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Insulin response of Diabetic Pregnant Women: Analysis of saliva by FTIR study

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Abstract

This paper presents the FTIR spectral study of saliva of normal and diseased (diabetes) and its use in differentiating those pregnant women on insulin therapy. FTIR saliva spectra were recorded over the region 4000-400 cm^{-1} on a Spectrum one Perkin Elmer FTIR spectrometer. Saliva samples were collected from 20 volunteers in each set of age group 25 to 35. From the spectral study, the absorbance values of the diseased and normal saliva samples were compared. The spectral differences of intensity ratio parameters were introduced and the results were discussed. In order to find the efficacy, the absorption values of the specific bands of the spectra of normal and diabetic pregnant women were compared and the internal ratio parameter R_1(I_{1653}/I_{1410}), $R_2(I_{2931}/I_{547})$, $R_3(I_{2931}/I_{1410})$, $R_4(I_{2931}/I_{3292})$,$R_5(I_{3292}/I_{547})$ were calculated , and the result observed for diabetic pregnant women after insulin therapy (i.e., 3 hrs and 7 days) was almost similar to that of the normal pregnant women. Besides, the intensity of absorption peaks of the diabetic pregnant women before administration of insulin, after intake of insulin (3hrs) and after insulin therapy (7 days) were considered in which the intensity ratio parameters R_1(I_{1544}/I_{1075}), R_2 (I_{1544}/I_{1224}), $R_3(I_{2931}/I_{1075})$, $R_4(I_{3292}/I_{1075})$, $R_5(I_{3292}/I_{1224})$, were calculated. The observed absorbance values increased in diabetic pregnant women initially, but slightly decreased after insulin therapy 3 hrs and further decreased after 7 days compared to normal pregnant women. Thus, striking spectral differences (in terms of intensity value) observed between saliva of normal, diseased subjects when analysed after insulin therapy. The comparison of these values showed that the spectra of normal and diabetic pregnant women before and after therapy are different and the vibrational analyses were carried out. It is concluded that FTIR may be applied to indicate the changes in the salivary pattern of the diabetic pregnant women with insulin therapy.

Keywords: Saliva, normal pregnant women, diabetic pregnant women, insulin, FTIR spectroscopic analysis.

Introduction

The greatest challenge of salivary diagnostics is to identify disease diagnostic markers and successfully translate these research efforts from the laboratory into the clinic. To empower salivary diagnostics to become an approach for health surveillance, established robust scientific platforms for saliva biomarker discovery, validated potential candidates, and developed point-of-care technologies for high throughput, efficiency, and accurate clinical applications. Saliva is a complex fluid containing a variety of enzymes, hormones, antibodies, antimicrobial constituents, and growth factors. (Zelles *et al.,* 1993; Rehak *et al.,* 2000). Today, a growing number of proof-of-principle assays have been established using saliva to monitor diseases or bodily conditions such as HIV infection (Emmons, 1997; Malamud 1997) immune responses to viral infections (*e.g.*, hepatitis A, B, & C), (Chaita *et al.*, 1995; Ochnio *et al.,*1997; El-Medany *et al.,* 1999) systemic levels of drugs, and the detection of illicit drug use (Cone,1993, Kidwell *et al.,*1998). Salivary diagnostics would enable clinicians to monitor diseases frequently and easily and would have impact on the future medical research and therapy.

Pregnancy involves complex hormonal interactions, which cause profound physiologic changes. Some changes are more evident than others. The changes that occur are the result of increasing maternal and fetal requirements for the growth of the fetus and the preparation of the mother for delivery. An increase in the secretion of female sex hormones, estrogen by 10-fold and progesterone by 30-fold, is important for the normal progression of pregnancy. Increased hormonal secretion and fetal growth induce several systemic, as well as local physiologic and physical changes in a pregnant woman. Changes in salivary pattern in normal and diabetic pregnant women in each trimester have been compared using FTIR spectroscopy both qualitatively and quantitatively (Raziya Sultana *et al.,* 2011).

Early detection of disease plays a crucial role in successful therapy. Since the advantages of saliva as a diagnostic tool were revealed, the use of saliva for surveillance of disease and general health has become a highly desirable goal in healthcare research and promotion. However, the full power and potential of saliva in medical applications was only recently recognized when saliva was shown to reflect the spectrum of health and disease states and to offer distinctive advantages over serum (Slavkin, 2004; Mandel,1993). Pregnancy does not cause periodontal disease but does worsen an existing condition (Tilakaratne *et al.,* 2000). Female sex hormones (estrogen, progesterone, and human gonadotrophin) are secreted primarily by the placenta.

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These hormones are responsible for most of the physiologic changes during pregnancy. The main salivary changes in pregnancy involve its flow, composition, pH, and hormone levels.

