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Salivary amino acids quantification using RP-HPLC during normal menstrual cycle

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The present study was carried out to detect amino acids profile in women saliva in order to establish the qualitative and quantitative differences that might have potential value in detection of ovulation by noninvasive methods. For the collection of sample, the stages of menstrual cycle were decided by the physical and morphological examination of salivary fern pattern. The saliva from various reproductive phases (prepubertal, preovulatory, ovulatory, postovulatory phases and menopause) was collected and analyzed by reverse phase high performance liquid chromatography (HPLC) after precolumn derivitization of amino acids using O-Pthaldehyde (OPA) by means of RP-HPLC amino acid analyzer. Among the various amino acids identified the compounds such as tryptophan, arginine and phenylalanine were comparatively found to be higher during ovulatory phase when compared to that of other phases. The increase in amino acid concentration during ovulatory phase may be due to the circulation of steroid hormones. Thus, the presence of specific amino acids in ovulatory saliva makes the possibility to develop a biomarker for detection of ovulation by noninvasive methods.

Key words: Ovulation steroid hormones, O-Pthaladehyde, chromatography, amino acids.

INTRODUCTION

A number of techniques such as basal body temperature, ultrasound examination and plasma LH (Lutenising hormone). But these methods are not sufficient to detect the time of ovulation accurately. It has been recently reported that women facial attractiveness increases during follicular phase than the luteal phase (Roberts et al., 2004). The exact time of ovulation is important because it would help to identify the fertile period and thereby give treatment in fertile therapy. The prediction of ovulation period still remains a challenge for the investigators.

Human saliva an easily available biological fluid, which shows cyclic variation in its composition during the menstrual cycle (Tandra and Bhattacharaya, 1989). Historica-

lly, salivary analyses of female sex hormones were used for fertility monitoring (Read, 1993; Hofman, 2001). However, recent findings indicate that these assays may be useful beyond the study of reproductive concerns.

Estradiol, which was at high levels in women during their reproductive years, may cause increased immunoreactivity responsible for these phenomena (Markovic, 2001). Normally, the day of ovulation is determined by observing the change in physical characteristics of human cervical mucus (Moghissi et al., 1972; Billings, 1981). In 1969, Cassals reported that the saliva would fern or crystallize during hormonal changes, almost identically to the changes observable in cervical mucus. These changes in cervical mucus have helped to predict when a woman is about to ovulate. Further the salivary ferning showed that the saliva could also help to predict ovulation in some extent (Berardono et al., 1993). Cervical

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mucus is not an easily available body fluid and at the same time needs patient motivation, where as saliva has practical advantages over cervical mucus for the detection of ovulation.

Cyclic changes in various physical properties and biochemical constituents of saliva are known to reflect accurately the hormonal changes associated with a menstrual cycle and may be utilized clinically to determine the time of ovulation. For example, the biochemical substances like salivary amino acids as well as hormones like estrogen and progesterone have been found to fluctuate during the ovulatory period of the menstrual cycle (Landau and Lugibihil, 1967; Lyons et al., 1989). In blood plasma, the large amount of neutral amino acids exhibited maximum reduction and remained low during postovulatory periods (Wall and Truswell, 1991). To date, analysis of amino acid in blood is considered as a valuable diagnostic tool in cases of suspected inborn errors of amino acid metabolism. The presence of a characteristic pattern of elevated amino acids is very useful in diagnosis of these rare disorders. To overcome this, the preliminary reports prove the changes in the salivary amino acids during reproductive phases of menstrual cycle provide an evidence for detection of ovulation. These applications have led to an increase in the number of salivary amino acid determination and the need for a cost-effective, rapid, reliable and automated method for the prophecy of ovulation through noninvasive methods.

MATERIALS AND METHODS

Collection of samples

Saliva was collected according to the spitting method (Navazesh, 1993; Bosch et al., 1996). The stages of reproductive phases were confirmed through salivary fern pattern (Alagendran et al., 2007) and the sample was processed in preweighed ice-chilled tubes and the collection period was about 10 min. The saliva was collected from 20 different women volunteers during various periods viz, pre-ovulatory, ovulatory, postovulatory phases (aged 22 - 35 years old) and also from prepubertal (7 - 9 years) and menopause stages (aged 45 - 55 years).

The volunteers were instructed to abstain from smoking and drinking 10hrs prior to testing. And also the volunteers were asked for tooth brushing to prevent minimal gingival bleeding.

