Serum leptin and erythropoietin during menstruation

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Summary

Objective: The potential influence of leptin and erythropoietin on the angiogenesis and bleeding of the endometrium during normal menstrual cycles and possible correlations between them.

Study design: Serum concentrations of leptin and erythopoietin were measured at menstrual days 20, 1 and 3 or 4 in healthy, non-obese, normal menstruating women.

Results: Mean leptin and erythropoietin concentrations showed no significant alteration over time ($F = 0.588$, $p = 0.563$ and $F = 0.654$, $p = 0.528$, respectively). There was, however, a strong negative linear relationship between the concentration of the two substances on days 1 and 3 or 4 ($p = 0.018$ and $p = 0.028$, respectively).

Conclusions: If the two substances affect endometrial angiogenesis, they may do this in a locally limited way, so that peripheral concentration changes cannot be observed. Their inverse correlation prompts further study with receptor determination.

Key words: Leptin; Erythropoietin; Endometrium; Menstruation; Angiogenesis.

Introduction

Apart from their established role in food intake regulation and hematopoiesis, much attention has been paid recently to the potential role of leptin and erythropoietin in female reproductive function.

Leptin deficiency is associated with reversible hypogonadism and sterility in rodents [1]. In healthy human females, leptin levels fluctuate throughout the menstrual cycle, showing a peak during the luteal phase [2, 3], whereas its receptor (OB-R) is expressed in human endometrium [4]. Leptin has been shown to possess hematopoietic [5] and angiogenic [6] properties.

Erythropoietin (Epo) is the major hematopoietic hormone. Provided that no excessive menstrual bleeding occurs, menstruation may not influence hematocrit values and may not impose an excessive need for hemopoiesis, and thus for erythropoietin [7]. However, Epo also has angiogenic properties in vitro and in vivo [8] and its mRNA is expressed in normal human endometrium [9]. There are data showing that Epo promotes endometrial angiogenesis in an estrogen-dependent manner in experimental settings [10], although its levels do not change during the menstrual cycle in humans [7].

Vasoconstriction and angiogenesis are the major perimenstrual events that contribute to endometrial shedding and regeneration. The classic studies of Markee [11] show that an ischemic phase caused by vasoconstriction of the arterioles and spiral arteries precedes the onset of menstrual bleeding by four to 24 hours. Bleeding occurs after arterioles and arteries relax, leading to hypoxia/reperfusion injury. The repair of the endometrium begins as early as 36 hours after the onset of bleeding, while menstrual desquamation is still in process [12]. Hypoxic and hormonial stimuli trigger the secretion of several factors, particularly vascular endothelial growth factor (VEGF), that induce angiogenesis [13, 14]. Although human endometrium has angiogenic potential throughout the menstrual cycle, the angiogenic response seems to be increased during the late menstrual/early proliferative [15, 16] and late secretory [16] phase. However, other data suggest that levels of endothelial cell proliferation in human endometrium do not show any consistent pattern across the different stages of the menstrual cycle [17].

The present study measures serum leptin and erythropoietin during the mid-luteal and menstrual phase of a normal cycle. This targeted double screening aims at simultaneously exploring within- and between-subjects variability of two substances with potential influence on the angiogenic mechanism of normal menstrual cycles, and at looking for possible correlations between them.

Materials and Methods

The study sample included 14 healthy women, aged from 20 to 25 years, who were recruited during their visit for regular Pap smears and gave their informed consent. All women had a normal menstrual history, defined as a menstrual cycle of 26 to 30 days, stable during the previous 12 months. Transvaginal ultrasound was performed during the periovulatory days in order to assess follicular development and ovulation and to exclude polycystic ovaries. A complete blood count was performed and women were included if they had hematocrit or hemoglobin values greater than 36% or 12 mg/dl, respectively. Polycystic ovarian syndrome (PCOS) was formally excluded by measuring FSH, LH, SHBG, testosterone and DHEAS on menstrual day 3. Selection criteria also included normal body weight, defined by a body mass index (BMI) < 24.

Menorrhagia was excluded by self-assessment, taking into account the number of pads/day, the presence of clots and the

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need for more than one protection method (e.g. tampon plus pad), with the understanding that this kind of assessment may be rather poor (for a review see Salamonsen [18]). Women did not receive any hormonal treatment or vitamin supplements during the prior three months.

