Medicina Sportiva, Vol. 7 (EE3): E83–E93, 2003

THE EFFECTS OF CIRCULATING OVARIAN HORMONES, GLUCAGON, CORTISOL, TESTOSTERONE AND GLUCOSE ON PLASMA INSULIN LEVEL IN PREMENOPAUSAL REGULARLY MENSTRUATING FEMALES WITH ANOVULATORY AND OVULATORY MENSTRUAL CYCLES

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ABSTRACT

It is well known that female sex hormones exert pronounced effects on endocrine pancreas function, insulin sensitivity and glucoregulation. The aim of the present study was to examine the predictors of circulating insulin in regularly menstruating premenopausal women with anovulatory menstrual cycles in comparison with age-matched women with ovulatory cycles. A total of 37 regularly menstruating women participated in the study, 16 classified as non-ovulating and 21 as ovulating on the basis of 3 months monitoring of basal body temperature and subsequent ovarian hormone determination in plasma between day 5-8 and again between 19-22 of the menstrual cycle. In ovulating subjects in the follicular phase circulating insulin was significantly related to plasma glucose and progesterone levels (partial r=0.462 and 0.469 for glucose and progesterone, respectively, model adjusted R^2=0.292, p<0.017). In the luteal phase of normal menstrual cycle plasma insulin levels were correlated with plasma glucose and glucagon concentrations (partial r=0.691 and r=0.431 for glucose and glucagon, respectively, model adjusted R^2 = 0.439, p<0.0021). In non-ovulating women in the follicular phase of the cycle circulating insulin was correlated with plasma levels of cortisol and testosterone (partial r=0.622 and r= -0.518 for cortisol and testosterone, respectively, model adjusted R^2 = 0.348, p<0.024). In contrast, in the luteal phase of anovulatory menstrual cycle circulating insulin was not related to any of the variables included into the model (glucose, glucagon, cortisol, testosterone). Anovulatory menstrual cycles irrespective to slight and insignificant effect on plasma insulin levels, markedly affect the predictors of circulating insulin and probably induce the lack of contribution of glucose, progesterone and glucagon to the regulation of plasma insulin levels.

Key words: insulin; ovarian hormones; menstrual cycle; glucagon; cortisol; testosterone

Introduction

There is growing evidence that anovulation in premenopausal females has become a worldwide problem (19). It is well established that ovarian hormone secretion is affected by many factors including weight loss, eating disorders and excessive physical activity, leading to luteal deficiency, hypoestrogenism, anovulation and even to amenorrhea (8). During the last years exercise-related menstrual irregularities (ERMI) have focused much attention mainly with respect to increased risk of developing bone demineralization in female athletes (2, 25). However, data concerning the relationship between circulating insulin, insulin action and ovarian hormones in regularly menstruating pre-menopausal women are scarce and inconclusive. Diamond et al. (12) have demonstrated that neither counterregulatory response of adrenaline, noradrenaline, cortisol and glucagon to hypoglycemia, nor the rate of hepatic glucose production and rates of the whole-body glucose appearance and utilization differ in the follicular and luteal phases of the menstrual cycle. In addition, Thorton et al. (52) have reported that the administration of micronized estradiol and progesterone to regularly menstruating reproductive-age women did not affect plasma glucose utilization, hepatic glucose production and insulin sensitivity. In contrast, Valdes et al. (53) and Escalante Pulido and Alpizar Salazar (16) have revealed that in regularly menstruating females insulin sensitivity is higher in the follicular than in the luteal phase of the menstrual cycle.

Numerous studies have indicated that disturbances in female hormone secretion in polycystic ovary syndrome, gonadal dysgenesis, hypogonadotrophic hypogonadism and menopause cause insulin resistance, and subsequent glucose intolerance (10, 36, 55). On the other hand, in the rat hyperinsulinemia has been found to depress the development of gonads and to disturb normal reproductive cycling (11, 41). Recently it has been suggested that in rats the absence of female steroid hormones gives rise to a decreased insulin sensitivity (34). In addition, insulin gene expression in rat pancreas during the estrous cycle has been significantly correlated with circulating progesterone and 17β-estradiol (38). In obese women hyperinsulinemia has been noted to affect ovary function and to induce anovulation (37). Moreover, in cultured human ovarian cells insulin has stimulated progesterone production in a dose-dependent manner (43). The association between female reproductive system and insulin action is further supported by the studies which have demonstrated that hormonal replacement therapy improves insulin sensitivity and glucose tolerance in postmenopausal women (48, 51). Furthermore, insulin-sensitizing agents (metformin, rosiglitazone) have restored ovulation in women with polycystic ovary syndrome (7, 29).

