Original Article

DOI: 10.5582/ddt.2017.01008

Ultrastructural and physico-chemical characterization of saliva during menstrual cycle in perspective of ovulation in human

Ganesan Saibaba[1], Mahalingam Srinivasan[1], Archunan Priya Aarthy[2], Velliyangiri Silambarasan[1], Govindaraju Archunan[1,*]

[1] *Centre for Pheromone Technology, Department of Animal Science, School of Life Sciences, Bharathidasan University, Tiruchirappalli, Tamilnadu, India;*
[2] *Tagore Medical College and Hospital, Rathinamangalam, Chennai, India.*

Summary **Human saliva is a potential diagnostic fluid and any alteration in body might be reflected in saliva so that saliva is considered as "mirror of the body". Variations in salivary hormone level, ultra structure, pH, flow rate, buffering capacity and electrolytes level are found during menstrual cycle in regard to ovulation. Thirty healthy volunteers were used for the assessment of physico-chemical changes in saliva. Reproductive cycle was categorized as pre-ovulation phase (5 to 12 days), ovulation phase (13 or 14 days) and post-ovulation phase (15 to 25 days) according to salivary arborization test and hormonal analysis. Estradiol and luteinizing hormone was gradually increased and attained peak at the level of 2.28 ± 0.20 pg/mL and 1.35 ± 0.41 mIU/mL respectively during the ovulation phase. The electrolytes result clearly indicates that the influx of common electrolytes is important for crystallization and help to induce clear fern ing pattern in ovulation phase. Sodium (Na) and chloride (Cl) were found to be high during ovulation phase only. Average salivary pH was 7.5, 7.1, and 7.3 during ovulation, pre- and post-ovulation phases respectively. Buffering capacity of saliva was normal during pre- and post- ovulation phases. In contrast, in ovulation phase the buffer capacity was slightly higher. At the first time, the scanning electron microscopy (SEM) studies revealed the ultra structure difference of saliva during menstrual cycle. During ovulation phase a compact network-shaped mesh was appeared; such structure was not appeared in pre- and post ovulation phases. Additionally, we observed the saliva is arrayed as a fine mosaic-like structure during ovulation. Based on physico-chemical properties and hormonal levels may lead to develop a detection kit/sensor for detecting the ovulation phase in human.**

Keywords: Buffer capacity, SEM, luteinizing hormone, estradiol, electrolytes

1. Introduction

Saliva is secreted from three paired extrinsic salivary glands in humans such as parotid, submandibular, and sublingual glands under the control of both the parasympathetic (PNS) and sympathetic nervous systems (SNS) (*1*). Parotid glands and submandibular glands are contributing around 25% and 70%, whereas the sublingual glands account for only 5% of total salivary output (*2,3*). In recent years, more blood borne substances have been detected in saliva, which leads to saliva as an essential, noninvasive diagnostic medium. On the other hand, saliva is composed of 95% water and 5% of various minerals, electrolytes, hormones, enzymes, immunoglobulins, cytokines, *etc*. (*4*).

Ovulation is a complex mechanism in which mature ova is likely to respond to surge of luteinizing hormone (LH) and rupture to release fertilizable oocytes. The LH-induced transition of ovarian tissue is prerequisite for the process of ovulation which is caused by

Released online in J-STAGE as advance publication April 14, 2017.

**Address correspondence to:*
Dr. Govindaraju Archunan, Centre for Pheromone Technology, Department of Animal Science, Bharathidasan University, Tiruchirappalli 620024, Tamilnadu, India.
E-mail: garchu56@yahoo.co.in

multi-gene and multi-step process. Particularly, LH surge initiates a cascade of proteolytic events that control ovulation. Mainly, the LH gene activates the progesterone receptor (*5*). During ovulation numerous cell types get altered namely granulosa, theca cells, fibroblasts and endothelial cells, as well as the ovarian surface epithelium (*6*). Additionally, various protease enzymes mediate the ovulation during LH surge; for instance, proteins are thought to be mediators of ovulation such as: plasminogen activators (PAs), matrix metalloproteinases (MMPs), kallikreins and tissue-specific inhibitors of MMPs (TIMPs) (*7*).

