Ventilatory, metabolic, and thermal responses to hypercapnia in female rats: effects of estrous cycle, ovariectomy, and hormonal replacement

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The aim of this study was to examine how estrous cycle, ovariectomy, and hormonal replacement affect the respiratory [ventilation (Ve), tidal volume, and respiratory frequency], metabolic (V̇O₂), and thermoregulatory (body temperature) responses to hypercapnia (7% CO₂) in female Wistar rats. The parameters were measured in rats during different phases of the estrous cycle, and also in ovariectomized (OVX) rats supplemented with 17β-estradiol (OVX+E₂), or with corn oil (OVX+H11001, vehicle). All experiments were conducted on day 8 after ovariectomy. The intact animals did not present alterations during normocapnia or under hypercapnia in Ve, tidal volume, and respiratory frequency. Ve and V̇E/V̇O₂, and Ve/V̇O₂ in the different phases of the estrous cycle. However, body temperature was higher in female rats on estrus.

Hormonal replacement did not change the ventilatory, thermoregulatory, or metabolic parameters during hypercapnia, compared with the OVX animals. Nevertheless, OVX+E₂, OVX+E₂P, and OVX+O presented lower hypercapnic ventilatory responses compared with intact females on the day of estrus. Also, rats in estrus showed higher Ve and Ve/V̇O₂ during hypercapnia than OVX animals. The data suggest that other gonadal factors, besides E₂ and P, are possibly involved in these responses.

CO₂; estrogen; progesterone; cycling rats; breathing

SEX HORMONES, INCLUDING androgens, estrogens, such as 17β-estradiol (E₂), and progestogens, such as progesterone (P), are present in both males and females, although marked differences in the plasma levels occurs throughout the lifespan of the animals (3). In female rats, E₂ levels are low on estrus, elevated on metestruus and diestruus, and reach a peak during the proestruus, falling again as the next cycle starts (18). On the other hand, levels of P increase between the night of metestruus and early morning of diestruus, and fall thereafter (18). Then, on the afternoon of proestruus, P levels increase again, reaching a second peak, and then return to baseline levels on estrus (43).

These hormones affect neuromodulatory systems that influence the central respiratory network (3), acting directly on respiratory motor or adjacent neurons (5, 13), brain stem premotor neurons (13), rhythm generating neurons (3). They can also influence peripheral or central chemoreceptors (5), which suggests an important hormonal role in the breathing control (3). Most of the experiments in respiratory control, however, are performed in male rats. When females are used, the studies do not consider the natural hormonal oscillations that occur during the estrous cycle.

In women, studies demonstrated that the ventilatory responses to hypoxia and hypercapnia vary in the different phases of the menstrual cycle (34, 48). For example, the ventilation (Ve) and tidal volume (Vt) are increased in the luteal phase, compared with the follicular phase (13, 35, 41), and consequently arterial partial pressure of CO₂ is reduced. Additionally, central and peripheral chemosensitivity are increased in luteal phase (16, 35, 48), and administration of medroxyprogesterone acetate, a synthetic variant of P, stimulates the ventilatory system (40, 52), suggesting that the increased ventilatory response during the luteal phase is related to the elevated plasmatic concentrations of P.

Wenninger et al. (47) reported that female rats present a significantly higher Ve/V̇O₂ compared with male rats. Variations in the body temperature (Tb) and V̇O₂ also occur during the menstrual and estrous cycle (11, 28, 33), but this is still a poorly explored issue. The hormonal variations in different stages of the estrous cycle may present additional challenges to data collection and interpretation.

To the best of our knowledge, no study has examined the influence of the estrous cycle on ventilatory, metabolic, and thermoregulatory parameters in normocapnic and hypercapnic conditions in rodents. We hypothesized that hormonal fluctuations observed during the estrous cycle have an impact on basal Ve, V̇O₂, and Tb. We also assessed the possible role of E₂ and P in those influences. To this end, we measured Ve, V̇O₂, and Tb in intact, as well as ovariectomized (OVX) without replacement and OVX with E₂ and P replacement female rats.

