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Fluctuations of Unbound Whole Blood Polyamine Levels during the Menstrual Cycle (39333)

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The polyamines, putrescine, spermidine (Spd), and spermine (Spm), appear to be ubiquitous in animal tissues. The only known route of polyamine biosynthesis in mammals involves the decarboxylation of ornithine to form putrescine, which reacts with the *N*-propylamine moiety of decarboxylated *S*-adenosylmethionine to form spermidine, and in a similar manner spermidine reacts with decarboxylated *S*-adenosylmethionine to form spermine. Although the physiological role of these cations remains to be eluciated, they have been implicated in playing an important role during growth and cellular transformation. Their level increases dramatically along with their respective biosynthetic enzymes at the onset of various growth processes (1). Several hormones, including luteinizing hormone (2), estradiol (3, 4), and testosterone (5) directly affect polyamine biosynthesis; furthermore, studies (6, 7) indicate that one or more of the polyamines, at physiological concentrations, can substitute for hormones in mediating specific biologic responses.

Recent clinical reports (8, 9) have demonstrated that various neoplastic states are accompanied by increased levels of tissue and circulating polyamines; and, in some cases, successful therapy leads to a decrease in the concentration of these compounds (9). Another possible medical application of polyamine determinations relates to identification of cystic fibrosis homo- and heterozygotes (10, 11). In particular, our laboratory observed that male, but not female, homo- and heterozygotes for cystic fibrosis (CF), the most common lethal inherited disease affecting Caucasians (12), were characterized by a significantly higher Spd/Spm ratio in unhydrolyzed whole blood when compared to control subjects (11). The observation that male, but not female, CF patients displayed this abnormality is not consistent with the accepted mode of inheritance of this genetic disease: i.e., autosomal recessive inheritance (12). Close inspection of the polyamine levels and Spd/Spm ratios from female controls and female homo- and heterozygotes for CF suggested that their ratios are subject to greater amounts of variability than male values. This observation suggested that a sex-related hormone(s) may be influencing the female Spd/Spm ratio during the menstrual cycle. For this reason, we have examined unhydrolyzed whole blood Spd and Spm concentrations and Spd/Spm ratios in four normal females, three normal males, and one ovariectomized female, over a period of 4 weeks, to encompass the menstrual cycle.

Materials and methods. Subjects. Blood samples were obtained from normal volunteers who were housed at the National Institutes of Health for the duration of these studies. A routine medical examination showed all to be in good health. The ages of the normal males were 19, 19, and 20. The normal females, ages 19, 19, 19, and 20, had regular menstrual cycles of 23, 27, 27, and 31 days. One of the four was placed on an oral contraceptive containing 1 mg of ethynodiol diacetate and 50 μg of ethinyl estradiol (Demulen, Searle & Co., Chicago) 30 days after the initial studies, and her Spd and Spm concentrations were determined for an additional 22 days.

The ovariectomized female available for these studies was 25-yr old, in good health, and not taking hormonal supplements. Sixteen months previously, she had been found to have a differentiated adenocarcinoma of the endometrium and shortly thereafter underwent total abdominal hysterectomy, bilateral salpingo-oophorectomy, and appendectomy.

Polyamine extraction. Whole blood was obtained in a fasting state from all the individuals and extracted within 30 min of collection. Extraction of whole blood polyamines was carried out as follows, using plastic containers when possible. Whole blood (10 ml) was drawn into a syringe containing 1.0 ml of 3.0% sodium citrate, mixed, added to 10 ml of 3.0% $HClO_4$, allowed to stand at 5° for 20 min and centrifuged at 4500 *g* for 20 min. The supernatant was decanted and stored, and the pellet re-extracted as above. The supernatants were pooled, then neutralized with 4 *N* KOH and stored at 4° for 16 hr. After decanting, 2.0 ml of 3.0% sulfosalicylic acid was added (to assure precipitation of any residual protein), the resulting precipitate removed by 10,000 *g* centrifugation, and the extract concentrated to dryness by lyophilization. Samples were then resuspended in 2.0 ml of sodium citrate buffer (pH 2.2, 0.2 *M*) and centrifuged at 4500 *g* for 10 min to remove residual $KClO_4$. Previous studies using [^{14}C]Spd and [^{14}C]Spm, added to whole blood and carried through the extraction procedure, had shown that recoveries averaged 66% and varied by less than 5% for either polyamine.

