20, female HACont = 18 and HApNorm = 19. *$P < 0.05$, HACont vs. HApNorm for corresponding sex. #$P < 0.05$, males vs. females for corresponding group. * or # above a bar indicates a group or sex effect without interaction.

females ($P = 0.05$). For $\dot{V}_E/\dot{V}_{O_2}$, there was a sex × group interaction ($P = 0.03$), without other significant effect.

DISCUSSION

This study shows that rats living at high altitude are profoundly affected by the period of postnatal hypoxic exposure. By performing a "deletion experiment" by which the influence of hypoxia is removed during development, we have been able to reduce Hct and Hb values, alleviate the RV hypertrophy (a direct index of pulmonary hypertension), and favor adequate lung development, despite the fact that these rats subsequently spent the major part of their life under chronic hypoxia. These results strongly indicate that HACont have a pronounced hypoxemia compared with HApNorm. In males, this is likely explained by the altered development of lung tissue, whereas in females we have not been able to detect relevant changes in lung morphology. In addition, the fact that the resulting hypoxemia is not restored by enhanced

ventilation suggests an impaired chemoreflex function. Much to our surprise, body weight was similar between all age groups, though we expected that normoxic exposure would allow faster growth in pups.

Methodological Considerations

Lung fixation. We have not performed lung fixation under constant positive pressure to ensure adequate distension of alveoli, which is standard for lung morphology studies (7, 10). However, this method of fixation has been used successfully in a previous study, yielding reliable results (43). In our study, air spaces were well delineated, and visual examination of the lungs of HACont male rats clearly showed enlarged air spaces, as quantified by the morphometric analysis. Although this is not a thorough morphometric analysis, it gives a reliable estimate of the impact of postnatal normoxia in high-altitude rats. Furthermore, the results of lung morphology are consistent with the rest of our data and
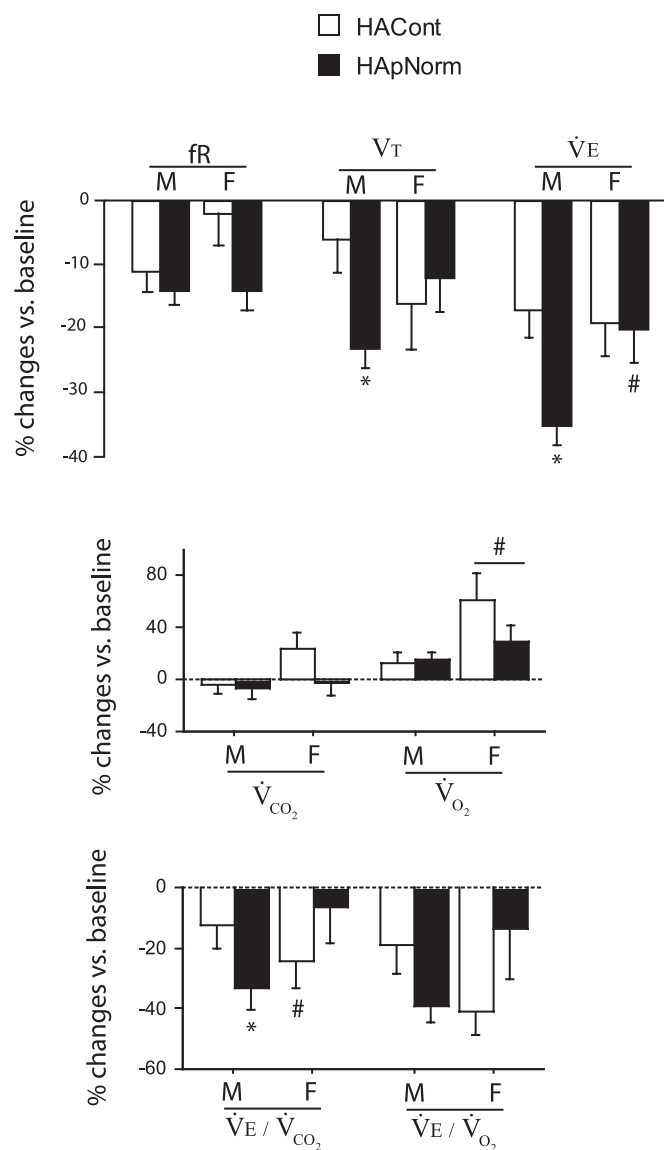
Fig. 6. Normalized responses to 32% O_2 as %changes from baseline for fR, V_T, and $\dot{V}_E$ in male and female HACont and HApNorm rats. Values are means ± SE; n for each sex and group are same as in Fig. 5 legend. *$P < 0.05$, HACont vs. HApNorm for corresponding sex. #$P < 0.05$, males vs. females for corresponding group. * or # above a bar indicates a group or sex effect without interaction.

agree with the effects reported in the literature on long-term consequences of postnatal hypoxia (6, 50).

