Synergism between psychosocial and metabolic stressors: impact on reproductive function in cynomolgus monkeys

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Williams NI, Berga SL, Cameron JL. Synergism between psychosocial and metabolic stressors: impact on reproductive function in cynomolgus monkeys. Am J Physiol Endocrinol Metab 293: E270–E276, 2007. First published April 3, 2007; doi:10.1152/ajpendo.00108.2007.—The role of energy imbalance versus psychosocial stress in the pathogenesis of female reproductive dysfunction characterized by anovulation and amenorrhea remains controversial. In women, functional hypothalamic amenorrhea can develop in the absence of significant weight loss, excessive exercise, or profound psychosocial disruption. We posited, therefore, that commonplace, seemingly minor stressors that alone would have minimal impact upon reproductive function might interact synergistically such that combinations of stressors would cause a greater impairment of the reproductive axis than any single stressor alone. We then developed a monkey model to test this hypothesis. Adult female cynomolgus monkeys with normal menstrual cycles were randomized into three experimental groups and studied over four menstrual cycles. The groups were: low-level psychosocial stress (i.e., moving to a new housing environment; Move, n = 8), moderate energy imbalance (Exercise + Diet, n = 9); and all stressors in combination (Move + Exercise + Diet, n = 10). Food intake, body weight, menstrual cyclicity, and reproductive hormones were assessed for two control menstrual cycles followed by two experimental cycles during which the monkeys experienced the stressors. Abnormal cycles were considered to be abnormally long or anovulatory cycles. Few abnormal cycles occurred in the Move group (1 of 8 monkeys) and in the Exercise + Diet group (1 of 9 monkeys). In contrast, 7 of 10 monkeys in the Move + Exercise + Diet group displayed at least one abnormal cycle (χ² = 9.61, P = 0.008). These findings suggest that infertility due to hypothalamic hypogonadism can result from the combination of commonplace, seemingly minor stressors that often escape clinical attention.

exercise; diet; psychosocial stress; reproduction

CLINICALLY RECOGNIZED FORMS of stress-induced reproductive dysfunction include functional hypothalamic amenorrhea (FHA), anorexia nervosa, bulimia nervosa, and exercise-associated amenorrhea. The proximate cause of these forms of reproductive compromise is a functional and theoretically reversible reduction in central drive to the reproductive axis provided by the hypothalamic neuroendocrine gonadotropin-releasing hormone (GnRH). The presence of clinical forms of stress-induced reproductive dysfunction heightens the risk for other diseases, including cardiovascular disease, osteoporosis, depression, and other psychiatric conditions as well as infertility (1, 15, 20, 28). Stress exposure during pregnancy can also have a negative impact on fetal development. Potential fetal consequences include preterm labor, poor neurodevelopment, and compromised psychosocial development (26, 31, 35).

Although it was previously believed that different forms of stress-induced reproductive dysfunction develop subsequently to a fairly discrete form of stress (i.e., FHA: psychosocial stress; anorexia nervosa and bulimia nervosa: nutritional compromise; exercise-associated amenorrhea: excessive exercise), there is a growing recognition that each of these syndromes develops in response to exposure to combinations of psychogenic and metabolic stresses. FHA can develop in the absence of profound psychosocial disruption. Compared with eumenorrheic women, women with FHA display mild indexes of “psychological stress” in the form of dysfunctional attitudes, difficulty coping with daily hassles, higher dependence on interpersonal relationships, and a higher incidence of past psychiatric disorders (4, 17). However, neuroendocrine abnormalities associated with FHA also indicate metabolic stress (21), and these patients exhibit a high incidence of subclinical eating abnormalities (19, 33, 37). Similarly, reproductive dysfunction occurring in anorexia nervosa was considered to be primarily the result of severe nutritional stress; yet the propensity to exercise intensively adds to the metabolic imbalance, and food restriction itself has a behavioral, seemingly volitional component that reflects altered cognitions that predispose to psychosocial stress. Furthermore, the high percentage of patients with anorexia nervosa in which weight gain does not restore reproductive function highlights the potential contribution of psychological stress in the pathogenesis of reproductive compromise (19, 29, 34). Cross-sectional studies of subjects with exercise-associated amenorrhea have documented reduced resting metabolic rate (27) and endurance indexes of chronic energy deficiency (14, 22, 23). However, we (40) have recently shown that menstrual disturbances induced by a diet and exercise program are associated with energy deficiency but also are associated with a significant increase in perceived stress. Thus, virtually all common forms of stress-induced reproductive dysfunction most likely involve simultaneous exposure to a combination of metabolic and psychosocial stress.