Blood samples were collected in fasting women at menstrual days 20, 1 and 3 or 4, between 10 and 12 AM and serum leptin and erythropoietin levels were measured. A complete blood count was obtained at days 20 and 3/4. Serum separator tubes (SST) allowed samples to clot for 30 minutes until centrifugation. An SST was used for each sample, kept at room temperature while clotting. Centrifugation followed at 1000 x g for ten minutes. Specimens were stored at –20°C until assayed. Samples were vortexed gently after thawing and were afterwards analyzed.

Serum leptin concentration was determined by the quantitative sandwich enzyme immunoassay technique with an intra-assay and inter-assay CV of 2.4-4.6% and 2.2-4.2%, respectively, (sensitivity < 7.8 pg/ml, Quantikine, R&D Systems, Abingdon, UK).

Serum erythropoietin levels were measured by the immunometric assay technique with an intra-assay and inter-assay CV of 2.2-4.0% and 0.2-2.0%, respectively, (sensitivity 0.24 mU/ml, Immulite, Euro/DPC, Gwynedd, UK).

The individual changes in the concentrations of the two substances over the three measurements were assessed using either within-subjects General Linear Model/ANOVA, or, when the assumption of normality of the residuals was violated, using Friedman's two-way analysis of variance. Scheffé's test was used for post hoc multiple comparisons when appropriate. Correlations between the two substances for every time-point were also assessed using Pearson's r test.

Results

The mean age of the women was 22.3 years (SD 1 year). The mean hematocrit value was 40.8% (SD 1.7%) and 40.1% (SD 1.8%) on days 20 and 3/4 respectively, showing no significant fluctuation.

The individual curves of leptin changes over time are shown in Figure 1. The measurements of four individual curves (the upper curves) showing a unique pattern were considered as outliers and were excluded from the calculations. Mean leptin concentration was 7720 (SD 2952) μg/ml on day 20, 7959 (SD 2911) μg/ml on day 1 and

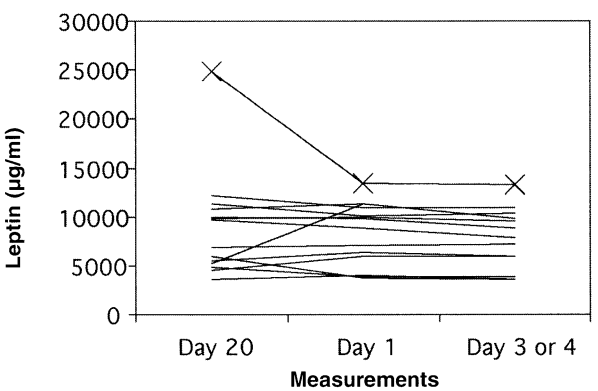

Figure 1. — Leptin concentration on menstrual days 20, 1 and 3 or 4.

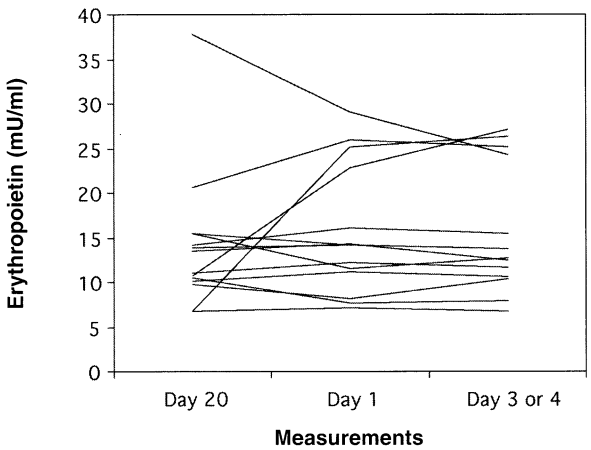

Figure 2. — Erythropoietin concentration on menstrual days 20, 1 and 3 or 4.

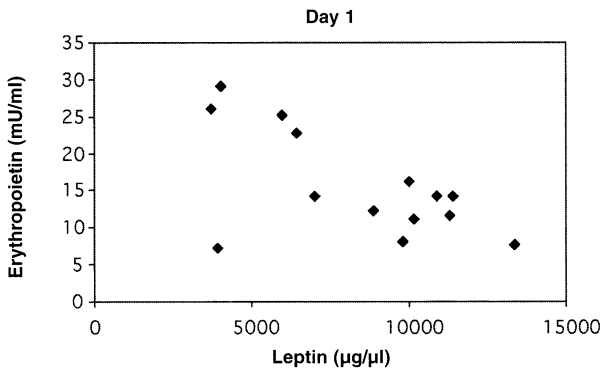

Figure 3. — Leptin vs erytropoietin concentration on menstrual day 1 (EPO = 27.55-0.14 leptin).