Total amino acids (acid hydrolysis)

1 ml aliquots of saliva were deproteinized with 50 mg sulfosalicyclic acid (Sigma st, Louis, USA) and centrifuged for 10 mins at 4000 xg at 4°C. The pellet was washed with distilled water. A known weight of the pellet was transferred to a hydrolysis tube. 5 ml of 6 N HCl was added to the pellet and incubated the tube at 110°C for 18 h. Transferred the contents to a china dish after hydrolysis. Acid vapors were completely removed by keeping the china dish over a boiling water bath by repeated evaporation using distilled water. The residue was made up to a known volume and kept in the refrigerator.

Extraction of samples (total free amino acids estimation)

The sample was extracted with 80% ethanol (if necessary the mixture was heated at 70 - 80°C for 30 min in a water bath) and centrifuged at 10,000 xg for 10 min. The clear supernatant was concentrated and used for the assessment of amino acids. Standard amino acids were also run parallel with the unknown samples. Amino acid standard samples were prepared by mixing 95 L of the 250 pmol/ L amino acid standard mixture with 5 L of 10 mM norvaline and analyzed directly by RP-HPLC, within 24 h from preparation. Solutions for linearity study were prepared in duplicate by diluting the 1 nmol/ L amino acid standard solution, and contained 20, 50, 120, 220 or 500 pmol/ L of amino acid standard mixture together with 0.5 mM norvaline (The internal standard l-norvaline was obtained from Sigma-Aldrich). From the standard profile, the amino acid concentration in saliva was quantified (Gnanou et al., 2004) through RP-HPLC. Due to technical constraints, rest of the amino acids in the samples was not quantified. For derivatization 10 µl of supernatant was used.

Instrumentation

The gradient HPLC system was used an LC- 10AT VP (Shimadzu Corporation, Kyoto, Japan) attached with auto injector. Separations were performed at 40°C on a 5 µm Luna C19 column (250 x 4.6 mm (i.d) from Phenomenx (St. Torrance, CA, USA)), protected by a reverse phase guard column (4.0 mm x 3.0 mm (i.d)) from the same supplier. The C-19 column was placed in the column oven (Mayura Analytical Pvt. Ltd, Bangalore, India) and maintained at 40°C. Peak monitoring was performed with a model RF-10Axl fluorescence detector (Shimadzu Corporation, Kyoto, Japan), excitation, wavelength 350 nm and emission cutoff filter at 450 nm. LC workstation CLASS-VP software from the same supplier was used for data processing.

Derivatization procedure

The fully automated precolumn derivitization was performed using SIL- 10AD VP autoinjector ((Shimadzu Corporation, Kyoto, Japan). The auto injector rack contained 2 reagents working OPA reagent and neutralization buffer. The samples 10 µl were loaded on to sample vials and dervitization was started by transferring 90 µl of working OPA reagent. The sample and OPA reagent were assorted by two cycles of aspiration and dispensing. After incubation for 3 min at room temperature. 100 µl of neutralizing buffer was added to the sample OPA reagent mixture, then added 20 µl if the final mixture was injected on to the column. During the chromatography of one sample, the next sample was being derivatized.

HPLC conditions

Mobile phase A was 40 mM NaH_2PO_4, adjusted to pH 7.8 with NaOH, while mobile phase B was acetonitrile/methanol/water (45/45/10 v/v/v). The separation was obtained at a flow rate of 2 mL/min with a gradient program that allowed for 1.9 min at 0% B followed by a 16.3-min step that raised eluent B to 53%. Then washed at 100% B and equilibration at 0% B was performed in a total analysis time of 40 min. Fluorometric detection was done using an excitation wavelength of 350 nm and an emission cut off filter of 450 nm. Amino acid concentrations were calculated using the determination and peak areas relative to the area of the internal standard.

Statistical methods

Data are expressed as Mean ± SEM. Those means in the same vertical column that are not marked with the same superscript letters are significantly different at $p < 0.05$. The relationship between changes in amino acid concentration was explored by means of

Table 1. Amount of amino acids present in the saliva at various stages of menstrual cycle.