$\dot{V}_{O_2}$ *and* $\dot{V}_{CO_2}$. Recording of $\dot{V}_{O_2}$ or $\dot{V}_{CO_2}$ using a standard open-flow system is viewed as a reliable tool to indirectly assess metabolic rate (29), i.e., the amount of energy produced by an organism under a particular condition (rest, exercise, hypoxia, etc.). However, this remains indirect, and potential errors between indirect and direct estimations of energy production might be large (51). Accordingly, in our specific conditions, and because $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ are, respectively, higher and lower in HApNorm males compared with control, we cannot conclude whether these differences correspond to different metabolic rate (i.e., energy production) between groups.

With these discrepancies, and because $\dot{V}_E$ is similar between groups, the $\dot{V}_E/\dot{V}_{O_2}$ and $\dot{V}_E/\dot{V}_{CO_2}$ give different impressions

regarding respiratory control. Under the highly specific conditions of this study, we suggest that the lower $\dot{V}_{O_2}$ of adult HACont male rats compared with HApNorm reflects limitation of gas exchange through the lungs: for each breath more O_2 is left in the less efficient alveoli, expired fraction of inspired O_2 is higher, thus reducing measured $\dot{V}_{O_2}$ in our system. Since CO_2 has a much higher solubility than O_2, it diffuses ~20 times faster than O_2 through the blood-gas interface (54). Accordingly, if HACont males are also in hypoventilation due to defective respiratory control, then arterial P_{CO_2} rises, leading to higher alveolar and expired P_{CO_2}, as reflected in our measures; thus the high $\dot{V}_{CO_2}$ rate observed in HACont male rats vs. HApNorm appears as a reliable estimate of hypoventilation. On the other hand, chronic lactic acidosis might also contribute to increase CO_2 release from bicarbonate, which might also contribute to increase $\dot{V}_{CO_2}$.

This is in line with the rest of our data, but also with what is observed in chronic obstructive pulmonary disease patients, where, in front of low-arterial P_{O_2}, due to impaired lung diffusion, some patients have normal arterial P_{CO_2}, while other have high arterial P_{CO_2}. The patients with high arterial CO_2 have an intense dyspnea and low ventilatory response to CO_2 (34, 53), i.e., they are clearly in hypoventilation. Interestingly, this also suggests that these differences are linked to neonatal hypoxic exposure and subsequent long-term effects on respiratory control.

Finally, it is worth mentioning that a respiratory exchange ratio close to 1 in HACont males indicates that glucose is the main substrate for oxidative pathways, whereas the much lower value observed in HApNorm indicates that fatty acids are used as the main source of metabolic substrate for oxidative pathways. At sea level, respiratory exchange ratio is generally between 0.85 and 0.95 in adult male rats (19, 47); accordingly, it appears slightly more elevated in HACont male rats and largely reduced in HApNorm males. While relative hyperventilation and improved lung diffusion in HApNorm males might contribute to bring arterial and expired P_{CO_2} down, thus reducing respiratory exchange ratio, it is quite remarkable that a high amount of glucose oxidations ensures the highest number of ATP molecules synthesized for each molecule of O_2 consumed (18), and that one of the main biochemical pathway induced by hypoxia inducible factor-1 favors glucose utilization, while hypoxia inducible factor-2 reduces the utilization of fatty acid as metabolic substrate (32).

Effect of postnatal hypoxia in high-altitude rats. During postnatal development, intense structural and functional changes occur, both in the lungs and in the respiratory control system. Studies in rats have highly contributed to our knowledge of alveolar formation, which occurs during a critical period, starting around postnatal *day 4* (12). At this age, the lungs are made of large, thick-walled saccules in a relatively small number. This stage is followed by progressive projection of ridges into the air spaces, forming the contours of the future alveoli (septation phase). During the second postnatal week, the alveolar walls become thinner, and alveolar capillary are properly organized, allowing the formation of a fully mature lung (alveolarization phase) (6, 8, 11, 12). This process allows the formation of alveoli, which are more numerous, smaller, and thin-walled compared with the saccules seen in the newborn. The development of pulmonary vessels occurs in parallel to these events. Postnatal formation of alveoli is altered by exposing newborn rats to hypoxia, resulting in lung tissue showing fewer and larger alveoli,