Recently, attention has been paid to the development of noninvasive methods for ovulation detection (*8,9*). Saliva is a good investigative tool for various diagnostic purposes. It is reported that the sympathetic nervous system, parasympathetic nervous system, hypothalamic-pituitary-adrenal axis and immune system are response to change their biomolecules in saliva due to stress (*10*). Ovulation has been linked to an inflammatory-like response (*11*) and well considered as stress to women. Perhaps, one or more salivary proteins would serve as a potential non-invasive biomarker that would be characterized to predict ovulation (*12*). Report revealed that, uridine diphosphoacetylglucosamine pyrophosphorylase (UDP)-N-acetylglucosamine pyrophosphorylase was found to be a specific protein differentially expressed in human saliva during the ovulation phase (*13*). Most recently, the salivary protein expression differs particularly the high 14.5 kDa protein expression were high in ovulation phase (*14*). In general, LH assay in serum and ultrasonography methods are used to predict the ovulation time, even though, these methods are invasive, high cost and needs technically skilled persons. In the present scenario, the time of ovulation in women is highly essential to avoid unwanted pregnancy and *in vitro* fertilization to effective implementation of assisted reproductive technology (ART). However, presently there is no reliable, cost effective indicator for detection of ovulation in women accurately. As of now, there is an increasing demand to promote cost effective method or tool to predict fertile period in women. The present study was undergone to analyze LH and estradiol levels, pH, buffer capacity, flow rate, and electrolytes during the phases of menstrual cycle. Furthermore, for the first time the ultra-structure of saliva in different phases of menstrual cycle was spotted using scanning electron microscopy.

2. Materials and Methods

2.1. *Ethics statement*

Sample collection from local women volunteers and all experimental protocols were approved by Institutional Ethical Committee (IEC) (Approval No: DM/2014/101/38), Bharathidasan University, Tiruchirappalli, India. All the methods were carried out in accordance with the Indian Council for Medical Research (ICMR, New Delhi) approved guidelines and regulations. Volunteers were duly informed and subsequently given a written consent.

2.2. *Volunteer's inclusion criteria*

Thirty healthy female volunteers (ranges from 18-30 years) without any chronic diseases of oral system, infectious diseases, systemic illness, cardiac, renal, respiratory or hepatic failure, ovarian dysfunction or any medications known to alter sex hormone levels were selected. Volunteers were excluded from the study if they did not meet the above criteria.

2.3. *Saliva sampling and process*

The saliva samples were collected without any stimulus in the morning (8.00 to 9.00 a.m.) from female volunteers. The volunteers were instructed to brush the teeth 30 min before the collection of saliva. Further, all subjects abstained from eating or drinking for a minimum of 1 h before saliva collection. The saliva samples were collected by expectorating into polypropylene tubes within 5 min according to the protocol followed by Navazesh (*15*). The samples were centrifuged at 16,000 g for 15 min to remove insoluble materials and cells, if any. The samples were stored at –80˚C until further use.

2.4. *Confirmation of ovulation*

The saliva samples were assigned among the three phases, preovulatory (day 6 to 12), ovulatory (days 13 or 14) and postovulatory (day 15 to 26) phases according to the salivary hormone concentrations and fern pattern analysis (*13*). In addition, the ovarian follicle status was assessed with ultrasonography to validate the day of ovulation. The concentration of hormones such as estradiol, LH (luteinizing hormone), and FSH (follicle-stimulating hormone) were determined by enzyme immunoassay (EIA) using commercial kits (Pathozyme Oestradiol OD477 EIA kits, Omega House, Scotland, UK).

2.5. *pH, buffer capacity and flow rate*

Saliva pH was measured using a pHTestr® 30 (Thermo Scientific Eutech products, Ayer Rajah Crescent, Singapore). To measure buffering capacity of saliva, after measuring pH, 1 mL of 0.1 N HCl was added to 1 mL of saliva and pH was recorded (*16*). Buffer capacity was calculated according to changes in pH. For flow rate, the sample tubes were first weighed and reweighed with tubes containing saliva samples.