On the basis of these observations, we tested the hypothesis that a combination of metabolic and psychosocial stressors will...
act synergistically to cause a greater impairment of reproductive function than would occur if an individual experienced single stressors alone. To test this hypothesis, we performed a study using female cynomolgus monkeys (*Macaca fascicularis*), which display monthly menstrual cycles with no seasonal variation, much like women. We modeled our stress paradigms on the mild levels of psychosocial stress and the diet and exercise habits reported by women with FHA (5, 17, 25).

**EXPERIMENTAL PROCEDURES**

**Animals.** Twenty-seven adult female cynomolgus monkeys, weighing 2.72–5.26 kg, were used for this experiment. Monkeys were housed at the University of Pittsburgh Primate Research Laboratory in individual cages. Lights were on from 0700 to 1900; temperature was maintained at 24 ± 2°C. Animals were fed a single daily meal at 1100 (~300 kcal, no. 5045 chow; Ralston-Purina, St. Louis, MO) and a quarter piece of fresh fruit (~25 kcal) in the afternoon. They also received novel items, such as toys or noncaloric foods approximately twice a week as part of a psychological enrichment program in accordance with US Department of Agriculture guidelines. Water was available ad libitum. Food intake was recorded daily. Experiments were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

**Experimental protocol.** Animals were randomly assigned to 1) a group (*n* = 8) that experienced only a low level of psychosocial stress [Move group, in which monkeys were housed in individual cages but moved to a new room where they were then surrounded by unfamiliar monkeys, a procedure that leads to a transient increase in heart rate and plasma cortisol secretion (32)], 2) a group (*n* = 9) that exercised for 1 h a day, 5 days/wk, at a moderate intensity and experienced a 20% restriction in caloric intake [Exercise + Diet group, with the level of these stressors designed to approximate levels documented in women seeking treatment for FHA (5, 17, 25)], or 3) a Move + Exercise + Diet group (*n* = 10) that experienced all three stressors in combination (Fig. 1).

Monkeys were studied for four consecutive menstrual cycles, two control cycles (C1, C2), and two experimental cycles in which they were exposed to the stressors (E3, E4). Throughout C1 and the follicular phase of C2, they remained in their home cages and were fed their standard meal of 325 kcal/day. During the mid-luteal phase (at day 20 ± 2) of C2, monkeys in the Exercise + Diet group and the Move + Exercise + Diet group began acclimating to the treadmill and learning to walk. The purpose of the acclimation period was to reduce any variability between monkeys associated with learning to run on the treadmill, so that all monkeys could begin the first experimental menstrual cycle at approximately the same training level. Caloric intake was reduced by 20% on day 20 of C2. Caloric restriction was initiated at the same time as the acclimation period for treadmill walking/running, so that exposure to training and diet were equal for a given monkey. With the next menses, all monkeys in the Move group and the Move + Exercise + Diet group were moved to a novel room, where they were housed in single cages surrounded by unfamiliar monkeys. They remained in this new room until their next menses, when they were moved again (E4) and studied until the next menses occurred. However, one monkey in the Move + Exercise + Diet group was studied for only one experimental cycle (E3), as this monkey had a cycle greater than 100 days in E3; all other monkeys were studied for two experimental cycles (E3 + E4). Monkeys in the Exercise + Diet group remained in their home cages and exercised daily throughout E3 and E4. For monkeys in the Exercise + Diet and Move + Exercise + Diet groups, the exercise and diet stressors were applied throughout the entire menstrual cycle for both E3 and E4. All experimental groups were studied concurrently and over a similar time frame.

**Blood sample collection.** Blood samples for serum LH, FSH, estradiol (E2), and progesterone (P4) were collected from awake animals every other day, prior to exercise, throughout the study, as previously described (38). Samples were allowed to clot at room temperature for 1 h, were refrigerated for 2 h, and then were centrifuged at 2,500 rpm for 10 min. Serum was then stored at −20°C in glass vials until assays were performed. Every 6 wk, animals were given a 0.5-cm³ intramuscular injection of iron dextran to maintain normal hematocrit. Monkeys were weighed at the time of each blood sample collection.