Circulating insulin and hormone availability to the skeletal muscle and adipose tissue have a pronounced contribution to the regulation of carbohydrate and lipid metabolism (for review see ref. 33). Peripheral tissue resistance to insulin and/or deficient insulin secretion lead to severe metabolic disturbances as for example type 2 diabetes (40).

However, there is a paucity of data concerning the effects of the reproductive system dysfunction in regularly menstruating premenopausal females on the predictors of circulating insulin.

Therefore, the aim of this work was to compare the contribution of ovarian hormones as well as glucose, glucagon, cortisol and testosterone to variability in plasma insulin concentrations in regularly menstruating women with ovulatory and anovulatory menstrual cycles.

Material and Methods

Subjects

All the women participating in the study were regularly menstruating, healthy non-smokers, not taking any medication on a regular basis. All the subjects were physical education students not engaged in any specific sport. However, their physical activity was high (11 h/week) due to study's program, which included swimming, track and field, volley ball, gymnastics and martial arts. The participants were recommended to follow their habitual diet and lifestyle throughout the study. The procedures of this study were approved by the Ethics Commission of the Academy of Physical Education and all the subjects gave their written consent prior to participation. The prospective subjects monitored their basal body temperature (BBT) for 3 months prior to the study (26). The subjects with incomplete BBT records were excluded from the study. Plasma 17β-estradiol and progesterone concentrations were determined twice per cycle - between the 5th and 8th day of the cycle and again between the 19th and 22nd day of the cycle. In 21 women with bi-phasic BBT pattern plasma progesterone concentration between the 19th and 22nd of the cycle exceeded 19 nmol•L^{-1}. These women were classified as ovulating (4). In 16 women a lack of increase in BBT was observed and plasma progesterone concentration between the 19th and 22nd of the cycle did not differ from that determined between the 5th and 8th of the cycle. Those women were classified as non-ovulating. Subject characteristics are given in Table 1.

Blood samples

The subjects were instructed to consume their last meal or snack not later than 10:00-10:30 P.M. on the day preceding blood sampling. Fasting venous blood was drawn between 8:30-9:00 a.m. using disposable syringes and needles under aseptic conditions into lithium heparin tubes on the same cycle days as for ovarian hormone determination. The erythrocytes and plasma fractions were separated by centrifugation (15 min/4000 rpm) and plasma was stored at - 70^{o}C until analyzed. All analyses in plasma obtained from one subject were performed on the same day.

Plasma glucose and hormone assay

Plasma glucose was measured at 500 nm by means of the glucose oxidase method using Randox commercial kits (Randox Laboratories Ltd., United Kingdom) with intra-and inter- coefficients of variation of 4.3 % and 5.8%, respectively. Plasma 17β-estradiol, progesterone, insulin, testosterone and cortisol concentrations were assayed by standard radioimmunoassay techniques using commercial kits (OBRI-ORION, Poland). Intra- and inter-

Table 1. *Characteristics of ovulating and non-ovulating women*

	Ovulating women (n=21)	Non-ovulating women (n=16)
Age (years)	20.5 ± 1.2	20.3 ± 1.0
Body mass (kg)	63.3 ± 7.8	62.1 ± 8.2
Body height (cm)	172.4 ± 6.7	169.8 ± 7.0
BMI (kg/m^2)	21.0 ± 2.1	20.8 ± 2.1
Cycle length (days)	27.8 ± 1.9	30.2 ± 3.5*