2.6. *Electrolytes measurement*

Electrolytes (Na, Cl, K and Ca) were analyzed in Atomic Absorption Spectrophotometer (AAS) (AAS iCE 3000 series, Thermo Fischer, Waltham, Massachusetts, USA) after calibrating for each element using standard solution of known concentration

2.7. *Scanning electron microscopy analysis*

Saliva samples were fixed in 3% glutaraldehyde solution in 0.25 M cacodylate buffer at 4°C for 48 h. After the fixation period, the specimens were washed twice in a 0.25 M cacodylate buffer for 30 min each time, to remove the remaining fixer. Then they were dehydrated in an ascending gradient in acetone concentration. For this, the specimens went through 30%, 50%, 70%, 90% and 100% (v/v) concentration acetone solutions, remained in each solution for 120 min as described in Barros *et al*. (*17*). Followed by auto gold coating, the samples were examined at 10.00K x magnification at 10 kV. SEM Observations were made with a ZEISS FE-SEM (Carl Zeiss NTS Ltd, Oberkochen, Germany) scanning electron microscope.

2.8. *Energy-dispersive X-ray spectroscopy*

Energy-dispersive X-ray spectroscopy (EDX) analysis was applied to the area nearest to the smear surfaces of the samples. EDX analysis was performed using ZEISS FE-SEM instrument equipped with EDX.

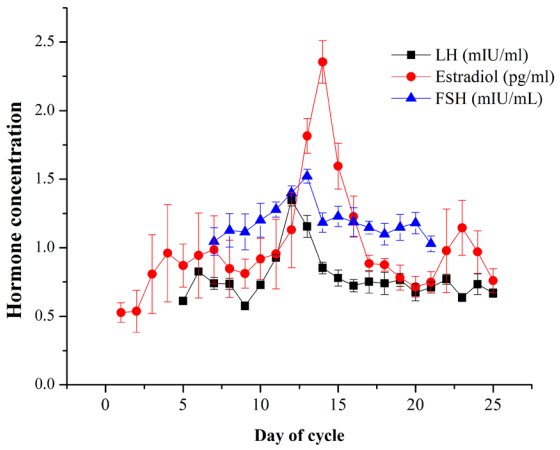

Figure 1. Salivary LH and estradiol level during menstrual cycle. The daily pattern of LH and estradiol level was recorded in subject's saliva sample (n = 30). The timing of LH and estradiol peak were appears around 12th and 14th day of menstrual cycle respectively. Data are presented as mean±SD (p < 0.05).

2.9. *Statistical analysis*

All data represented as mean ± SE and analyzed using one-way analysis of variance (ANOVA) with LSD post-hoc comparisons. A p value ≤ 0.05 was considered statistically significant. All statistical analysis was performed using the software package Statistical Package for Social Sciences (SPSS) for Windows, version 16.0 (SPSS Inc., Cary, NC, USA).

3. Results

3.1. *Hormones level in saliva*

The levels of salivary estradiol and LH during various phases of menstrual cycle were shown in Figure 1. A peak LH was observed during menstrual cycle at 12th day, whereas, the mean concentration of estradiol began to increase after LH shut down and reached its peak level during 14th day of cycle (Figure 1). The FSH concentration also differed in a significant level (p < 0.05) during the menstrual cycle which was highest during the ovulatory phase. The concentrations of hormones varied in preovulatory, ovulatory and postovulatory phases according to their physiological condition. The mean level of salivary estradiol, LH are during ovulation phase 2.35 ± 0.15 pg/mL and 1.34 ± 0.04 mIU/mL respectively.

3.2. *pH, buffer capacity and flow rate*

The results show that the pH was significantly (7.53 ± 0.073, p = 0.01) increased during ovulation phase as compared to pre- and postovulation phases (Figure 2A, Table 1). Normal buffer capacity was observed in phases of menstrual cycle but a slight change was found in ovulation phase (2.21 ± 0.23) as compared to other phases, but the difference was not significant (p = 0.145) (Figure 2B, Table 1). The mean value of flow rate was decreased significantly in ovulation phase (2.09 ± 0.18 mL/5 min, p = 0.01) as compared to pre- and postovulation phases are 2.23 ± .09 mL/5 min and 2.47 ± 0.01 mL/5 min respectively (Figure 2C, Table 1). A significant positive correlation was found between

Table 1. Physico-chemical properties during ovulation phase

Physico-chemical properties	Pre-ovulation	Ovulation	Post-ovulation	p value
pH	7.18 ± 0.08	7.53 ± 0.07*	7.35 ± 0.07	0.01
Buffer capacity	2.13 ± 0.20	2.21 ± 0.23	1.65 ± 0.20	-
Flow rate (ml/5 min)	2.23 ± 0.09	2.09 ± 0.18*	2.47 ± 0.06	0.00

* Differences (p < 0.05) with pre- and post-ovulation phases. Data are presented as mean±SE.