**Monitoring reproductive function.** All animals were previously accustomed to daily checks for menses, i.e., swabbing the vaginal area with a cotton-tipped applicator. Throughout the study, day 1 of a cycle was considered to be the first day of menses. Each monkey was monitored before the study until it had at least three normal menstrual cycles. A cycle was considered normal if it was ovulatory, 25–44 days in length, and exhibited midcycle surges of E2, LH and FSH, and a luteal rise in P4 >1 ng/ml. Although the mean cycle length of monkeys in this study was 30 ± 0.8 days, one monkey consistently displayed ovulatory cycles that were 44 days in length for several months prior to the study. This monkey maintained long cycles throughout the study and therefore was included in the analyses. Anovulatory cycles were defined as cycles that began with observable menses but did not display normal ovulatory patterns of reproductive hormones. For the purposes of this study, we defined abnormal cycles to be those that were anovulatory or cycles that were outside 25–44 days in length whether or not they were ovulatory.

**Exercise training.** Animals were trained to run on standard treadmills for human use (Precor, Bothell, WA), using previously published techniques (38). Speed and duration were individually increased until the animals were running 1 b/day at a rate of 3.2–4.8 km/h. Training-induced increases in cardiorespiratory fitness were documented by having monkeys complete a maximal graded exercise test within the first 2 wk of their training and then again at the end of the study. The use of a maximal graded exercise test to assess cardiorespiratory fitness is well documented in humans (41). The exercise testing protocol began at a low speed (1.3 km/h) and then increased by 0.32 km/h every 2 min thereafter until the monkey exhibited signs of fatigue (i.e., running at the back of the Plexiglas...
box). Changes in fitness level were determined by the change in maximum speed attained during the maximal graded exercise test from the pretraining to posttraining. Training speeds for daily exercise bouts were individually calculated at 80% of the highest speed reached during the pretraining maximal graded exercise test. Both heart rate and cortisol are known to increase significantly in humans exercising at this intensity (16, 41).

Psychosocial stress paradigm. The form of psychosocial stress used in this study consisted of moving the animals to a novel room where they were housed in individual cages surrounded by unfamiliar monkeys. Previous work in this laboratory has shown that this form of stress leads to an elevation of heart rate and cortisol levels and can be associated with a disruption of normal menstrual cyclicity in a small percentage of monkeys (32). Experimental conditions during E3 and E4 in the Move group varied only with respect to the room in which monkeys were housed.

Hormone assays. Serum E2, P4, FSH, and LH concentrations were measured by radioimmunoassay (RIA) by the RIA Core Laboratory of the Center for Research in Reproductive Physiology at the University of Pittsburgh, using previously described methods (39). The sensitivities of the E2 assays ranged from 2.20 to 3.88 pg/ml (8.08 to 14.2 nmol/l), and the intra- and interassay coefficients of variation for the E2 assays were 6.1 and 7.7%, respectively. The sensitivities of the P4 assays ranged from 0.05 to 0.14 ng/ml (0.16 to 0.44 nmol/l), and the intra- and interassay coefficients of variation for the P4 assays were 5.0 and 6.4%, respectively. The sensitivities of the FSH assays ranged from 1.2 to 3.7 ng/ml (1.2–3.7 IU/l), and the intra- and interassay coefficients of variation for the FSH assays were 6.8 and 8.0%, respectively. Over the course of this study, LH was assayed using two different RIAs, with either purified monkey LH or recombinant monkey LH as a standard (2, 39). Conversion factors for the appropriate range of the standard curve were used to convert all data collected using the second assay to values in the first assay. The sensitivities of the LH assays ranged from 7.4 to 12.2 ng/ml (7.4–12.2 nmol/l), and the intra- and interassay coefficients of variation for the LH assays were 7.2 and 9.0%, respectively.