It should be noted that "polyamines" in this study refer to free, as opposed to total, blood polyamines which would also include those known to be covalently bound. Determination of total blood polyamines requires acid hydrolysis with 6 *N* HCl at 110 *c* or a similar procedure and is the technique most often utilized when studying polyamine concentrations in fluids from cancer patients (1). We have elected not to hydrolyze in view of the following: (i) the original observation that polyamine levels are abnormal in the blood of cystic fibrosis patients was made on unhydrolyzed blood (10); (ii) our subsequent conformation of the original observation was made on unhydrolyzed blood (11); and (iii) the initial suggestion that blood polyamine levels may fluctuate in females was obatined on unhydrolyzed blood.

Polyamine separation and quantification. Polyamines were separated and quantitated using a KCl–potassium citrate H_2O buffer, as previously described (13). Of the above supernatant, 0.5 ml (equivalent to 2.5 ml whole blood) was applied to an amino acid analyzer column (0.9 × 23 cm), containing PA-35 resin (Beckman Instrument Co., Palo Alto, Calif.). Replicate samples routinely showed greater than 95% agreement between runs.

Hormone determination. Plasma samples collected at the same time as whole blood samples for polyamine determinations were analyzed for estradiol and progesterone by Bio-Science Laboratories (Van Nuys, Calif.).

Statistics. Female Spd/Spm ratios appeared to be cyclic in nature, suggesting that it may be unreasonable to assume that an individual female ratio belongs to a known frequency distribution, thereby precluding the use of a parametric test for statistical comparison of male to female ratios. To compare female to male ratio variance we have used the Wilcoxon-Mann-Whitney *U* test, a nonparametric test.

Results. Male ratios and concentrations. Male Spd/Spm ratios and concentrations are shown in Fig. 1A and B, respectively. The

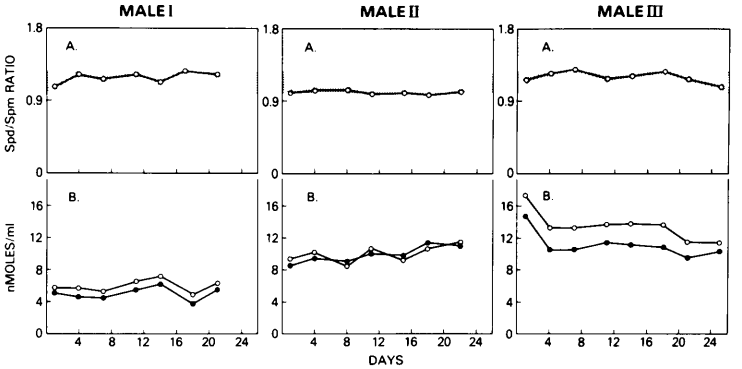

FIG. 1. (A) Male Spd/Spm ratio profiles. The shaded areas indicate the cumulated 1 SD interval obtained for Males I, II, and III. (B) Male Spd (-○-) and Spm (-●-) concentrations.

mean and 1 SD for the cumulated (n = 22) male ratios is 1.14 ± 0.11. Of the 22 individual male ratios, all but one fell within the 1 SD interval. Although showing more fluctuation than their respective ratios, the actual Spd and Spm concentrations (Fig. 1B) appear to be stable for a given individual. However, unlike the individual ratios, which varied little from one male to the next and are similar to our previous results (11), the individual Spd and Spm concentrations varied from one male to the next (Fig. 1B). For example, the mean ± SE for Spd concentrations of Male I and Male III were 5.76 ± 0.25 and 13.69 ± 0.67 nmole/ml, respectively.

Normal female ratios and concentrations. In contrast to the male Spd/Spm ratios, the four normal females showed substantial fluctuation in their ratios (Figs. 2A and 3A) and when compared to male values were found to be significantly different (P < 0.029). The mean cumulative (n = 36) female ratio is 1.34 ± 0.25. Eighteen (64%) of the female ratios fell above the 1 SD interval for males (shaded areas, Figs. 3A and 4A) and 11 (39%) were greater than 3 SD. However, only two ratios fell below the male 1 SD interval.

Figure 2B provides the estradiol and progesterone levels determined for three of the normal females. The highest values obtained for the midcycle peak of estrogen and the progesterone surge are indicated by vertical solid lines and hatched lines, respectively (Fig. 2B). Female ratios appear to rise at some point after the last day of menses and fall before the onset of the next menstrual cycle. As was typical of the male Spd and Spm concentrations (Fig. 1B), the female Spd and Spm concentrations (Fig. 2C) varied from one individual to the next. However, unlike the individual male concentrations, which appear to be stable over the period of the study, individual females, when compared to males, show a high degree of fluctuation from one sampling to the next.