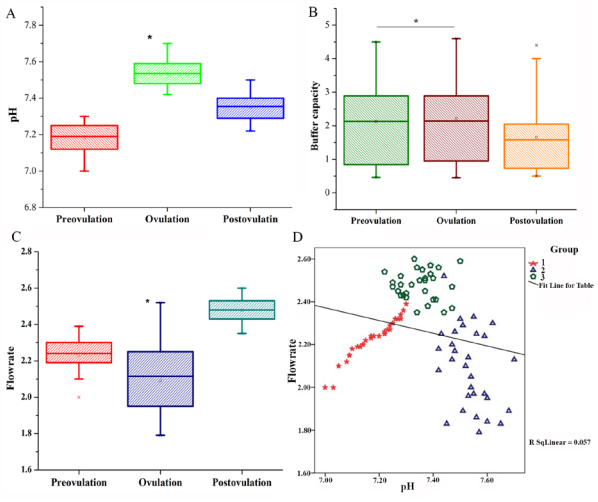

Figure 2. Physico-chemical properties of saliva. A) pH level of saliva during different phases of menstrual cycle, **B)** Buffer capacity of saliva, **C)** Flow rate of saliva, **D)** Correlation slope for saliva between pH and flow rate.

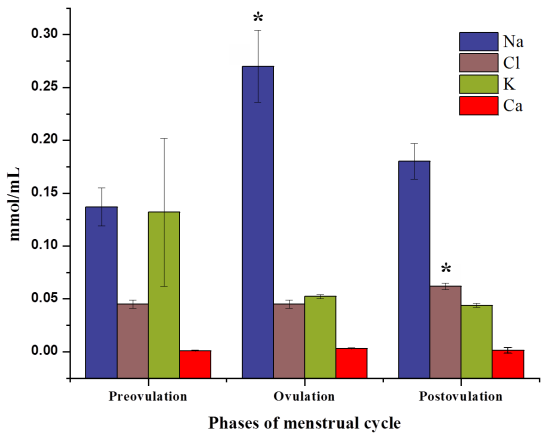

Figure 3. Major salivary electrolytes level. Bar diagram shows that level of Na, Cl, K, Ca electrolytes in saliva during menstrual cycle. The Na and Cl level were significantly high during ovulation and post-ovulation phase respectively compared to other phases using Fisher's least significant difference post-hoc comparisons ($p < 0.05$). Data are presented as mean±SE.

salivary pH and flow rate ($r = 0.751$ and $r^2 = 0.565$, $p < 0.05$) (Figure 2D).

3.3. *Major electrolytes level*

The salivary electrolytes were observed around the menstrual cycle (Figure 3). The concentrations of sodium in saliva significantly increased during ovulation phase (0.27 ± 0.08 mmol/mL, $p = 0.002$), whereas the chloride concentration was significantly less during preovulation phase and rapidly increased during ovulation phase (0.045 ± 0.010 mmol/mL, $p = 0.05$) and reached maximum in postovulation phase (0.062 ± 0.009 mmol/mL, $p = 0.008$). The mean potassium and calcium level in saliva did not alter during the phases of menstrual cycle. The potassium and calcium levels during ovulation phase were observed as 0.05 ± 0.005 mmol/mL, $p = 0.292$ and 0.003 ± 0.001 mmol/mL, $p = 0.195$.

3.4. *Scanning electron microscopy analysis*

The ultra-structure of saliva revealed by SEM analysis and a significant structural change were observed in different phases of menstrual cycle. Three different saliva structures were found in saliva such as, micro globules, compact network-shaped mesh and network-shaped mesh with pores at 10 K X magnification. The micro globules were exclusively found in pre-ovulation phase saliva with 2 µm scale (Figure 4A). Likewise, the ovulation phase saliva shows a unique compact network-shaped mesh structure, which is recorded as the highest relative abundance (Figure 4B). In post-ovulation, the network-shaped meshes denatures and pores were developed (Figure 4C), which indicates that mesh structure started to lose their structural arrangement due to decrease the ion content.

3.5. *SEM-EDX analysis*

In saliva, the presence of elements was recorded using EDX analysis. The following elements were detected namely: calcium, sodium, chloride, potassium and magnesium. Interestingly, the Cl element was appeared exclusively during ovulation phase and it may have vital role in crystals formation during ovulation phase (Figures 4D-4I).