Data analysis. The effects of the experimental treatments on reproductive hormone secretion were characterized by measuring LH and FSH in each sample obtained every other day over the whole reproductive cycle and P4 during the second half of the cycle. The following parameters were calculated in ovulatory cycles: (i) LH surge (i.e., highest P4 level measured during the luteal phase); (ii) peak E2 (i.e., highest E2 value immediately preceding the LH surge); (iii) average follicular-phase E2 (i.e., the average of all follicular phase E2 concentrations measured); (iv) peak luteal-phase P4 (i.e., the highest P4 level measured during the luteal phase); (v) average luteal-phase P4 (i.e., the average of all luteal phase P4 concentrations measured); (vi) late-luteal FSH (i.e., the average of the last 2 FSH concentrations measured in the luteal phase); and (vii) average luteal-phase LH (i.e., the average of the LH concentrations measured during the luteal phase). Values for energy intake, body weight, and daily exercise training were averaged for each animal over each of the experimental periods. Results for primary outcome variables did not differ between the C1 and C2 time periods; thus all parameters calculated for the C1 and C2 periods were averaged together and reported as C1 + C2 (C1 + 2) values. Likewise, results for primary outcome variables also did not differ between the E3 and E4 cycles; thus all parameters calculated for the E3 and E4 cycles were averaged together and are reported as E3 + E4 (E3+4) values.

Prior to analysis, all data were checked for normality. For normally distributed data, changes over time were analyzed using repeated-measures analysis of variance (ANOVA). Baseline comparisons were performed using a one-way ANOVA. Post hoc testing utilized the least significant squares test. If assumptions of normality were violated, nonparametric tests appropriate for repeated measures were performed, i.e., the Wilcoxon signed rank test. For nonparametric comparisons between groups at baseline, the Kruskal-Wallis test was used followed by a Mann-Whitney U-test to determine where effects occurred. For data comparing the proportions of abnormal cycles between groups, χ2 analyses were used. For these analyses, an abnormality in either or both cycles during the experimental phase (E3 or E4) was counted as a single abnormality when E3 and E4 were combined (E3+4). For all analyses, P ≤ 0.05 was considered significant. All analyses were performed using SPSS v. 13.0 (SPSS, Chicago, IL). All data are reported as means ± SE.

RESULTS

The exercise and diet stressors used in this study led to a moderate increase in cardiovascular fitness, as expected with the implementation of low-level fitness training. In the exercising animals, maximal speeds attained during the exercise test were not different between groups at baseline and increased similarly (19–29%) with training [F(2,10) = 32.5, P = 0.001], i.e., from 5.8 ± 0.99 to 7.2 ± 0.12 km/h in the Exercise + Diet group and from 4.8 ± 1.49 to 6.2 ± 1.77 km/h in the Move + Exercise + Diet group. Daily training distances of individual monkeys ranged from 3.1 to 5.7 km/day during E3+4 for the two groups that exercised, and there was no significant difference in daily exercise level between these two groups at any time point. Training increased significantly from C1+2 to E3+4 in both exercising groups [F(1,15) = 531.8, P < 0.0001], and there was no difference between groups in this increase (Fig. 2). Food intake was not different between groups during C1+2 [F(2,26) = 1.87, P = 0.175] but decreased significantly over time [14–18% reduction; F(2,22) = 11.1, P = 0.0005] in the Exercise + Diet (P = 0.0003) and Move + Exercise + Diet groups (P = 0.002; Fig. 2). Body weight was not different between groups during C1+2 [F(2,26) = 0.31, P = 0.736]. No changes in body weight were observed in the Move group or in the Move + Exercise + Diet group, but weight in Exercise + Diet decreased significantly over time, from 3.56 ± 0.23 kg in C1+2 to 3.29 ± 0.20 kg in E3+4 [F(2,24) = 7.5, P = 0.003; Fig. 2].