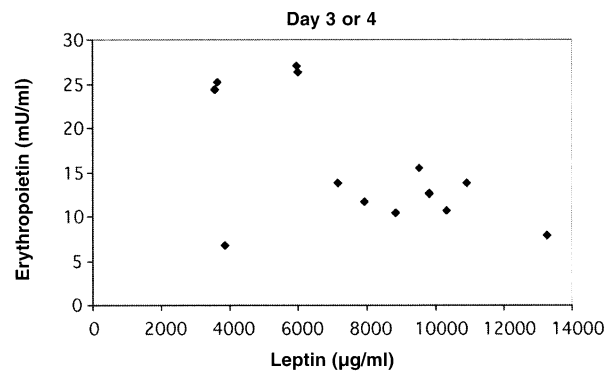

Figure 4. — Leptin vs erythropoietin concentration on menstrual day 3 or 4 (EPO = 26.52-0.14 leptin).

7488 (SD 2652) μg/ml on days 3 and 4. Leptin levels showed little variation during the study period. Application of within-subjects GLM confirmed this finding by not demonstrating any statistical significance (F = 0.588, p = 0.563).

The individual changes in Epo concentration over time are shown in Figure 2. Mean Epo concentration was 14.03 (SD 7.7) mU/ml on day 20, 15.7 (SD 7.2) mU/ml on day 1 and 15.6 (SD 7.02) mU/ml on days 3 and 4.

Although for most individuals the levels of Epo remain relatively stable, it is difficult to establish a constant pattern. Application of within-subjects General Linear Model showed that Epo concentrations did not alter significantly over time ($F = 0.654$, $p = 0.528$).

Explorations for correlations between the levels of the two substances at every time-point yielded two significant results: leptin and erythropoietin concentrations demonstrated a significant negative linear correlation on days 1 (E = 27.55-0.14 l) and 3,4 (E = 26.52-0.14l) ($p = 0.018$ and $p = 0.028$, respectively), (Figures 3 and 4).

Discussion

Mean blood loss during normal menstruation is 43 ml, whereas 10% of women may lose at least 100 ml of blood per month [18]. Normal menstrual flow is not likely to induce a hematocrit fall and thus need for excessive hematopoiesis. Erythropoietin, however, apart from its hematopoietic action, has demonstrated direct angiogenic properties in vivo and in vitro [8]. Studies in rodents have shown that Epo protein and its mRNA were produced in an estrogen-dependent manner in endometrial cell cultures, and injection of Epo into the endometrial cavity of ovariectomized mice promoted endometrial vessel formation [10]. Epo receptor protein has been found in the endothelium of human endometrial vessels [9]. Having in mind that endometrial repair and angiogenesis begins during menstruation [15, 16], one would expect that erythropoietin concentration might increase during the last days of menstruation.

Our results showed no significant difference in hematocrit, hemoglobin or erythropoietin values before and during menses. This is in agreement with others [7, 19] and may imply either that erythropoietin does not significantly affect endometrial angiogenesis or that it does so without significantly raising its peripheral concentration. Indeed, Yasuda *et al.* [10] suggested that Epo is locally and transiently produced in the endometrium of ovariectomized mice in response to E2 and induces local angiogenesis without affecting peripheral Epo concentrations. They also assumed that peripheral blood Epo is probably not involved in human endometrial angiogenesis, since the ligand affinity to EpoR in endometrial cells is much lower than that in erythroid precursor cells. A third perspective is highlighted by Rogers *et al.* [17], who found that angiogenesis occurs continuously throughout the menstrual cycle, without the formation of vascular sprouts, whereas erythropoietin induces sprouting [8].

Leptin concentration showed a fairly stable pattern, in contrast with what has been described. In other studies it has been described that serum leptin values are decreased in amenorrheic-anorectic women [20] and elevated in obese women [21] but without changes in normally menstruating women [22]. More specifically, leptin was shown to fluctuate during menstrual cycles, reaching a peak at the mid-luteal phase and then rapidly declining [2, 3], whereas at all instances its values were higher in obese women [2]. Our sample included normal-weight, normal menstruating women, however none of them demonstrated any significant changes in leptin levels during the 12-day observation period. In the original study concerning leptin's action as an angiogenic factor in mice [6], it was demonstrated that leptin induced formation of elongated, bifurcating tubules in vitro, and caused a vigorous angiogenic response in vivo; in both instances, leptin-induced angiogenesis had the same features as VEGF-induced angiogenesis. Moreover, Kitawaki *et al.* showed that OB-R isoforms are expressed in human endometrium, demonstrating a cyclical variation with higher levels at early secretory and lower levels at early proliferative phases, a finding that authors interpreted as having a relationship with the implantation process [4]. This pattern of receptor expression and our findings may suggest that the role of leptin in endometrium is other than angiogenesis.