Female receiving oral contraceptives. The Spd/Spm ratios of a fourth normal woman were determined for a period of 32 days, after which she was placed on the oral contraceptive Demulen and the Spd/Spm ratios determined for an additional 22 days. Ratios before receiving the oral contraceptive (Fig. 3A) had the same appearance as the other three women's (Fig. 2A) and were characteristic of an increase well above the male 1 SD (shaded area Fig. 3A). As shown, this increase occurred approx 15 days after the first day of menses and fell substantially at about Day 20. After receiv-

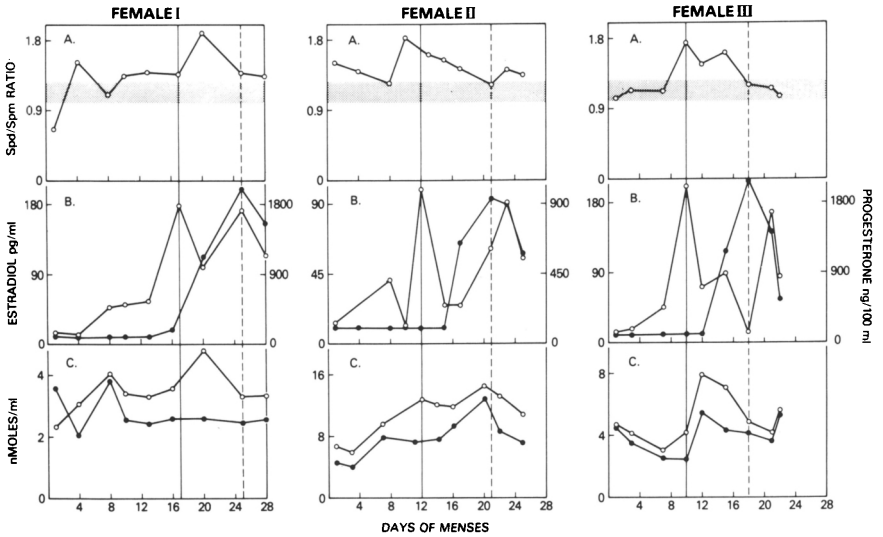

FIG. 2. (A) Female Spd/Spm ratio patterns with male cumulated 1 SD interval indicated (shaded areas). Day 1 equals the first day of menses. (B) Plasma estradiol (-○-) and progesterone (-●-) levels. The highest values obtained for the first estradiol peak and the highest value obtained for progesterone during the menstrual cycle are indicated by the vertical solid and hatched lines, respectively. (C) Female Spd (-○-) and Spm (-●-) concentrations.

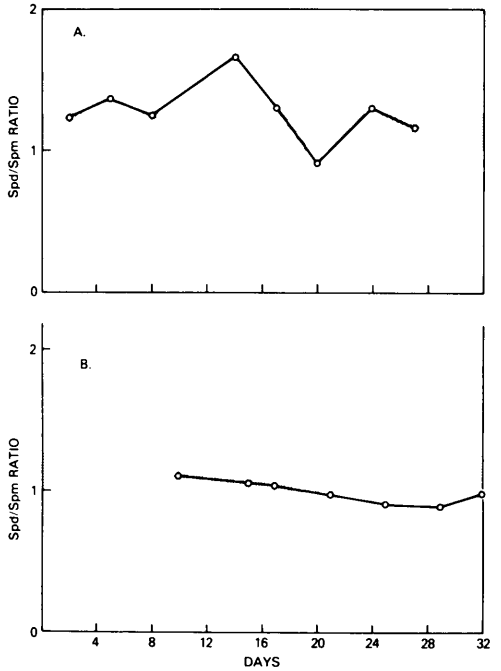

FIG. 3. (A) Female Spd/Spm ratio profile before receiving oral contraceptive. Day 1 equals first day of menses. The shaded area indicates the cumulated male 1 SD interval. (B) Female Spd/Spm ratio profile after receiving oral contraceptive (shaded area as in A).

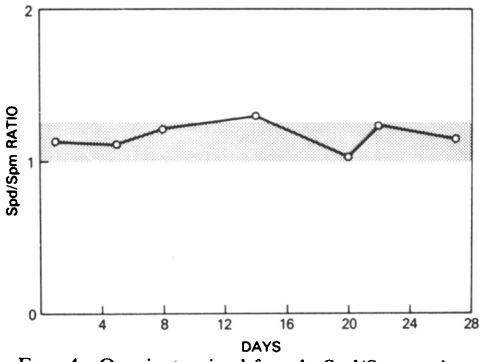

FIG. 4. Ovariectomized female Spd/Spm ratio pattern. The shaded area indicates the cumulated male 1 SD interval.

ing the oral contraceptive, however, her ratio pattern showed very little fluctuation: the characteristic finding obtained with males (Fig. 1A). The mean Spd/Spm ratio, as well as the SEM (a reflection of ratio variance), while on hormonal supplements, was significantly ($P < 0.02$) lower than the mean figure ± SEM obtained in the first study (0.99 ± 0.08 vs 1.25 ± 0.19, respectively).