Data are means ± SD
Significantly different vs ovulating women, $p<0.01$

assay coefficients of variation for 17-β-estradiol were 3.8% and 7.9%, for progesterone - 4.3% and 5.0%, for insulin - 6.8% and 9.3%, for testosterone - 5.3% and 5.4% and for cortisol - 3.6% and 6.8%, respectively. Plasma glucagon was determined by a standard radioimmunoassay method using commercial kits (BioChem Immuno-System, Italy) with intra- and inter-assay coefficients of variation (CV) 8.8% and 8.7%, respectively. All assays were run in duplicate. The detection limits of plasma hormone determination were 0.1 nmol•L^{-1}, 4 nmol•L^{-1}, 0.2 nmol•L^{-1} and 20 pmol•L^{-1} for testosterone, cortisol, progesterone and 17β-estradiol, respectively.

Statistical analysis

The results were expressed as mean ± standard deviation (SD). The variable distribution was evaluated using Shapiro-Wilk test. Mann Whitney U test and Wilcoxon matched pairs test were used for comparison of variables with non-Gaussian distribution (glucagon, insulin, testosterone, progesterone and 17-β-estradiol concentrations in the plasma). The means of normally distributed variables (anthropometric data, cycle length, plasma glucose and cortisol) were compared using the Student t-test for paired and unpaired data. Data with non-Gausian distribution were logarithmically transformed (ln) before they were introduced into regression analysis. Pearson product-moment correlations were calculated between plasma insulin levels and all other variables. These variables were then used in multivariate analysis by use of forward stepwise regression, to determine the model which best predicts plasma insulin concentrations in ovulating and non-ovulating subjects. All calculations were performed using STATISTICA 6.0 (StatSoft, Inc. USA). Differences were considered significant at $p<0.05$.

Results

Ovulating and non-ovulating women did not differ in respect to mean values of anthropometric data (table 1). However, in non-ovulating women the mean menstrual cycle was significantly longer ($p<0.01$) than in their ovulating counterparts.

In ovulating women the mean plasma progesterone concentration between day 19-22 increased 17.5 times ($p<0.001$) in comparison with day 5-8 of the menstrual cycle. On the contrary, in non-ovulating women the mean plasma progesterone level between day 19-22, though significantly higher than between day 5-8 ($p<0.02$), was much lower ($p<0.001$) than in ovulating ones.

Table 2. *Plasma glucose and hormone concentrations in ovulating and non-ovulating women*

	Ovulating women (n=21)		Non-ovulating women (n=16)	
	Day 5 - 8	Day 19 - 22	Day 5 - 8	Day 19 - 22
17β-estradiol (pmol•L^{-1})	206.5 ± 103.9	525.8 ± 122.3[a]	94.9 ± 22.0[c]	395.5 ± 172.0[a,d]
Progesterone (nmol•L^{-1})	2.2 ± 0.6	38.5 ± 13.1[a]	2.1 ± 0.6	5.1 ± 3.8[b,c]
Glucose (mmol•L^{-1})	4.6 ± 0.6	4.6 ± 0.6	4.2 ± 0.3[e]	4.4 ± 0.3
Insulin (pmol•L^{-1})	51.6 ± 21.6	55.7 ± 22.4	43.4 ± 21.7	45.2 ± 20.7
Glucagon (pmol•L^{-1})	24.9 ± 9.3	32.8 ± 11.6[a]	33.2 ± 13.5	34.1± 17.2
Cortisol (nmol•L^{-1})	439.4 ± 106.3	416.5 ± 143.3	472.9 ± 152.8	415.5 ± 102.6
Testosterone (nmol•L^{-1})	2.71 ± 0.77	2.70 ± 1.10	2.68 ±1.14	2.78 ± 1.12

Data are means ± SD

Significantly different vs day 5-8, [a] $p<0.001$, [b] $p<0.02$

Significantly different vs respective values in ovulating women, [c] $p<0.001$, [d] $p<0.05$, [e] $p<0.01$

Table 3. *Pearson product-moment correlations between logarithmically transformed (natural logarithm) plasma insulin level and other variables in ovulating and non-ovulating women in the follicular and luteal phases of the menstrual cycle*