MATERIALS AND METHODS

Animals

Experiments were performed in unanesthetized adult female Wistar rats weighting 250–300 g. The age of the animals ranged from 90 to 120 days. The animals were fed ad libitum and housed in a temperature-controlled chamber, maintained at 24–26°C (ALE 9902001; Alesco, Monte Mor, Brazil), with a 12:12-h light-dark cycle (lights on at 6:00 AM). All experimental procedures were in compliance with the Brazilian College of Animal Experimentation guidelines and approved by the local Animal Care and Use Committee (protocol no. 007827-09).

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We used cycling female rats on the days of proestrus (n = 8), estrus (n = 6), metestrus (n = 4), and diestrus (n = 4), and OVX rats treated with corn oil (OVX+O; n = 6), E2 (OVX+E2; n = 6), or E2 and P (OVX+E2P; n = 5). We used 39 animals in total.

Ventilatory, Tb, and Metabolic Measurements

The V̇e measurements were obtained using the barometric method (whole body plethysmography) (2), as previously described by Biancardi et al. (6), de Carvalho et al. (12), and Lopes et al. (25). Tb was recorded by the use of a temperature datalogger (SubCue, Calgary, AB, Canada) implanted in the abdominal cavity, as previously described in the literature (6). The datalogger was programmed to acquire data every 5 min.

The metabolic rate was measured by indirect calorimetry (V̇O2) using a closed respirometry system, as described by Almeida et al. (1). At the end of the normocapnic and hypercapnic periods, the airflow in the chamber was interrupted for 2 min, and the air was continuously sampled by an O2 analyzer (PowerLab System. ADInstruments/Chart Software, version 7.3, Sydney, Australia). While the chamber was sealed, the oxygen fraction did not drop to <19%, and CO2 did not increase >0.7%. The percentage of oxygen decay inside the chamber was plotted against time, and the slope of the resulting curve corresponded to the rate of V̇O2. The values are presented in milliliters per kilogram in STPD (standard conditions of temperature, pressure, and dry air).

Vaginal Smears

The animals were subjected to daily vaginal lavages to monitor the progression through the estrous cycle. Vaginal smears were performed daily at 9:00 AM to verify estrous cycle regularity. Only females exhibiting at least four consecutive regular cycles were used in the experiments. The estrous cycle stage was determined using the criteria used by Freeman (18).

Blood Collection for Plasma Hormone Assays

After the hypercapnic challenge, and just before euthanasia, a blood sample of ~1 ml was collected from the heart of anesthetized rats into heparinized syringes. The plasma was separated by centrifugation at 3,000 rpm for 20 min at 4°C and stored at −20°C for posterior quantification of P and E2 by radioimmunossay. Plasma P and E2 concentrations were determined by double-antibody radioimmunossay using MAIA kits provided by Biochem Immunosystem (Bologna, Italy). The lower limits of detection for E2 and P were 5.0 pg/ml and 0.02 ng/ml, respectively.

Ovariectomy and Hormonal Replacement

Before the ovariectomy surgery, animals were anesthetized, via intraperitoneal injection of 100 mg/kg of ketamine (Union National Pharmaceutical Chemistry S/A, Embu-Guáçu, São Paulo, Brazil) and 10 mg/kg of xylazine (Laboratories Calier S/A, Barcelona, Spain). A temperature datalogger (SubCue, Calgary, AB, Canada) was also implanted in the abdominal cavity through a midline laparotomy. After this procedure, bilateral ovariectomies were performed using an incision 1.5 cm inferior to the palpated rib cage. ovaries and surrounding fat tissue were removed; the incision was closed by suturing the muscles and stapling the skin. After surgery, animals were treated with two doses of enrofloxacin (10 mg/kg intramuscular) and flunixin meglumine [2.5 mg/kg subcutaneous (SC)] to prevent infection and postoperative discomfort, respectively. Eight days after ovariectomy, rats were treated with corn oil (0.2 ml SC) or E2 (10 μg/0.2 ml SC, 17β-estradiol cypionate; Pfizer, São Paulo, Brazil), at 9 AM for 3 days. On the 4th day, oil-treated rats received a final oil injection (OVX+O group), while E2-treated rats received an injection containing corn oil (OVX+E2 group) or P (2.5 mg/0.2 ml SC, OVX+E2P group; Sigma, St. Louis, MO), and the experiments were performed 2 h later. The regimens of hormonal treatment used yielded physiological levels of plasma E2 and P, as described by Szawka et al. (44). On the day of experiment, vaginal cytology was performed to monitor the effectiveness of the hormonal treatment. Animals in which hormone replacement was effective presented cornified epithelial cells in the vaginal cytology (27). In contrast, when the hormonal replacement was not efficient, the smears displayed predominance of small leukocytes, similarly as found in the cytology of OVX+O rats, and the animals were not used in the experiments.