We defined abnormal cycles as those that were anovulatory or cycles that were outside 25–44 days in length, whether or not they were ovulatory. Baseline prevalence of menstrual cycle abnormalities (the incidence of long menstrual cycles or anovulatory cycles) was very low and not significantly different between groups. Only one monkey had an abnormal cycle (in C2); all other control cycles were of normal cycle length and ovulatory and thus not abnormal. When menstrual cycle abnormalities were examined in each group across time, there were no significant effects of the intervention in either the Move (Z = −1.00, P = 0.317) or the Exercise + Diet (Z = −1.00, P = 0.317) groups (Fig. 3A). Menstrual cycle abnormalities occurred at a low frequency in the Move group (1 of 8 monkeys displayed an abnormal cycle during E4) and in the Exercise + Diet group (1 of 9 monkeys displayed an abnormal cycle during E3+4). In marked contrast, the intervention significantly disrupted menstrual cycles in the Move + Exercise + Diet group (Z = −2.449, P = 0.014). In this group, 7 of 10 monkeys displayed abnormal cycles in either E3 (4 of 10) or E4 (4 of 9), with one monkey displaying abnormal cycles during E3+4. No differences between groups existed in
the proportion of monkeys experiencing an abnormality during C1 + 2, \( (\chi = 1.765, P = 0.414) \). During E3 + 4, the proportion of cycle abnormalities was highly dependent on group (\( \chi = 9.613, P = 0.008 \)). The Move + Exercise + Diet group was significantly different from both the Move group (\( P = 0.025 \)) and the Exercise + Diet group (\( P = 0.025 \)).

The higher proportion of abnormal cycles in the Move + Exercise + Diet group was marked by a significant increase in menstrual cycle length across time (\( Z = -2.668, P = 0.008 \); Fig. 3B). There were no significant changes in cycle length across time in the Move or Exercise + Diet groups. Specifically, we found that there were eight long experimental menstrual cycles (>45 days in length) in the Move + Exercise + Diet group, with three cycles over 100 days in length, whereas there were one long menstrual cycle in the Move group and two long menstrual cycles in the Exercise + Diet group, with no cycles over 100 days in length in these groups. The change in cycle length in the Move + Exercise + Diet group was predominantly due to an increase in follicular phase length (\( Z = -2.666, P = 0.008 \)), although luteal length also changed significantly in this group (\( Z = -2.254, P = 0.024 \)). When cycle length was compared between groups, no differences were found at baseline in total cycle length, follicular length, or luteal length (\( C1 + 2: \chi = 1.684, P = 0.431; \chi = 0.789, P = 0.674; \chi = 1.397, P = 0.497 \)), respectively. However, during E3 + 4, group differences were noted for total length (\( \chi = 8.64, P = 0.013 \)) and follicular length (\( \chi = 9.73, P = 0.008 \)), such that total and follicular lengths for the Move + Exercise + Diet group were significantly greater than for both the Move and the Exercise + Diet groups (\( P < 0.025 \)).

Changes in reproductive hormones over time are shown in Table 1. Average follicular phase E2 levels declined significantly over the course of the study [\( F(1,21) = 12.01, P = 0.002 \)]; however, there was no group \( \times \) time interaction. Peak E2 concentrations decreased similarly [\( F(1,21) = 6.91, P = 0.016 \)]. Average and peak luteal phase P4 levels also declined significantly, although again no time \( \times \) group interaction was observed [\( F(1,21) = 9.41, P = 0.045; F(1,23) = 7.03, P = 0.014 \), for average and peak P4, respectively]. No significant differences were noted between groups or over time in LH or FSH.

**DISCUSSION**

Our findings show that exposure to low-level stressors that are common in everyday human life (including mild psychosocial stress, mild dietary restriction, and moderate exercise), when experienced alone, disrupt reproductive function in relatively few individuals (~10% of the population). However, combinations of these same low level stressors (e.g., combined psychosocial plus metabolic stress) synergized to compromise

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**Fig. 2.** Changes in body weight (top), kilometers run per day (middle), and daily calorie intake (bottom) across the course of the experiment for each experimental group. \( ^{a} P < 0.025, C1 + 2 \) vs. E3 + 4.