Name of Amino acids (mg/dl)	Prepubertal (Age: 7-9 yr)	Preovulatory phase (6-10 day)	Ovulatory phase (13-14 day)*	Postovulatory phase (17-26 day)	Menopause (Above 45 yr)
Glycine	4.69 ± 0.68fg	5.90 ± 0.40e	7.13 ± 0.44f	5.20 ± 0.61c	2.78 ± 0.24f
Serine	7.30 ± 0.57bc	7.94 ± 0.29c	9.04 ± 0.39de	6.00 ± 0.36a	4.03 ± 0.61cd
Lysine	4.75 ± 0.30f	5.52 ± 0.20f	5.56 ± 0.47h	4.38 ± 0.42ef	3.31 ± 0.39ef
Glutamic Acid	6.82 ± 0.56cd	5.41 ± 0.47fg	7.65 ± 0.46e	4.50 ± 0.53c	3.37 ± 0.36e
Aspartic Acid	5.54 ± 0.64e	6.42 ± 0.38d	6.47 ± 0.70g	2.57 ± 0.77de	0.66 ± 0.21de
Tyrosine	6.95 ± 0.40c	7.40 ± 0.36cd	9.22 ± 0.49d	3.66 ± 0.80d	2.15 ± 0.80c
Phenylalanine	4.24 ± 0.41b	8.89 ± 0.82b	10.74 ± 0.96c	5.20 ± 0.43bc	3.84 ± 0.34d
Tryptophan	6.57 ± 0.46d	9.74 ± 1.25a	13.86 ± 1.03a	3.86 ± 0.38ab	2.71 ± 0.74a
Arginine	8.22 ± 0.54a	9.30 ± 0.80b	12.74 ± 0.93b	4.35 ± 0.30b	1.16 ± 0.65b

*Data are expressed as Mean ± SEM. Those means in the same vertical column that are not marked with the same superscript letters are significantly different at $p < 0.05$. The relationship between changes in amino acid concentration was explored by means of using one way ANOVA followed Duncan multiple range test (DMRT analysis).

using one way ANOVA followed DMRT analysis (Duncan multiple range test) using SPSS Software package version 5.0 (SPSS Inc, Chicago, USA)

RESULTS

The changes in the level of salivary amino acids during various stages of menstrual cycle are shown in Table. 1 and Figure 1 is the chromatogram peak. In all the (prepubertal, preovulatory, ovulatory, postovulatory phases and menopause) samples, the ovulatory phase of human saliva showed higher concentration of total free amino acids when compared to that of other phases due to the formation of ovarian hormones principally estrogen. Among the various amino acids profile, tryptophan, arginine and phenylalanine were found to be higher in ovulatory phase as compared to that of other amino acids due to the formation of food deprivation and estrogenic fluctuations are mainly involved in throughout the cycle. In prepubertal saliva, the amino acids like serine, tyrosine and arginine are markedly increased in concentration. The amino acid concentration of preovulatory sample is similar to that of prepubertal samples.

By contrast the amino acids were found to be decreased after ovulatory phase, that is, in postovulatory and menopause. The concentration of amino acids particularly aspartic acid and arginine showed significantly reduction during menopause. The other amino acids present in the human saliva appear to be less in concentration and those are not evident.

DISCUSSION

The present results showed the variation in concentration of amino acid varied in the reproductive phases. The prepubertal and menopause samples contain nine different amino acids but the concentrations of amino acids are comparatively lesser when compared to that of ovulatory samples. Among the amino acids identified in the present

study, tryptophan and arginine showed the maximum concentration in human saliva. It is interesting to note that the concentration of amino acids in plasma seems to be higher in ovulatory phase, which may be due to the regulation of steroid hormones synthesis during menstrual cycle (Craft and Wise, 1969). This is consisting with our findings that the specific salivary amino acids like arginine and tryptophan raised during ovulation.

Like the increase of amino acids during ovulation, earlier reports stated that the level of salivary glucose (Davis and Balin, 1973), phosphate (Ben-Aryeh et al., 1976) contents increases during the time of ovulation. Further, Boyer and France (1976) reported that there is an increase in salivary alkaline phosphatase at the time of ovulation, whereas Cockle and Harkness, (1978) did not find any changes on alkaline phosphatase at ovulatory phase. The contradiction in the observations is probably due to the variations in the method of collection and treatment procedures of saliva samples (Tandra and Bhattacharaya, 1989).