4. Discussion

This is the first approach to investigate the salivary physico-chemical properties and ultrastructure in relation to the ovulation phase during menstrual cycle. Ovarian hormones cause the cyclic changes in the endometrium of uterus; particularly, estradiol produced by a mature ovarian follicle which triggers a surge release of GnRH (gonadotropin-releasing hormone) from the hypothalamus through positive feedback mechanism. Saliva samples have been demonstrated to enable differentiation between the follicular and luteal phase by assessing progesterone (*18*) and estradiol (*19*). Chatterton *et al*., (*20*) found that the mean estradiol 2 level in the follicular phase across three consecutive cycles was 22.1 ± 2.7 pmol/L while mean luteal phase progesterone was 436 ± 34 pmol/L. Similarly, the present study confirms the variation in hormonal level around the menstrual cycle with peak concentration for estradiol (2.28 ± 0.20 pg/mL) and LH (1.35 ± 0.41 mIU/mL) during ovulation phase. Comparing our results with previous findings estradiol and LH are found to be equal concentrations in saliva during ovulation phase (*8,21*). It is well reported that the ferning pattern in women saliva was formed due to NaCl (sodium chloride), which is cyclically increased

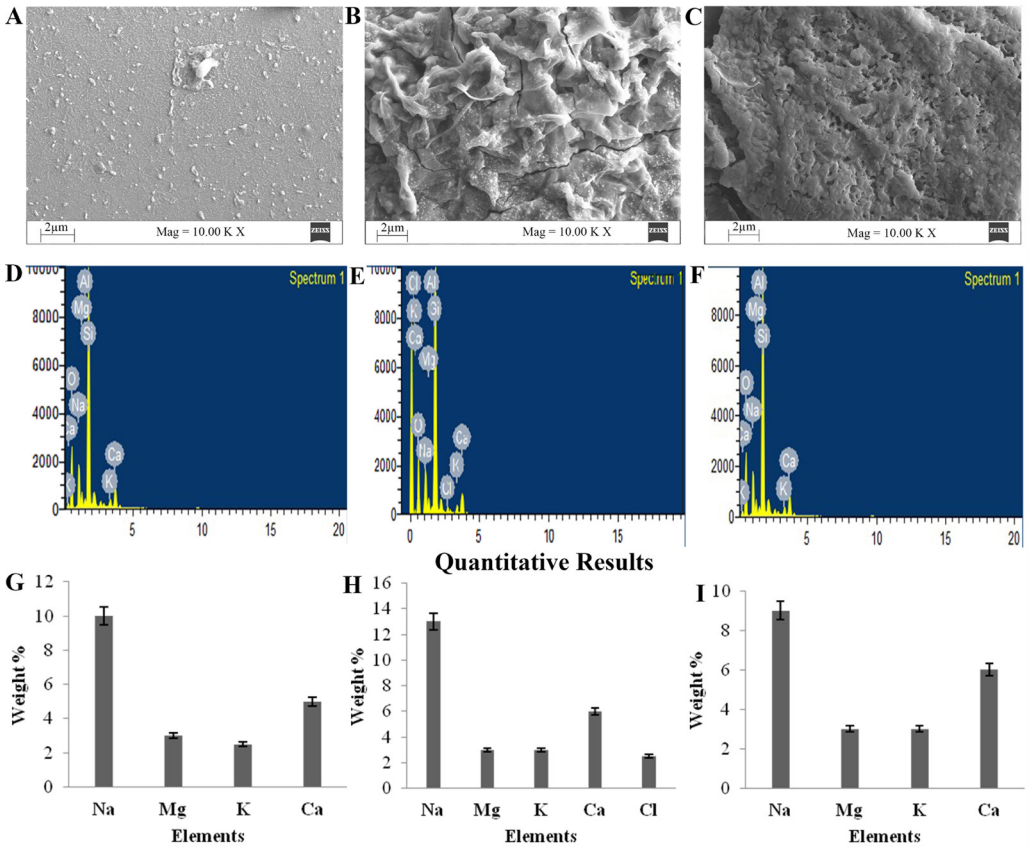

Figure 4. Scanning electron microscopic and SEM–EDX analysis of saliva during menstrual cycle. SEM analysis of **A)** pre-ovulation phase shows microglobules, **B)** Compact network-shaped mesh of ovulation phase, **C)** Network-shaped mesh with formation of pores of post-ovulation phase, at 10.00 K X in magnification and Bar = 2 µm. **D**, **G)** EDX electrolytes analysis of the pre-ovulation phase. **E**, **H)** EDX electrolytes analysis of ovulation phase. **F**, **I)** EDX electrolytes analysis of the post-ovulation phase saliva.

under the influence of estrogen (*22-24*). Barbato *et al*. (25) reported that the strong relationship is found among salivary ferning pattern and physiological condition during menstrual cycle.