**Fig. 3.** A: percentage of monkeys showing abnormal menstrual cycles (i.e., either cycles >44 days in length or anovulatory cycles) in E3 + 4 for each of the 3 experimental groups. \( ^{a} \)Significant difference over time comparing E3 + 4 with C1 + 2 (\( Z = -2.449, P = 0.014 \)) and significant difference between groups when comparing E3 + 4 (Move + Exercise + Diet vs. both Move and Exercise + Diet, \( \chi = 9.613, P = 0.025 \)). B: menstrual cycle length (top), follicular phase length (middle), and luteal phase length (bottom) across the course of the experiment for each experimental group. \( ^{a} \)Significant difference within group from C1 + 2 (\( P < 0.05 \)). \( ^{b} \)Significant difference between groups (Move + Exercise + Diet vs. both other groups, \( P < 0.05 \)).
Folliculogenesis. The treatments in this study also produced an of anovulatory cycles caused by exposure to a combination of significant alterations in menstrual cycle length and occurrence preterm delivery, is expensive, and is stressful itself. The reproduction, which carries the risk of multiple gestation and infertility patients than immediately proceeding to assisted effective, inexpensive, and less risky management strategy for combinations of subthreshold stressors that might easily not receive or changes in activity levels (6). Indeed, the current monkey reproductive restoration was not accompanied by weight gain or problematic attitudes syndromal psychiatric conditions such as depression and eating disorders (17, 25). Furthermore, cognitive behavior therapy aimed at remediating problematic attitudes that contribute to psychosocial stress has been found to restore ovarian function in the majority of women so treated, and reproductive restoration was not accompanied by weight gain or changes in activity levels (6). Indeed, the current monkey results suggest that ovarian dysfunction can result from combinations of subthreshold stressors that might easily not receive clinical attention (20). Treating first the predisposition to be easily stressed with psychological interventions might prove an effective, inexpensive, and less risky management strategy for infertility patients than immediately proceeding to assisted reproduction, which carries the risk of multiple gestation and preterm delivery, is expensive, and is stressful itself. The significant alterations in menstrual cycle length and occurrence of anovulatory cycles caused by exposure to a combination of low-level stressors reflect a suppression and significant delay in folliculogenesis. The treatments in this study also produced an overall small but significant decline in ovarian E2 and P4, which mostly occurred in the Exercise + Diet and the Move + Exercise + Diet treatments. These hormonal changes were not accompanied by clinical changes in menstrual cyclicity unless all three stressors were combined, i.e., in the Move + Exercise + Diet group. Although our Exercise + Diet group lost weight, the degree of metabolic stress and the duration over which it was applied are much less than in our earlier study in cynomolgus monkeys where prolonged amenorrhea was produced with an average of 14.3 ± 2.2 mo of daily exercise that averaged 12.3 ± 0.9 km/day (38). Effects of mild metabolic stress on reproductive hormone secretion, but not fertility, have been demonstrated with short-term fasting in men (8) and male monkeys (9). Other work supports a dose-response effect of metabolic stress on reproductive function when energy deficiency is below a particular threshold (24). Thus, it is not surprising that this relatively mild metabolic stress had little impact on menstrual cycles when it was the only stress animals were exposed to.

Our study involved exposure to multiple stressors over a fairly short time frame, i.e., two menstrual cycles, and thus it is not known whether the effects observed would worsen with continued exposure to the stressors or perhaps be reversed or ameliorated if individuals accommodated to the stressors. Some indication of accommodation to the stressors is evident in our study. Two of five monkeys that had abnormal cycles in E3 had normal cycles in E4, perhaps indicating some accommodation to the stress. Both of the monkeys that exhibited abnormally long cycles during both E3 and E4 experienced a reduction in cycle length from E3 to E4. Clearly, future studies that examine the impact of these stressors over longer time periods are necessary to determine whether accommodation occurs and to determine the underlying mechanism for such adaptation.