An interesting finding of our study, however, is that leptin levels demonstrated a significant negative correlation with erythropoietin concentration during menstruation, i.e., on days 1, and 3 or 4. This may be suggestive of their synergistic action on the endometrium. Menstruation triggers the need for angiogenesis in order to support the endometrium that is to regenerate, so this may be the field of action of the two substances. Moreover, leptin and Epo action on endometrium may have a different 'weight' in each individual, since neither OB-R nor Epo are found constantly in all endometrial specimens [4, 9].

The only other context in which the possible relationship between leptin and Epo levels has been surveyed refers to hemodialyzed patients. Based on the rationale that both Epo and leptin exert hemopoietic activity, the influence of Epo administration on leptin levels of hemodialysed patients was studied. The results were controversial as either no relationship [22] or a significant negative relationship [24] was found between Epo and serum leptin levels, whereas a single dose of rHuEPO did not influence plasma leptin levels in such patients [25].

In conclusion, the finding that neither leptin nor erythropoietin increase during the first menstrual days, cannot be supportive of their role in endometrial angiogenesis during that period. However, the fact that both of those substances exert angiogenic activity and express receptors in human endometrium, together with the finding that their levels are inversely correlated during the first menstrual days, give an indirect clue suggesting that they may exhibit some action on the endometrium of that phase of cycle. As already suggested for Epo in animal models [10], such an action may be limited in a strictly local level, something that seems to be supported by the combination of unaltered peripheral levels with an inversely correlated concentration of the two substances. Further studies are needed in order to correlate expression of both receptors with vascular density in separate individuals.

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References

[1] Chehab F.F., Lim M.E., Lu R.: "Correction of the sterility defect in homozygous obese female mice by treatment with the human recombinant leptin". *Nat. Genet.,* 1996, *12,* 318.

[2] Ouinton N.D., Laird S.M., Okon, M.A., Li T.C., Smith R.F., Ross R.J.M., Blakemore A.I.F.: "Serum leptin levels during the menstrual cycle of healthy fertile women". *Br. J. Biomed. Sci,* 1999, *56,* 16.

[3] Riad-Gabriel M.G., Jinagouda S.J., Sharma A., Boyadjian R., Saad M.F.: "Changes in plasma leptin during the menstrual cycle". *Eur. J. Endocrinol.,* 1998, *139,* 528.

[4] Kitawaki J., Koshiba H., Ishihara H., Kusuki I., Tsukamoto K., Honjo H.: "Expression of leptin receptor in human endometrium and fluctuation during the menstrual cycle". *J. Clin. Endocrinol. Metab.,* 2000, *85,* 1946.

[5] Cioffi J.A., Shafer A.W., Zupancic T.J., Smith-Gbur J., Mikhail A., Platika D., Snodgrass H.R.: "Novel B219/OB receptor isoforms: possible role of leptin in hematopoiesis and reproduction". *Nat. Med.,* 1996, *2,* 585.

[6] Sierra-Honigmann M.R., Nath A.K., Murakami C., Garcia-Cardeña G., Papapetropoulos A., Sessa W.C. *et al.*: "Biological action of leptin as an angiogenic factor". *Science,* 1998, *281,* 1683.

[7] Makinoda S., Mikumi M., Sogame M., Kobamatsu Y., Furuta I., Yamada H. *et al.*: "Erythropoietin, granulocyte-colony stimulating factor, interleukin-1 beta and interleukin-6 during the normal menstrual cycle". *Int. J. Obstet. Gynecol.,* 1996, *55,* 265.

[8] Ribatti D., Presta M., Vacca A., Ria R., Giuliani R., Dell'Era P. *et al.*: "Human erythropoietin induces a pro-angiogenic phenotype in cultured endothelial cells and stimulates neovascularization in vivo". *Blood,* 1999, *15,* 2627.