A previous study revealed that the sodium and chlorine concentration level increased in sweat and saliva of cystic fibrosis (CF) patients (*26,27*). Likewise, a decreased potassium concentration level was found in rheumatoid arthritis patients (*28*). Recently, the level of sodium, potassium, chloride, calcium and phosphorus were identified as higher in Down syndrome patients saliva compared to healthy individuals (*29*). The concentration of sodium (Na), potassium (K) and chloride (Cl) ions in saliva was higher in diabetic patients when compared to that of non-diabetic patients those are having dental caries (*30*). In the present study, higher levels of sodium and chloride were found in saliva during ovulation phase compared to that of other phases. Our previous report showed that the salivary electrolytes are responsible for fern pattern formation during the ovulation phase (*23*), the present study further affirms the elevated level of salivary electrolytes during the ovulation phase.

The results obtained in this study indicated that significant difference was recorded in salivary pH and buffer capacity during ovulation phase compared to other phases of menstrual cycle. Preethi *et al*., (*31*) showed that the buffering capacity of the saliva was lower in children having caries as compared to healthy children. According to our data, salivary flow is lesser in ovulation phase of women, which may lead to oral dryness seen in females (*32*). It is found that women have a low saliva flow rate than men for stimulated and un-stimulated parotid saliva (*33*). Possibly female sexual hormones, specifically estrogen, have a significant role in the suppression of saliva flow (*31,34*). Recently, it is reported that individuals with increased inorganic salivary calcium, phosphate, pH and increased flow rate may chance for periodontitis formation (*35*). Additionally, findings from the present study suggest that the sexual hormones may influence the changes in salivary flow rate and pH during ovulation phase. Electrolyte level, pH, buffer capacity, flow rate and ultra structure have been studied in saliva in reference to several diseases and physiological conditions (*26-30,33,36-42*). The present study revealed the difference in the electrolytes, pH, buffer capacity and flow rate of saliva during the ovulation phase. Likewise, the salivary protein expressions are varies in regard to ovulation phase (*14*).

Commonly, the typical endocrine pattern of the menstrual cycle controls the functions of the cervix and, thereby controls the biophysical properties of the cervical mucus (*38*). At earlier period the mesh type morphology was demonstrated in cervical mucus during ovulatory phase (*43*). Even though, in the human salivary ultra structure is not yet studied during menstrual cycle in regard to ovulation phase. Now, the SEM-EDX study reveals the difference in elements concentration during ovulation phase as compared to other phases.

On the basis of our findings, we suggest that human saliva has distinctive variation in physico-chemical properties during ovulation phase, which would help to set a reference for the identification of fertile period in women. Further, studies are required to understand the composition of saliva during the fertile period in women. In conclusion, micro and macro molecules which involved in the ultra structural variation and electrolytes would lead to the development of biosensor for ovulation detection.

Acknowledgements

G.S. thanks the Indian Council of Medical Research (ICMR-New Delhi, India; File No. 45/5/2014-BIO/BMS) for the award of Senior Research Fellowship. The facility availed from Bharathidasan University, ICAR-National Fund, DST-FIST-Phase-I (Stage-II) and UGC-SAP-DRS-II is greatly acknowledged. Authors thank Dr. A.S. Rao, Honorary Professor, Department of Biotechnology, Bharathidasan University, Tiruchirappalli-620024 for critical reading of manuscript.