Experimental Protocol

Each animal was individually placed in a plethysmograph chamber (5 liters), kept at 25°C and allowed to move freely, while the chamber was flushed with humidified room air. The baseline measurements of V̇e and V̇O2 were taken after animals acclimated for ~1 h. Subsequently, a hypercapnic gas mixture (7% CO2 in air, White Martins, Sertãozinho, São Paulo, Brazil) was flushed through the chamber for 30 min, and V̇e was measured at 30 min. V̇e was calculated before (time zero) and 30 min after hypercapnia or normocapnia. The percentage of CO2 and the exposure time were chosen based on pilot experiments and previous studies (6, 15). The V̇O2 was measured once again after the stimuli. The experiments were carried out from 900 to 1300. Tb was continuously measured during the experiment.

Statistical Analysis

Statistical analysis of the blood hormone concentration data was performed by one-way ANOVA followed by Tukey post hoc testing. V̇e, respiratory frequency (IR), V̇T, Tb, and V̇O2 data were compared using repeated two-way ANOVA. When interactions between the factors were observed, groups were compared using Bonferroni’s post hoc test. The significance level was set to P < 0.05. The statistical analysis was performed using computer software (SigmaStat; Systat Software, Point Richmond, CA). Data are presented as group means ± SE for each parameter investigated.

RESULTS

Effects of Estrous Cycle Stages on V̇e, V̇O2, and Tb

Vaginal cytology. Figure 1 shows the cytological observations characteristic of specific stages of the estrous cycle in cycling females. Proestrus vaginal smears showed nucleated epithelial cells (Fig. 1A), whereas estrous smears present densely stained cornified cells in addition to some leukocytes and nucleated cells (Fig. 1C). Finally, diestrous smears showed presence of leukocytes (Fig. 1D).

Hormonal profile. Figure 2 shows plasma E2 and P concentrations in cycling female rats. Plasmatic concentration of E2 was significantly higher in proestrus than in estrus, metestrus, and diestrus. The plasma concentration of P was lower on estrus compared with proestrus, metestrus, and diestrus.

Pulmonary V̇e and metabolism. In normocapnic conditions, no differences in V̇e were observed in different phases of the estrous cycle (Fig. 3). Hypercapnia caused an increase in VE in all groups [hypercapnia effect: P < 0.0001; F(3,22) = 131.7; no interaction] due to an increase in V̇T [hypercapnia effect: P < 0.0001; F(3,22) = 84.6; no interaction] and IR [hypercapnia effect: P < 0.0001; F(3,22) = 301.1; no interaction]. There was no difference in the hypercapnic ventilatory response between
groups in the different phases of the estrous cycle, as seen in Fig. 3.

All groups had lower \( \dot{V}O_2 \) during hypercapnia [hypercapnia effect: \( P = 0.0127; F_{3,22} = 6.8; \) no interaction], and no differences were observed between the groups in different stages of the estrous cycle. Hypercapnia caused a significant increase in the \( \dot{V}e/\dot{V}O_2 \) [hypercapnia effect: \( P < 0.001; F_{3,22} = 85.6; \) no interaction], and, again, no difference was found between groups (Fig. 4).

\( \text{Tb} \). As seen in Fig. 5, \( \text{Tb} \) of rats on estrus was higher than that of rats on other stages of the estrous cycle, during both normocapnia and hypercapnia [cycle effect: \( P < 0.001; F_{3,22} = 7.8; \) no interaction]. Hypercapnia caused a decrease in \( \text{Tb} \) only in estrous and proestrous rats [hypercapnia effect: \( P = 0.0003; F_{3,22} = 20.2; \) no interaction].