The present results suggested that there might be an interrelationship between the female hormones associated with reproduction and the role of Vitamin B6 in the metabolism of tryptophan. There is considerable evidence for an interrelationship between steroid hormones, particularly the cortical steroids and enzyme activity (Cox and Calame, 1978). However, in the preovulatory saliva, the amino acids such as tryptophan, arginine and serine were present in higher quantity than all other stages. The alterations in the flow of tryptophan through kynurenine pathway occur during the course of the menstrual cycle and are characterized by higher plasma and urinary kynurenine levels in the postovulatory relative to the preovulatory phase (Price et al, 1967). On the other hand, tryptophan is a precursor of serotonin and changes in their availability may modulate melatonin synthesis, which may suffer alterations during stress conditions in mammals (Kulczykowska, 2001; Larson et al., 2004). In prepubertal farm animals, the LH secretion indicates that,
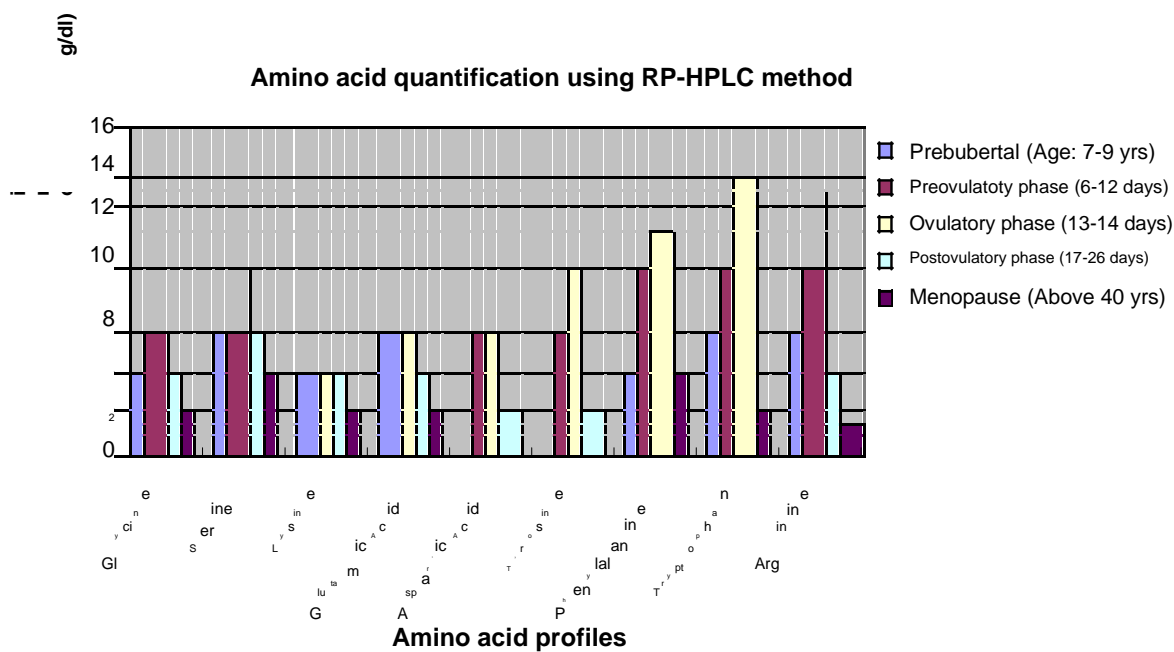
Figure 1. Amino acid quantification using RP-HPLC method.

arginine may be an important constituent for the activetion of hypothalamo-pituitary axis during pubertal development. For instance, arginine is involved in several important metabolic functions, including nitric oxide synthesis, which plays important roles in many diverse processes, as vasodilation, immune response, and neurotransmission (Wu and Morris, 1998). During the menopause period, glycine, phenylalanine and glutamic acid are moderately increased due to the stimulation of food intake and hormone fluctuations. Thus, the presence of specific amino acids content in saliva during ovulation makes the possibility to develop a biomarker for the detection of ovulation. The ferning pattern of saliva is also helpful to detect the female ovulation period. The ferning is caused by NaCl, which cyclically increases under the influence of estrogen (Alagendran et al, 2007; Fernando et al., 1988). These findings are similar to the result of Barbato et al. (1993) in which a strong relationship is found among salivary ferning patterns in menstrual cycle.

Salivary glands are very metabolically active (Warren et al., 1994). Selective metabolism of amino acids during passage from plasma to saliva may contribute to the difference in the relative concentrations of free amino acids in plasma and saliva (Lindsay et al., 1969) . Results of this study showed that the level of arginine were so high next to tryptophan. This may be due to the stimulatory effect of arginine on (GnRH) gonadotropin releasing hormone and LH secretion (Foster et al., 1986). In addition, arginine may stimulate GH release (Davenport et al., 1985), which inhibit the negative feedback of estradiol (Ramaley and Phares, 1980) and increase ovarian sensitivity to gonadotropins (Ojeda and Advis, 1980). Furthermore, tyrosine, glutamic acid and aspartic acid at postovulatory and menopause periods were significantly decreased in agreement with previous findings (Craft and Peters, 1971). This finding indicates that the qualification of amino acids in saliva through RP-HPLC may be considered as one of the parameters in revealing of ovulation in human by noninvasive profiling. However, the salivary amino acid detection in ovulatory phase like tryptophan and arginine seems to be marker for ovulation prediction.

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