[9] Yasuda Y., Fujita Y., Musha T., Tanaka H., Shiokawa S., Nakamatsu K. *et al.*: "Expression of erythropoietin in human female reproductive organs". *Ital. J. Anat. Embryol.,* 2001, *106* (suppl. 2), 215.

[10] Yasuda Y., Masuda S., Chikuma M., Inoue K., Nagao M., Sasaki R.: "Estrogen-dependent production of erythropoietin in uterus and its implication in uterine angiogenesis". *J. Biol. Chem.,* 1998, *273,* 25381.

[11] Markee J.E.: "Menstruation in intraoccular endometrial transplants in the rhesus monkey". *Cont. Embryol.,* 1940, *177,* 220.

[12] Ludwig H., Metzger H., Frauli M.: "Endometrium: tissue remodelling and regeneration". In: D'Arcangues C., Fraser I.S., Newton J.R. *et al.* (eds.): Contraception and Mechanisms of Endometrial Bleeding. Cambridge: Cambridge University Press, 1990, 441.

[13] Smith S.K.: "Angiogenesis, vascular endothelial growth factor and the endometrium". *Hum. Reprod. Update*, 1998, *4,* 509.

[14] Popovici R.M., Irwin J.C., Giaccia A.J., Giudice L.C.: "Hypoxia and cAMP stimulate vascular endothelial growth factor (VEGF) in human endometrial stromal cells: potential relevance to menstruation and endometrial regeneration". *J. Clin. Endocr. Metab.,* 1999, *84,* 2245.

[15] Graubert M.D., Ortega M.A., Kessel B., Mortola J.F., Iruela-Arispe M.L.: "Vascular repair after menstruation involves regulation of vascular endothelial growth factor-receptor phosphorylation by sFLT-1". *Am. J. Pathol.*, 2001, *158,* 1399.

[16] Maas J.W., Groothuis P.G., Dunselman G.A., de Goeij A.F., Struyker-Boudier H.A., Evers J.L.: "Endometrial angiogenesis throughout the human menstrual cycle". *Hum. Reprod.,* 2001, *16,* 1557.

[17] Rogers P.A.W., Lederman F., Taylor N.: "Endometrial microvascular growth in normal and dysfunctional states". *Hum. Reprod. Update,* 1998, *4,* 503.

[18] Salamonsen L.A., Kovacs G.T., Findlay J.K.: "Current concepts of the mechanisms of menstruation". *Bailliere's Clin. Obstet. Gynecol.,* 1999, *13,* 161.

[19] Reeves J.T., Zamudio S., Dahms T.E., Asmus I., Braun B., Butterfield G.E. *et al.*: "Erythropoiesis in women during 11 days at 4,300 m is not affected by menstrual cycle phase". *J. Appl. Physiol.,* 2001, *91,* 2579.

[20] Di Carlo C., Tommaselli G., De Filippo E., Pisano G., Nasti A., Bifulco G. *et al.:* "Menstrual status and serum leptin levels in anorectic and in menstruating women with low body mass index". *Fert. Steril.,* 2002, *78,* 376.

[21] Maruyama S., Minami S., Kaseki H., Ishihara K., Araki S., Suzue R.: "A comparison of serum leptin concentrarions in obese and normal weight Japanese women with regular menstrual cycles". *J. Nutr. Sci Vitaminol.*, 2001, *47,* 87.

[22] Yamada M., Irahara M., Tezuka M., Murakami T., Shima K., Aono T.: "Serum leptin profiles in the normal menstrual cycles and gonadotropin treatment cycles". *Gynecol. Obstet. Invest.,* 2000, *49,* 119.

[23] Malszko J., Zbroch E., Wolzynki S., Malszko J.S., Hryszco T., Mysiviec M.: "Leptin and serum erythropoietin in hemodialyzed and peritoneally dialyzed uremic patients during rHuEPO therapy". *Am. J. Nephrol.*, 2000, *20,* 180.

[24] Kokot F., Wiecek A., Mesjasz J., Adamczac M., Spiechowicz U.: "Influence of long-term recombinant human erythropoietin (rHuEPO) therapy on plasma leptin and neuropeptide Y concentration in hemodialyzed uraemic patients". *Nephrol. Dial. Transplant,* 1998, *13,* 1200.

[25] Kokot F., Samardzija G., Klimek D., Wiecek A.: "Intravenous single dose of erythropoietin (rHuEPO) does not influence plasma leptin concentration in hemodialyzed patients with chronic renal failure (CRF)". *Clin. Nephrol.*, 1999, *5,* 389.

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