Effects of Ovariectomy and Hormonal Replacement on \( \dot{V}e, \dot{V}O_2, \) and \( \text{Tb} \)

Pulmonary \( \dot{V}e \) and Metabolism. There was no difference in the ventilatory parameters between groups during normocapnia (Fig. 6). Hypercapnia induced an increase in \( \dot{V}e \) in all groups [hypercapnia effect: \( P < 0.0001, F_{3,19} = 160.7; \) interaction: \( P < 0.0001; F_{3,19} = 17.1 \)], which was a result of an increase in \( \dot{V}t \) [hypercapnia effect: \( P < 0.0001, F_{3,19} = 85.6; \) interaction: \( P = 0.0064; F_{3,19} = 4.8 \)] and \( \text{fR} \) [hypercapnia effect: \( P < 0.001, F_{3,19} = 94.8; \) no interaction]. OVX animals (OVX\(_O\), OVX\(_E2\), and OVX\(_E2P\)) showed an attenuated hypercapnic ventilatory response (43% lower for all groups) compared with rats on estrus [hormone effect: \( P < 0.0001, F_{3,19} = 30.1; \) interaction: \( P < 0.0001; F_{3,19} = 17.1 \)] due to lower \( \dot{V}t \) [hormone effect: \( P = 0.0001, F_{3,19} = 9.2; \) interaction: \( P = 0.0064; F_{3,19} = 4.8 \)] and \( \text{fR} \) [hormone effect: \( P = 0.0030, F_{3,19} = 5.5; \) no interaction]. However, the hormonal replacement did not affect the ventilatory responses to CO2, as they were similar across all OVX groups.

In normocapnia, \( \dot{V}O_2 \) of the estrus group was higher compared with OVX\(_E2P\) animals [hormone effect: \( P = 0.0017, F_{3,19} = 6.2; \) no interaction] (Fig. 7). No difference in \( \dot{V}O_2 \) was
observed between the groups under hypercapnic conditions [hypercapnia effect: \( P = 0.9756; F(3,22) = 0.00095 \), no interaction], and hormone replacement did not affect \( \dot{V}O_2 \) during CO2 exposure (Fig. 7).

\( \dot{V}E/\dot{V}O_2 \) was not different between groups during normocapnia (Fig. 7). Hypercapnia increased \( \dot{V}E/\dot{V}O_2 \) in all groups [hypercapnia effect: \( P = 0.001, F(3,22) = 69.4 \), interaction \( P = 0.0083; F(3,22) = 5.4 \)] (Fig. 7); however, the effect was greater in \( \dot{V}E/\dot{V}O_2 \) of the estrus group, compared with OVX groups [hormone effect: \( P = 0.0059, F(3,22) = 5.9 \), interaction \( P = 0.0083; F(3,22) = 5.4 \)].

\( T_b \). \( T_b \) of rats on estrus was higher than that of the OVX groups during normocapnia [hormone effect: \( P = 0.0070, F(3,22) = 4.8 \), no interaction], but not under hypercapnia (Fig. 8). Exposure to hypercapnia caused a drop in \( T_b \) in all groups, except for the OVX+E2 animals [hypercapnia effect: \( P = 0.028, F(3,19) = 5.8 \), no interaction] (Fig. 8).

**DISCUSSION**

The present study demonstrates that, despite the hormonal fluctuations of cycling rats, \( \dot{V}E, \dot{V}O_2 \), and \( \dot{V}E/\dot{V}O_2 \) during normocapnia and hypercapnia were not different in different stages of the estrous cycle. As shown in previous studies, our measurements of \( T_b \) were higher in estrous rats in both normocapnic and hypercapnic conditions. Additionally, this study also shows that ovariectomy promotes an attenuation of the response to hypercapnia (in \( \dot{V}E \) and \( \dot{V}E/\dot{V}O_2 \)) compared with the response obtained from intact animals in estrus. Moreover, we also saw that hormonal replacement did not reverse these effects, suggesting that this treatment does not restore the ventilatory responses of an intact animal.
Estrous Cycle and \( \dot{V}E \), Metabolism, and Tb

Many studies have demonstrated that \( \dot{V}E \) in women is higher during the luteal than the follicular phase (22, 35, 41), and also that the administration of P promotes an increase in \( \dot{V}E \) (40, 52), which suggests that this increase in \( \dot{V}E \) is related to an augment of P levels. In the present study, no difference in \( \dot{V}E \) and \( \dot{V}O_2 \) was observed in the different groups under normocapnic conditions, which suggests that hormonal changes during estrous cycle were not sufficient to promote alteration in resting \( \dot{V}E \) and metabolism. In this regard, Brack et al. (7) performed experiments using anesthetized cycling female rats and reported no differences in respiratory rates in different stages of the estrous cycle, although \( \dot{V}T \) and \( \dot{V}E \) were not measured. In addition, a previous study demonstrated that phrenic nerve activity is not estrous cycle dependent (51).