reduced surface-exchange area, and limitations of respiratory gas exchange (6, 50). In humans, septation and alveolarization begin shortly before birth and develop at least until the end of the first or second year of life (11). Despite the strong evidences linking neonatal hypoxia to lung development in rats and mice, in humans, there is no evidence of altered respiratory gas exchange in life-long altitude residents (see Ref. 9 for review). On the other hand, adverse neonatal events, such as preeclampsia (associated to fetal hypoxia) (20) and transient perinatal hypoxia, can lead to exaggerated hypoxic pulmonary hypertension in adults (42). Our results are consistent with this phenomena and show that restoring normal O_2 levels in rats living at high altitude limits the deleterious consequences of postnatal hypoxia on pulmonary hypertension, but also on lung morphology and gas exchange function.

Furthermore, an immature respiratory control system at birth is characterized by poor ventilatory response to hypoxia, which fails to remain elevated for more than a few minutes in hypoxia (5, 38). During subsequent development, structural and biochemical changes within the peripheral chemoreceptors and their central projections allow the development of a fully mature ventilatory response and stronger carotid body response to hypoxia (1, 37, 38, 52). As observed for lung development, these maturational processes are also sensitive to ambient oxygen level (3, 15, 30, 39). Chronic hypoxia from birth delays the postnatal maturation of ventilatory response to hypoxia (17), which has also been reported in our laboratory's previous studies of high-altitude rats in La Paz (21), whereas in vitro studies have clearly demonstrated that the carotid body response to hypoxia and the interaction between hypoxia and hypercapnia are drastically reduced following chronic hypoxic exposure from birth (25). These experimental studies linking postnatal hypoxia to blunted chemoreflex responses help to explain results from human studies, showing that $\dot{V}_E$, but also hypoxic ventilatory response, are decreased in high-altitude compared with sea-level natives acclimatized to altitude (13, 23, 45, 46). The fact that acute exposure to 32% O_2 ($P_{O_2} = 160$ Torr) has a lesser effect on HACont rats than HApNorm suggests that carotid body contribution to ambient hypoxic respiratory drive is lower in HACont animals. This is consistent with some of the data available for sea-level rats reared in postnatal hypoxia (i.e., blunted hypoxic response in male rats exposed to postnatal hypoxia) (3).

Sex-specific effects. As previously reported by several studies in experimental conditions and in high-altitude human residents, HACont females had higher V_T (although $\dot{V}_E$ was not higher), lower Hct and Hb levels, and reduced RV hypertrophy compared with males (22, 27, 28, 40). These effects are mainly related to ovarian steroids. On one hand, progesterone acts as a respiratory stimulant through peripheral chemoreceptors (22, 48) and central respiratory system (4), and, on the other hand, estradiol reduces hypoxic-induced erythropoietin synthesis (36) and pulmonary hypertension (24, 49, 55). Importantly, however, hematological acclimatization to hypoxia in female rats is also modified by postnatal hypoxia, as observed in our laboratory's previous study at sea level (31). In these conditions, the fact that there was an important effect of postnatal normoxia on lung morphology (and gas exchange function) in male but not in female rats is puzzling and may be related to sex-specific events affecting lung development (44) and their potential interactions under hypoxia, which remain poorly documented. Alternatively, other components of the lung, such as blood vessels, might have been modified in HApNorm females, thus accounting for the difference observed on hematological values.

We conclude from this study that exposure to ambient hypoxia during early postnatal development in rats living at high altitude exerts an important life-long influence on the acclimatization processes taking place in these animals. When rats are raised at sea-level P_{O_2} during this period, they display an overall better acclimatization as adults with reduced Hct, Hb, pulmonary hypertension, and a clear tendency toward better survival for both males and females. In males, this process appears to be related to changes in alveolar structure of the lungs, whereas in HACont males there is an apparent hypoxemia and hypercapnia not corrected by the respiratory control system. The relevance of this finding for our understanding of the conditions favoring adequate responses and survival at high altitude is evident and supports the hypothesis of developmental origins of chronic mountain sickness.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: D.L., A.L., M.G., and G.V. performed experiments; D.L., A.L., M.G.I., and V.J. analyzed data; D.L., A.L., T.S., and V.J. interpreted results of experiments; D.L., A.L., and V.J. prepared figures; D.L. and V.J. drafted manuscript; D.L., M.G., T.S., and V.J. edited and revised manuscript; D.L., A.L., M.G., T.S., and V.J. approved final version of manuscript; T.S. and V.J. conception and design of research.

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