References

1. Marieb EN, Hoehn K. Human anatomy & physiology (8th ed.). Benjamin Cummings, San Francisco, USA, 2010.
2. Martini FH, Nath JL. Fundamentals of Anatomy and Physiology (8th ed.). Pearson/Benjamin Cummings, San Francisco, USA, 2009.
3. Farnaud SJ, Kosti O, Getting SJ, Renshaw D. Saliva: physiology and diagnostic potential in health and disease. ScientificWorldJournal. 2010; 10:434-456.
4. Edgar WM. Saliva: its secretion, composition and functions. Br Dent J. 1992; 172:305-312.
5. Robker RL, Russell DL, Yoshioka S, Sharma SC, Lydon JP, O'Malley BW, Espey LL, Richards JS. Ovulation: a multi-gene, multi-step process. Steroids. 2000; 65:559-570.
6. Espey LL, Lipner H. Ovulation. In: The Physiology of Reproduction (Knobil E, Neill JD, eds.). Raven Press, New York, 1994; pp. 725-780.
7. Nothnick WB, Keeble SC, Curry TE Jr. Collagenase, gelatinase, and proteoglycanase messenger ribonucleic acid expression and activity during luteal development, maintenance, and regression in the pseudopregnant rat ovary. Biol Reprod. 1996; 54:616-624.
8. Alagendran S, Archunan G, Prabhu SV, Orozco BE, Guzman RG. Biochemical evaluation in human saliva with special reference to ovulation detection. Indian J Dent Res. 2010; 21:165-168.
9. Martinez AR. Prediction and detection of the fertile phase of the menstrual cycle: an overview. Adv Contracept. 1997; 13:131-138.
10. Khaustova S, Shkurnikov M,Tonevitsky E, Artyushenko V, Tonevitsky A. Noninvasive biochemical monitoring of physiological stress by Fourier transform infrared saliva spectroscopy. Analyst. 2010; 135:3183-3192.
11. Espey LL. Ovulation as an inflammatory reaction-a hypothesis. Biol Reprod.1980; 22:73-106.
12. Saibaba G, Archunan G. Does salivary protein(s) act an ovulation indicator for women? A hypothesis. Med Hypotheses. 2015; 84:104-106.
13. Alagendran S, Saibaba G, Muthukumar S, Rajkumar R, Guzman RG, Archunan G. Characterization of salivary protein during ovulatory phase of menstrual cycle through MALDI-TOF/MS. Indian J Dent Res. 2013; 24:157-163.
14. Saibaba G, Rajesh D, Muthukumar S, Sathiyanarayanan G, Padmanabhan P, Akbarsha MA, Gulyás B, Archunan G. Proteomic analysis of human saliva: An approach to find the marker protein for ovulation. Reprod Biol. 2016; 16:287-294.
15. Navazesh M. Methods for collecting saliva. Ann N Y Acad Sci. 1993; 694:72-77.
16. Tulunoglu O, Demirtas S, Tulunoglu I. Total antioxidant levels of saliva in children related to caries, age, and gender. Int J Paediatr Dent. 2006; 16:186-191.
17. Barros C, Arguello B, Jedlicki A, Vigil P, Herrera E. Scanning electron microscope study of human cervical mucus. Gamete Res. 1985; 12:85-89.
18. Choe JK, Khan-Dawood FS, Dawood MY. Progesterone and estradiol in the saliva and plasma during the menstrual cycle. Am J Obstet Gynecol. 1983; 147:557-562.
19. Gröschl M, Rauh M, Schmid P, Dörr HG. Relationship between salivary progesterone, 17-hydroxyprogesterone, and cortisol levels throughout the normal menstrual cycle of healthy postmenarcheal girls. Fertil Steril. 2001; 76:615-617.
20. Chatterton RT Jr, Mateo ET, Hou N, Rademaker AW, Acharya S, Jordan VC, Morrow M. Characteristics of salivary profiles of oestradiol and progesterone in premenopausal women. J Endocrinol. 2005; 186:77-84.
21. Gandara BK, Leresche L, Mancl L. Patterns of salivary estradiol and progesterone across the menstrual cycle. Ann N Y Acad Sci. 2007; 1098:446-450.
22. Alagendran S, Archunan G, Achiraman S. Prediction of ovulation in women through the occurrence of salivary fern prototype. ICFAI J Life Sci. 2007; 1:7-15.
23. Alagendran S, Archunan G, Bonnila Armando EO, Guzman RG. Evaluation of salivary electrolytes during normal menstrual cycle with special reference to ovulation. Am J Appl Sci. 2010; 7:1066-1072.
24. Fernando RS, Regas J, Betz G. Ovulation prediction and detection with the CUE Ovulation Predictor. Hum Reprod. 1988; 3:419-424.
25. Barbato M, Pandolfi A, Guida M. A new diagnostic aid for natural family planning. Adv Contracept. 1993; 9:335-340.
26. Di Sant'agnese PA, Darling RC, Perera GA, Shea E. Abnormal electrolyte composition of sweat in cystic fibrosis of the pancreas; clinical significance and