	Ovulating subjects (n=21)		Non-ovulating subjects (n=16)	
	Day 5 - 8	Day 19 - 22	Day 5 - 8	Day 19 - 22
Glucose (mmol•L^{-1})	0,430[a]	0,617[b]	-0,160	0,005
17β-estradiol (pmol•L^{-1})	-0,100	0,098	0,120	0,134
Progesterone (nmol•L^{-1})	0,436[a]	0,080	0,420	-0,030
Glucagon (pmol•L^{-1})	0,004	0,187	0,216	-0,120
Cortisol (nmol•L^{-1})	-0,08	0,365	0,477	-0,110
Testosterone (nmol•L^{-1})	0,147	0,272	-0,280	-0,240

Except for cortisol and glucose data were logarithmically transformed (natural logarithm) before calculation
[a] $p<0.05$, [b] $p<0.003$

The mean plasma 17β-estradiol concentration between day 5-8 was significantly ($p<0.001$) higher than between day 19-22 of the menstrual cycle both in non-ovulating and ovulating women (Table 2). However, in non-ovulating women in both cycle phases it was markedly lower than in ovulating ones ($p<0.001$ and $p<0.01$ between day 5-8 and 19-22, respectively).

In ovulating women the mean plasma glucagon concentration between day 19-22 was significantly higher than between day 5-8 of the menstrual cycle ($p<0.001$). No effect of the menstrual cycle phase on the mean plasma glucagon level was noted in non-ovulating women.

There were no statistical differences in the mean plasma insulin concentration between ovulating and non-ovulating females (table 3). However, it should be stressed that plasma insulin concentration in ovulating females in both cycle phases tended to be higher than in non-ovulating counterparts (by 18.9% and 23.2% between day 5-8 and 19-22, respectively). Neither in ovulating nor in non-ovulating females insulin concentrations differ with respect to the menstrual cycle phase.

In ovulating and non-ovulating women plasma testosterone and cortisol levels were similar and did not differ with respect to the menstrual cycle phases. However, in non-ovulating ones plasma cortisol in the follicular phase tended to be higher (by 14%) than in the luteal phase of the menstrual cycle. Both in ovulating and non-ovulating females the mean plasma testosterone and cortisol levels were within manufacturer's physiological limits (0.3-3.6 nmol•L^{-1} and 146-715 nmol•L^{-1}) for testosterone and cortisol, respectively).

Pearson product-moment correlation revealed that in ovulating women both in the follicular and luteal phases plasma insulin was significantly and positively correlated with plasma glucose concentrations (Table 3). Additionally, in ovulating women plasma insulin was significantly and positively correlated with plasma progesterone levels, but such correlation was no noted in the luteal phase of the menstrual cycle.

Multivariate regression analysis revealed that in ovulating women the best model predicting plasma insulin levels differed with respect to the menstrual cycle phases. In the follicular phase of the menstrual cycle 29.2% of the variability in plasma insulin concentration was explained by the fluctuation in plasma glucose and progesterone levels (Table 4). The contribution of glucose in the variability of plasma in-

Table 4. *Partial and regression coefficients of the best models used to predict logarithmically transformed (natural logarithm) plasma insulin concentration in ovulating and non-ovulating females in the follicular and luteal phases of the menstrual cycle*

	Independent variable	Partial coefficient r	Regression coefficient B ± SE	Model adjusted R^2
Ovulating females (n=21)				
Day 5-8	Glucose	0.462	1.0166±0.4598	0.292[a]
	Progesterone	0.469	0.5611±0.2492	
Day 19-22	Glucose	0.691	2.0494±0.5056	0.439[b]
	Glucagon	0.431	0.3778 ± 0.1864	
Non-ovulating females (n=16)				
Day 5-8	Testosterone	-0.518	-0.58200± 0.2663	0.348[c]
	Cortisol	0.622	1.0809 ± 0.3772	
Day 19-22	–	–	–	–

Independent variables - in order of introduction into the model
Except for cortisol and glucose data were logarithmically transformed (natural logarithm) before introduction into the model
[a] $p<0.017$, [b] $p<0.0021$, [c] $p<0.024$

sulin for a given progesterone level was approx. 21.3%. For a given glucose level progesterone contributed to 21.9% of the variability in plasma insulin concentrations. In the luteal phase of the menstrual cycle 43.9% of the variability in plasma insulin was explained by the fluctuation in plasma glucose and glucagon concentrations, but the contribution glucose for a given glucagon level (47.7%) was much more pronounced than that of glucagon (18.6%) for a given glucose concentration.