The mechanism underlying the synergistic effects of individual stresses on reproductive function in this study is unknown. It is possible that the timing of exposure to stresses was important, in that the imposition of the psychosocial stress in the first experimental cycle occurred ~10 days after the monkeys had begun exercising and had their food intake reduced. Other investigators have found that the effect of a stress can be altered by prior exposure to low-level hypothalamic-pituitary-adrenal axis activation (30). It is likely that the suppression of reproductive function that we observed was due to a decrease in the activity of the hypothalamic GnRH neurons that provide the central drive to govern reproductive hormone secretion (10). A synergism between stresses might be expected to occur either by causing a larger effect on a single neurotransmitter system governing GnRH release or by each stress altering the central drive to govern reproductive hormone secretion in the activity of the hypothalamic GnRH neurons that provide the normal reproductive function that we observed was due to a decrease in ovarian function in the majority of women so treated, and reproductive restoration was not accompanied by weight gain or changes in activity levels (6). Indeed, the current monkey results suggest that ovarian dysfunction can result from combinations of subthreshold stressors that might easily not receive clinical attention (20). Treating first the predisposition to be easily stressed with psychological interventions might prove an effective, inexpensive, and less risky management strategy for infertility patients than immediately proceeding to assisted reproduction, which carries the risk of multiple gestation and preterm delivery, is expensive, and is stressful itself. The significant alterations in menstrual cycle length and occurrence of anovulatory cycles caused by exposure to a combination of low-level stressors reflect a suppression and significant delay in folliculogenesis. The treatments in this study also produced an overall small but significant decline in ovarian E2 and P4, which mostly occurred in the Exercise + Diet and the Move + Exercise + Diet treatments. These hormonal changes were not accompanied by clinical changes in menstrual cyclicity unless all three stressors were combined, i.e., in the Move + Exercise + Diet group. Although our Exercise + Diet group lost weight, the degree of metabolic stress and the duration over which it was applied are much less than in our earlier study in cynomolgus monkeys where prolonged amenorrhea was produced with an average of 14.3 ± 2.2 mo of daily exercise that averaged 12.3 ± 0.9 km/day (38). Effects of mild metabolic

Table 1. Changes in reproductive hormones with Move, Exercise + Diet, and Move + Exercise + Diet interventions

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Move</th>
<th>Exercise + Diet</th>
<th>Move + Exercise + Diet</th>
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<tr>
<td>Follicular phase</td>
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<tr>
<td>Average estradiol, pg/ml*</td>
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<td>94±13</td>
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<td>FSH, ng/ml</td>
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<td>7.5±0.8</td>
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<td>Luteal phase</td>
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<tr>
<td>Average progesterone, ng/ml†</td>
<td>5.8±0.7</td>
<td>8.6±0.9</td>
<td>5.4±0.6</td>
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<tr>
<td>Peak progesterone, ng/ml§</td>
<td>13.2±1.6</td>
<td>16.6±1.8</td>
<td>13.0±1.4</td>
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<tr>
<td>LH, ng/ml</td>
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<td>19.6±3.4</td>
<td>17.3±1.8</td>
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<tr>
<td>FSH, ng/ml</td>
<td>6.0±1.6</td>
<td>6.2±0.7</td>
<td>7.9±2.2</td>
</tr>
</tbody>
</table>

Values are means SE. C1, control groups 1 and 2; E3+4, experiments 3 and 4. *ANOVA significant time effect; F(1,21) = 12.01, P = 0.002; significant group effect F(2,21) = 4.23, P = 0.028; post hoc tests, Move vs. Move + Exercise + Diet (P = 0.014). †ANOVA significant time effect; F(1,21) = 6.91, P = 0.016. †ANOVA significant time effect; F(1,21) = 9.41, P = 0.045; significant group effect F(2,21) = 5.97, P = 0.008; post hoc tests, Exercise + Diet vs. Move + Exercise + Diet (P = 0.002). §ANOVA significant time effect; F(1,21) = 7.03, P = 0.014. Note: to convert estradiol pg/ml to nmol/l, multiply by 3.671; to convert progesterone ng/ml to nmol/l, multiply by 3.18; to convert LH and FSH ng/ml to IU/l, multiply by 1.0.