Ovariectomized female ratios. The Spd/Spm ratios obtained for an ovariectomized woman are provided in Fig. 4. Unlike the noraml women, the ovariectomized woman showed very little fluctuation and provided a Spd/Spm ratio pattern similar to the males'. Only one of the Spd/Spm ratios obtained for the ovariectomized woman fell outside the 1 SD interval for men (shaded area, Fig. 4) and it came close to being within this interval.

Discussion. These studies demonstrate that over extended periods of time, blood samples from normal women exhibit a higher Spd/Spm ratio variance than to specimens from men. The increased variance appears to be a reflection of the variation during the menstrual cycle, suggesting that a sex-related hormone(s) influences whole blood Spd and Spm concentrations.

This is also supported by the observations that: (i) a woman who possessed a Spd/Spm ratio pattern characteristic of three other women assumed a male ratio profile after taking an oral contraceptive containing a progesterone analog as the active ingredient, and (ii) an ovariectomized woman exhibited a Spd/Spm ratio profile very similar to men's.

Our findings suggesting an interrelationship between hormones and polyamines are in keeping with previous studies: (i) administration of 17-β-estradiol leads to increased putrescine, spermidine, and spermine, as well as their respective biosynthetic enzymes in castrated rat uterus (3); (ii) castrated rats are characterized by a marked reduction in polyamine concentrations as well as biosynthetic enzymes in ventral prostate, all of which increase after injection of testosterone propionate (5); (iii) in rat ovary, luteinizing hormone and human chorionic gonadotropin stimulate the first and rate-limiting enzyme in polyamine biosynthesis, ornithine decarboxylase (2); (iv) recent data suggest that ornithine decarboxylase may, in part, be regulated by cyclic AMP (14); (v) spermidine, at physiological concentrations, can substitute for glucocorticoid in promoting milk production in cultured mammary cells (6); and (vi) spermine and spermidine can mimic the action of insulin toward lipid and glucose metabolism in cultured adipose tissue cells (7).

It has been shown that polyamine concen-

trations are elevated in the blood and urine of individuals with a variety of different neoplasia (8, 9) and that the polyamine levels decline upon remission (9). These observations have prompted speculation concerning the utilization of polyamine measurements as an index of malignant disease (9, 15). In addition to abnormal levels in cancer, other diseased states such as cystic fibrosis (11) and polycythemia vera (16) appear to be associated with aberrant blood unbound polyamine concentrations and/or Spd/Spm ratios. An inherent requirement of utilizing a specific metabolite as a diagnostic tool, is that the normal population be clearly defined. Our data suggest that major differences exist with respect to whole blood Spd/Spm ratios and concentrations in men, as compared to women. Since female values fluctuate to a greater extent, it appears necessary to measure blood polyamines in women at a specific time in the menstrual cycle if valid data for clinical diagnoses are to be obtained. Men, on the other hand, show nearly constant Spd/Spm ratios over extended periods of time, suggesting that abnormal polyamines in whole blood may be useful in the diagnosis, or in following the clinical course, of some maladies such as cancer and cystic fibrosis.

Finally, in view of the fact (1) that polyamines exist in covalently bound as well as free forms, it will be of interest to determine if the magnitude of polyamine concentrations and spermidine/spermine ratio fluctuations observed in females during these studies on unhydrolyzed samples are even more pronounced after hydrolyzing samples. If so, it appears evident that the time samples are obtained from female cancer patients and control subjects for polyamine analysis must be coordinated with specific times in the menstrual cycle.

Summary. Perchloric acid-extractable whole blood spermidine and spermine concentrations were determined over a 4-week period in three men, four women, and one ovariectomized woman. Individual male spermidine/spermine ratios showed little fluctuation and similar values were obtained for each of the three males studied. Male spermidine and spermine concentrations, although stable for each male, varied from one subject to the next. Individual female spermidine/spermine ratios, as well as individual concentrations, fluctuated substantially when compared to those values obtained for males; female ratios appear to rise and fall as a function of the menstrual cycle. The spermidine/spermine ratios obtained from a normal female receiving oral contraceptive, as well as those from an ovariectomized female, were characteristic of values obtained from men. It is suggested that a sex-related hormone(s) influences both the spermidine/spermine ratio and spermidine and spermine concentrations in females.

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