In non-ovulating women in the follicular phase of the menstrual cycle 34.8% of the variability in plasma insulin concentrations was explained by the fluctuation in plasma testosterone and cortisol levels (Table 4). For a given cortisol level, testosterone explained 26.8% of the plasma insulin concentration. The effect of cortisol equalled 38.7% for given testosterone concentrations. It should be stressed that plasma insulin was positively related to plasma cortisol but inversly to plasma testosterone level. In the luteal phase there was no association between plasma insulin and any of the tested variables.

Discussion

According to our best knowledge the current study is the first which examined the relationship between circulating insulin, glucose, glucagon, cortisol and testosterone in regularly menstruating females with anovulatory and ovulatory menstrual cycles. It is worthy to note that numerous studies have focused on hormonal and metabolic disturbances in amenorrheic females characterized by more pronounced ovary dysfunction than our participants (35, 49, 54).

Our study revealed that there were only minor differences in glucagon and glucose plasma levels, the tendency to lower circulating insulin and no differences in circulating plasma testosterone and cortisol in non-ovulating subjects in comparison with ovulating ones. However, univariate and multivariate correlation analysis demonstrated significant effect of the distur-

bances in ovarian hormone secretion on the predictors of circulating insulin.

Plasma insulin is subjected to precise regulation by multiple factors including secretion from beta-cells, hepatic extraction as well as the whole-body sensitivity to insulin action (3, 15, 20, 46). Accordingly, plasma insulin determination does not identify the relative contribution of the above mentioned factors to the fluctuation in plasma hormone level. However, even within these limitations, the overall difference in the predictors of plasma insulin level between ovulating and non-ovulating women seems to shed more light on the association between ovarian hormones and endocrine pancreas.

Significant, positive correlation between circulating insulin and plasma glucose concentration in the follicular and luteal phases of the normal menstural cycle reflects islets sensitivity to glucose as well as the whole-body insulin sensitivity (for review see 40). Surprisingly, such correlation was not found in non-ovulating women. This may indicate that even subtle disturbances in ovarian hormone secretion accompanying anovulation alter islets response to glucose and/or insulin sensitivity. This finding is in agreement with other data concerning the association between ovarian hormones, endocrine pancreas and glucose disposal (6, 22, 47).

In regularly menstruating women with ovulatory menstrual cycles hormonal predictors of plasma insulin concentration markedly differed with respect to the menstrual cycle phase.

In the follicular phase of the menstrual cycle circulating insulin was significantly and positively correlated with plasma progesterone level. It could be postulated that this effect was due to well-known progesterone action depressing insulin sensitivity (34, 42, 50).

In contrast, in the luteal phase of the normal menstrual cycle plasma insulin was not affected by circulating progesterone, despite significant elevation in its plasma concentration. It could be presumed that the increase in plasma 17β-estradiol level in the luteal phase augmented insulin sensitivity and counteracted progesterone action noted in the follicular phase of the cycle (23, 34, 51).

However, in the luteal phase plasma insulin concentration in ovulating females was significantly and positively correlated with circulating glucagon. Furthermore, plasma glucagon level in the luteal phase was slightly, but significantly higher than in the follicular phase of the normal menstrual cycle. Peripheral glucagon level is regulated by hormone's secretion by the islets and its extraction by the liver (31, 32, 45). Therefore, elevated plasma glucagon may indicate either increased hormone secretion and/or depressed hepatic extraction. Under fasting conditions hormone extraction by the liver is elevated, since glucagon-stimulated liver glycogenolysis and gluconeogenesis are the major source of peripheral glucose (9, 46). Therefore, slighlty but significanlty increased plasma glucagon level in the luteal phase probably reflected increased hormone secretion.