Similarly, White et al. (48) showed that resting \( \dot{V}E \) is not different between follicular and luteal phases in women; however, \( \dot{V}T \) and end-tidal \( \text{PCO}_2 \) is higher in the luteal phase, possibly due to augmented levels of P. Hayashi et al. (22) observed higher values for basal \( \dot{V}E \) and \( \dot{V}T \) in the luteal phase, when comparing it with the follicular phase.

In this study, no changes in \( \dot{V}O_2 \) levels were observed under normocapnia in the different phases of the estrous cycle, suggesting that the hormonal fluctuations that occur during the cycle have a small effect (if any) in the metabolic rate under the present conditions. This has also been observed in women during the menstrual cycle (42, 45). Regarding Tb, our results show an increase on the day of estrus, similar to that previously

\[ \text{FIG. 6. } \dot{V}E, \dot{V}T, \text{ and } \dot{fR} \text{ of intact rats in estrus, ovariectomized (OVX) rats with corn oil replacement (OVX+O), OVX rats replaced with E}_2 \text{ (OVX+E}_2\text{), and OVX rats replaced with a combination of E}_2 \text{ and P (OVX+E}_2\text{P) during normocapnia (0\% CO}_2\text{) and hypercapnia (7\% CO}_2\text{). Values are means } \pm \text{ SE; } n, \text{ no. of rats. *Difference between estrus group compared with OVX+O, OVX+E}_2\text{, and OVX+E}_2\text{P groups (}P < 0.05).} \]

\[ \text{FIG. 7. } \dot{V}O_2 \text{ and } \dot{V}E/\dot{V}O_2 \text{ of intact rats in estrus, and OVX+O, OVX+E}_2\text{, and OVX+E}_2\text{P rats during normocapnia (0\% CO}_2\text{) and hypercapnia (7\% CO}_2\text{). Values are means } \pm \text{ SE; } n, \text{ no. of rats. *Difference between estrus group compared with OVX+O, OVX+E}_2\text{, and OVX+E}_2\text{P groups (}P < 0.05).} \]

\[ \text{FIG. 8. } \text{Tb of intact rats in estrus, and OVX+O, OVX+E}_2\text{, and OVX+E}_2\text{P rats during normocapnia (0\% CO}_2\text{) and hypercapnia (7\% CO}_2\text{). Values are means } \pm \text{ SE; } n, \text{ no. of rats.} \]
reported (23, 50). During estrus, Tb was ~0.6°C higher than in other phases. In women, Tb can increase up to 0.4°C after ovulation, whereas in rodents there is an elevation in Tb immediately before, rather than after presumed ovulation (33). Despite the fact that Tb was higher during estrus in this study, metabolism and $V_{\dot{E}}/V_{\dot{O}_2}$ of cycling rats did not vary in different phases of the cycle.

We found no difference in ventilatory responses to CO$_2$ during the different stages of the estrous cycle. In women, there is a controversy in the literature about the influence of menstrual cycle in the CO$_2$ drive to breathe. Some studies have demonstrated that alterations of sex hormones during the menstrual cycle have little or no effect on CO$_2$ sensitivity (48), while others have reported that, during the luteal phase, the hypercapnic ventilatory response is more pronounced than that in the follicular phase (16, 35). Shoene et al. (35) suggest that the increased ventilatory response during the luteal phase is related to the higher P concentration. This contradiction may reflect differences in study design and on the data analysis. Additionally, Preston et al. (32) demonstrated that CO$_2$ sensitivity was not different between pre- and postmenopausal women. In anesthetized rats, Zabka et al. (51) demonstrated that the short-term ventilatory response to hypoxia is similar across estrous phases. The present data show that the estrous cycle of rats does not interfere with the ventilatory responses to CO$_2$ in females with a regular cycle. Therefore, our findings, taken in conjunction with those of others, indicate that normally occurring alterations in sex hormones have very little or no effect on the sensitivity to CO$_2$.