The mechanism underlying the synergistic effects of individual stresses on reproductive function in this study is unknown. It is possible that the timing of exposure to stresses was important, in that the imposition of the psychosocial stress in the first experimental cycle occurred ~10 days after the monkeys had begun exercising and had their food intake reduced. Other investigators have found that the effect of a stress can be altered by prior exposure to low-level hypothalamic-pituitary-adrenal axis activation (30). It is likely that the suppression of reproductive function that we observed was due to a decrease in the activity of the hypothalamic GnRH neurons that provide the central drive to govern reproductive hormone secretion (10). A synergism between stresses might be expected to occur either by causing a larger effect on a single neurotransmitter system governing GnRH release or by each stress altering the central drive to govern reproductive hormone secretion in the activity of the hypothalamic GnRH neurons that provide the normal reproductive function that we observed was due to a decrease in ovarian function in the majority of women so treated, and reproductive restoration was not accompanied by weight gain or changes in activity levels (6). Indeed, the current monkey results suggest that ovarian dysfunction can result from combinations of subthreshold stressors that might easily not receive clinical attention (20). Treating first the predisposition to be easily stressed with psychological interventions might prove an effective, inexpensive, and less risky management strategy for infertility patients than immediately proceeding to assisted reproduction, which carries the risk of multiple gestation and preterm delivery, is expensive, and is stressful itself. The significant alterations in menstrual cycle length and occurrence of anovulatory cycles caused by exposure to a combination of low-level stressors reflect a suppression and significant delay in folliculogenesis. The treatments in this study also produced an overall small but significant decline in ovarian E2 and P4, which mostly occurred in the Exercise + Diet and the Move + Exercise + Diet treatments. These hormonal changes were not accompanied by clinical changes in menstrual cyclicity unless all three stressors were combined, i.e., in the Move + Exercise + Diet group. Although our Exercise + Diet group lost weight, the degree of metabolic stress and the duration over which it was applied are much less than in our earlier study in cynomolgus monkeys where prolonged amenorrhea was produced with an average of 14.3 ± 2.2 mo of daily exercise that averaged 12.3 ± 0.9 km/day (38). Effects of mild metabolic

stress on reproductive hormone secretion, but not fertility, have been demonstrated with short-term fasting in men (8) and male monkeys (9). Other work supports a dose-response effect of metabolic stress on reproductive function when energy deficiency is below a particular threshold (24). Thus, it is not surprising that this relatively mild metabolic stress had little impact on menstrual cycles when it was the only stress animals were exposed to.

Our study involved exposure to multiple stressors over a fairly short time frame, i.e., two menstrual cycles, and thus it is not known whether the effects observed would worsen with continued exposure to the stressors or perhaps be reversed or ameliorated if individuals accommodated to the stressors. Some indication of accommodation to the stressors is evident in our study. Two of five monkeys that had abnormal cycles in E3 had normal cycles in E4, perhaps indicating some accommodation to the stress. Both of the monkeys that exhibited abnormally long cycles during both E3 and E4 experienced a reduction in cycle length from E3 to E4. Clearly, future studies that examine the impact of these stressors over longer time periods are necessary to determine whether accommodation occurs and to determine the underlying mechanism for such adaptation.

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reproductive function (3, 5, 22). Recent studies in our laboratory indicate that monkeys that readily develop menstrual abnormalities when exposed to the combined metabolic plus psychosocial stress reported here have an elevation of corticotropin-releasing hormone gene expression in the caudal portion of the hypothalamic paraventricular nucleus (11). In contrast, metabolic stresses are known to alter other neuronal systems that are strong modulators of GnRH neuronal activity, including neuropeptide Y, a hypothalamic neuropeptide that is exquisitely sensitive to changes in metabolic fuel availability (13) that also modulates GnRH neuronal activity (10). Our study design was limited in our ability to examine these potential mechanisms, as our first goal was to develop a model to test whether synergistic effects of low-level stressors on reproductive function could be demonstrated. More recently however, we have found that female monkeys who readily develop stress-induced reproductive dysfunction when exposed to combined psychosocial and metabolic stresses have elevated levels of anxious behavior (18), depressed expression of a number of genes in the central serotonin pathway as well as increased GABAergic gene expression in the medial basal hypothalamus (12).

Our finding that low-level stressors exhibit synergistic effects on the reproductive axis was recently used to guide the development of a behavioral intervention for women with FHA (6). We reasoned that, if combined metabolic and psychosocial stress caused FHA, then simultaneously addressing both psychosocial and metabolic stressors would be necessary to reverse FHA. Our initial trial involving cognitive behavior therapy and consumption of small, frequent meals has supported this interpretation (6). Alternatively, one could conclude from our results that single stressors are less likely to impair reproductive function than combined stressors; thus any treatment that effectively removes at least one stress will be helpful in treating FHA. Nevertheless, it seems prudent to treat as many stressors as possible that could contribute to reproductive dysfunction in women seeking treatment for infertility. More broadly, the ability of low-level stressors to act synergistically suggests that low-level stressors, which are often overlooked when obtaining a clinical history or deciding a treatment plan, may have significant impact on a number of stress-related disease processes.

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