It is well recognized that glucagon secretion by the islets is regulated by both hormonal and neural factors with pronounced effect of insulin sensitively controlling alpha cells function (5, 14, 28, 30). It was established that alpha cells insulin resistance and reduced insulin-mediated suppression of glucagon secretion contribute to elevated glucagon secretion and plasma levels during oral glucose tolerance test (1). Unfortunately, data concerning insulin-glucagon association under basal conditions with respect to the fluctuation in ovarian hormones plasma levels are not available. However, it cannot be excluded that in the luteal phase of normal menstrual cycle insulin-induced inhibition of glucagon secre-

tion is less pronounced than in the follicular phase resulting is slightly, but significantly elevated glucagon secretion and plasma level.

Positive correlation between circulating glucagon and insulin in the luteal phase of normal menstrual cycle suggests closer link between alpha and beta cells function in the presence of high ovarian hormone secretion and plasma concentrations.

None of the variable contributing to the regulation of circulating insulin in ovulating subjects (glucose, progesterone, glucagon) was operative in non-ovulating women.

In the follicular phase of anovulatory menstrual cycle circulating insulin was positively correlated with plasma cortisol concentration, but inversely with plasma testosterone level.

Testosterone receptors have been identified in normal and castrated female rat pancreas suggesting testosterone contribution to the regulation of islets function (13, 44). Recently Morimoto et al. (39) demonstrated that testosterone both in vitro and in vivo has a direct effect upon pancreatic islet function in female rat by favouring insulin gene expression and hormone release. In addition, serum testosterone concentration positively correlated with insulin m-RNA levels and plasma insulin concentration. On the contrary, in euandrogenic premenopausal women glucose-stimulated insulin release was negatively correlated with serum testosterone level, however, neither glucose uptake nor insulin-mediated glucose disposal was affected by physiological testosterone levels (24).

Data concerning the association between fasting insulin and testosterone levels in plasma in euandrogenic women are not available. In the current study such a negative correlation was found in females with depressed ovarian hormone secretion.

It is well established that elevated plasma cortisol brings about insulin resistance and increases plasma insulin level (18, 27). In the present study plasma cortisol in non-ovulating subjects was within normal range, but significant and positive correlation between circulating insulin and cortisol may indicate that even physiological cortisol level in women with disturbed ovarian hormone secretion contributes to depressed insulin sensitivity.

The reason for the association between circulating insulin, testosterone and cortisol in the follicular phase of anovulatory menstrual cycle could be only speculated. However, it could be presumed that low plasma 17β-estradiol level unmasked cortisol and testosterone action on insulin secretion and/or sensitivity. It seems feasible, since insulin-sensitizing effect of 17β-estradiol and its derivatives was confirmed by others (17, 21, 22).

In the luteal phase of anovulatory menstrual cycle circulating insulin was not affected by any of the tested variables (glucose, ovarian hormones, testosterone, cortisol). The reason for this finding remains obscure, but it suggests that the lack of progesterone peak together with low 17β-estradiol even further disturbed the mechanisms responsible for the regulation of plasma insulin level.

It could not be excluded that other mechanisms (hormonal and/or neural) were mobilized to compensate for disturbed regulation in plasma insulin level due to anomalies in ovarian hormone secretion and subsequent anovulation. Consequently, in non-ovulating women circulating insulin was only slightly lower than in ovulating ones.

In conclusion, present study has revealed that ovarian hormone disturbances and resultant anovulatory menstrual cycle exert noticeable influence on predictors of circulating insulin in physically active premenopausal females. In non-ovulating females low plasma 17β-estradiol concentration in the folli-

cular phase of the cycle hinders the association between plasma insulin, glucose and progesterone. In the luteal phase, the lack of progesterone peak and low plasma 17β-estradiol level even further disturbs the regulation of circulating insulin, and hormone level is correlated neither with glucose nor glucagon plasma levels. In consequence, in non-ovulating women the tendency to lower plasma insulin level has been noted with unknown at present delayed metabolic consequences e.g. for lipid and carbohydrate metabolism both at rest and during exercise.

Acknowledgements

Supported by grants DS-50 and DS-42 from the Academy of Physical Education

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Received: July 28, 2003
Accepted: September 01, 2003

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