relationship to the disease. Pediatrics. 1953; 12:549-563.
27. Jiménez-Reyes M, Sánchez-Aguirre FJ. Sodium and chlorine concentrations in mixed saliva of healthy and cystic fibrosis children. Appl Radiat Isot. 1996; 47:273-277.
28. Syrjänen S, Lappalainen R, Markkanen H. Salivary and serum levels of electrolytes and immunomarkers in edentulous healthy subjects and in those with rheumatoid arthritis. Clin Rheumatol. 1986; 5:49-55.
29. Singh V, Arora R, Bhayya D, Singh D, Sarvaiya B, Mehta D. Comparison of relationship between salivary electrolyte levels and dental caries in children with Down syndrome. J Nat Sci Biol Med. 2015; 6:144-148.
30. Hegde MN, Tahiliani D, Shetty SS, Devadiga D. Salivary electrolyte as a biomarker in caries active type II diabetes-A comparative study. Nitte Uni J Health Sci. 2014; 4:85-89.
31. Preethi BP, Reshma D, Anand P. Evaluation of flow rate, pH, buffering capacity, calcium, total proteins and total antioxidant capacity levels of saliva in caries free and caries active children: An *in vivo* study. Indian J Clin Biochem. 2010; 25:425-428.
32. Sreebny LM. Saliva in health and disease: an appraisal and update. Int Dent J. 2000; 50:140-161.
33. Lukacs JR, Largaespada LL. Explaining sex differences in dental caries prevalence: saliva, hormones, and "life-history" etiologies. Am J Hum Biol. 2006; 18:540-555.
34. Temple DH. Variability in dental caries prevalence between male and female foragers from the Late/Final Jomon period: Implications for dietary behavior and reproductive ecology. Am J Hum Biol. 2011; 23:107-117.
35. Rajesh KS, Zareena, Hegde S, Arun Kumar MS. Assessment of salivary calcium, phosphate, magnesium, pH, and flow rate in healthy subjects, periodontitis, and dental caries. Contemp Clin Dent. 2015; 6:461-465.
36. Brunelli R, Papi M, Arcovito G, Bompiani A, Castagnola M, Parasassi T, Sampaolese B, Vincenzoni F, De Spirito M. Globular structure of human ovulatory cervical mucus. FASEB J. 2007; 21:3872-3876.
37. Ceric F, Silva D, Vigil P. Ultrastructure of the human periovulatory cervical mucus. J Electron Microsc (Tokyo). 2005; 54:479-484.
38. Elstein M. Cervical mucus: its physiological role and clinical significance. Adv Exp Med Biol. 1982; 144:301-318.
39. Gipson IK, Moccia R, Spurr-Michaud S, Argüeso P, Gargiulo AR, Hill JA 3rd, Offner GD, Keutmann HT. The Amount of MUC5B mucin in cervical mucus peaks at midcycle. J Clin Endocrinol Metab. 2001; 86:594-600.
40. Muthukumar S, Rajkumar R, Karthikeyan K, Liao CC, Singh D, Akbarsha MA, Archunan G. Buffalo cervico-vaginal fluid proteomics with special reference to estrous cycle: heat shock protein (HSP)-70 appears to be an estrus indicator. Biol Reprod. 2014; 90:97.
41. Vigil P, Cortés ME, Zúñiga A, Riquelme J, Ceric F. Scanning electron and light microscopy study of the cervical mucus in women with polycystic ovary syndrome. J Electron Microsc (Tokyo). 2009; 58:21-27.
42. Wentz AC, McCranie WM. Circulating hormone levels in a case of granulosa cell tumor. Fertil Steril. 1976; 27:167-170.
43. Menárguez M, Pastor LM, Odeblad E. Morphological characterization of different human cervical mucus types using light and scanning electron microscopy. Hum Reprod. 2003; 18:1782-1789.

(Received January 29, 2017; Revised March 25, 2017; Accepted April 3, 2017)