We observed a decrease in $V_{\dot{O}_2}$ during hypercapnic exposure in all phases of the estrous cycle. This was also observed in male rats during 5% CO$_2$ exposure (36). However, CO$_2$ exposure only decreased Tb of rats in estrus and proestrus. In many species, a decrease in Tb is observed during hypercapnia, including rats (19). Nevertheless, previous studies from our laboratory did not observe a Tb alteration during CO$_2$ challenge in male Wistar rats (6, 13, 14, 31).

Ovariotomy, Hormonal Replacement, $V_{\dot{E}}$, Metabolism, and Tb

In the present study, no differences in $V_{\dot{E}}$ were found during normocapnia and hypercapnia between spayed females treated with corn oil, E$_2$, or P. It has long been recognized that E$_2$ participates in the respiratory adjustments observed during late gestation (9, 29). Nevertheless, the administration of E$_2$ alone did not affect $V_{\dot{E}}$ in male rats (34). Some evidence indicates a synergistic effect of estrogen and P on $V_{\dot{E}}$ (37). In fact, as estrogen upregulates P receptors (24), this mechanism is likely involved with the respiratory effects of P. E$_2$ and P receptors (ER-α, ER-β; PR-Α, PR-Β) were identified in many respiratory regions of the brain, including the nucleus of the solitary tract (38, 39), the hypoglossal nuclei (4), and the motor nuclei of the phrenic nerve (4). In fact, some studies have reported an inhibitory effect of E$_2$ in some areas important for $V_{\dot{E}}$ (10, 44, 46, 49). Also, studies in OVX rats (8) and castrated cats (20, 21) have demonstrated that administration of P combined with estrogen promotes an increase in pulmonary $V_{\dot{E}}$ and hypercapnic ventilatory response through receptor-mediated mechanisms of action involving central and peripheral sites. The fact that we found no difference in $V_{\dot{E}}$ might be related to deficiencies in the hormonal replacement protocol. We choose not to use P treatment without E$_2$, since it is well known that P sensitivity is highly dependent on prior or concurrent estrogen exposure, once estrogens induce P receptor expression in the brain (4, 26, 30).

Interestingly, our results demonstrate that OVX rats that underwent hormonal replacement showed a ventilatory response to hypercapnia 43% lower compared with female rats on estrus. This comparison was made because lower levels of sexual hormones are observed during estrus. It is interesting to note that hormonal replacement is used in the literature for several purposes (3). However, at least in the perspective of respiratory control, this approach has its limitations, since hormonal replacement did not revert the effects of ovarioectomy in the ventilatory responses to hypercapnia. In this context, when studying female rats, it is important to consider the possibility that replacing hormones using the present protocol might not perfectly mimic the conditions of an intact cycling animal, despite the fact that said protocol yielded physiological plasmatic levels of E$_2$ and P, according to Szawka et al. (44). What is more, OVX+E$_2$ and OVX+E$_2$P rats presented plasmatic levels of E$_2$ higher than those in OVX+O rats.

In the present work, we show that hormonal replacement did not restore the ventilatory, metabolic, and thermal variables studied. Even though E$_2$ and P are the main female gonadal hormones, other gonadal hormones might play a role in $V_{\dot{E}}$, metabolism, and temperature. Gonadal hormones include sexual steroids and androgens, and also peptide hormones, such as anti-Müllerian hormone, activin, inhibin, and follistatin, which might be involved. However, there are no studies in the literature reporting the involvement of these hormones. Humans are able to easily manipulate the levels of their sexual hormones (with oral contraceptives and steroids), but the respiratory consequences of this are not fully understood (3).

In conclusion, despite the hormonal fluctuations during the estrous cycle, the ventilatory responses to hypercapnia in female rats were similar along the cycle, but OVX rats presented a reduced ventilatory response to CO$_2$. Our data demonstrate that hormonal replacement with E$_2$ or P (the main sexual hormones) did not alter the $V_{\dot{E}}$ under the present experimental condition, suggesting that other gonadal factors may be involved in these responses. This study contributes to the understanding of the respiratory and metabolic responses to CO$_2$ exposure in female conscious rats, being an important complement to the existing literature.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: D.A.M., D.d.C., R.E.S., J.A.A.F., K.C.B., and L.H.G. conceived and designed of research; D.A.M. and D.d.C. performed experiments;

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