Diabetes mellitus is a chronic illness, in which the body is exposed to continual high levels of blood glucose, a condition known as hyperglycemia. Glucose is a simple sugar and an important source of energy, especially for the brain. Almost all forms of diabetes stem from problems in the body's production and use of insulin, the hormone that is responsible for keeping blood glucose levels in check. One cause of diabetes is the inability to produce enough insulin; for this problem, treatments range from oral medications that increase insulin secretion (i.e., secretagogues, such as tolbutamide) to injections of insulin itself. Another cause of diabetes is the inability of body tissues to respond sufficiently to normal amounts of insulin, or insulin resistance.

Glucose is the source of quick energy, and we always need a certain minimum amount of glucose in the bloodstream. On the other hand, excess blood glucose can damage tissues. Insulin is the hormone that keeps blood glucose levels from getting too high, but diabetes disrupts the body's ability to use insulin effectively. Carbohydrates come in all sizes. Large carbohydrates such as polysaccharides (e.g., starch) are chains of individual sugar molecules. The smallest carbohydrates are monosaccharides, individual sugar molecules. Glucose, which is a small water-soluble molecule, is a monosaccharide (Nussey & Whitehead, 2001). Insulin is a protein molecule made in beta cells that are clustered in islets within the pancreas. Glucose is the main stimulus for insulin secretion, but the pancreas also releases insulin in response to elevated blood levels of amino acids or when signaled by the parasympathetic (vagal) nervous system.

In this the saliva samples are collected from diabetic pregnant women of 1st, 2nd and 3rd trimester and FTIR analysis were carried out. Early in pregnancy, increase in estrogen and progesterone level which lead to pancreatic beta cell hypertrophy and insulin excretion alters maternal carbohydrate metabolism. The placental peptide hormone human chorionic somatomamotropia known as human placental lactogen has been implicated in inducing insulin resistance as have prolactin corisol, estrogen and progesterone. The level of all these substances is significantly greater in pregnancy than in non pregnant states. Plasma cellulose responses to similar carbohydrate loads are higher in pregnant women than nonpregnant women (Frank & Patrick Duff, 2004).

The present work is attempted in the study of changes in salivary pattern in normal and diabetic pregnant women and the response in change in saliva on insulin therapy (after 3 hrs and after 7 days) are compared by using FTIR spectroscopy both qualitatively and quantitatively. The type of spectral signatures qualitatively differentiates the progesterone levels in pregnancy. The intensity ratio among the absorption bands characterizes it quantitatively. The present work is proposed to evaluate a new approach in the analysis of saliva in normal and diabetic pregnant women (before insulin) and the changes in salivary pattern during pregnancy and the response in change in saliva after (3 hrs and after 7 days) therapy in mid-IR spectroscopy.

Materials and methods

In this study, saliva samples were collected from normal and diabetic pregnant women from the Govt. RSRM Lying in Hospital, Chennai. The samples were collected from normal and diabetic pregnant women (after excluding other diseases) and the response in change in saliva of insulin therapy patients have been considered and chosen for the analysis. All the sampling procedures were performed between 12.00 am to 1.00 pm for uniformity, 20 samples of age group 25 to 35 were used for spectral analysis. After insulin therapy the response in diabetic patient were compared with normal patient and how long it takes for response were also analysed. In each category, diabetic pregnant women after insulin 3 hrs (since both insulin peak action in about 3-4 hours) and after insulin 7 days (to assess the control of the disease) – During the study period all diabetic pregnant women maintained in the same type of insulin. Informed consent were obtained from all subjects as approved by local ethics committee. Depending upon the blood sugar level, insulin is prescribed for the patients. In this analysis, short acting insulin and intermediate acting insulin were used. The FTIR spectral measurements of all the samples were carried out at Sophisticated Analytical Instrumentation Facility IIT, Madras, Chennai-36, using Spectrum-One Perkin- Elmer FTIR Spectrometer. The spectra are recorded in the mid infrared region of 4000 – 400 cm^{-1} in the absorption mode. 50 µL of each solution was spread evenly on the thallium bromide crystals window. The samples were air dried for water evaporation to eliminate the stray absorption bands due to water and holder was mounted in the sample window of the spectrometer. The spectrometer is equipped with a globar source, KBr beam splitter and DTGS cooled detector. The sampling window was scanned as the background and 32 scans are co added with a spectral resolution of 1 cm^{-1}. All the spectra were baseline corrected and normalized to acquire identical area under the curve.

Statistical analysis

Saliva samples of normal and diabetic pregnant women were analysed by means of Statistics **FTest** (0.05,"Series1","Series2") in which the parameter used was the alpha value (probability), the “first input series name” which means the name of the series object that stores the first group of data and the “second input series name” which indicates the name of the series object that stores the second group of data.

An **FTestResult** object, which has the following members: First series mean; Second series mean; First series variance; Second series variance; F value;

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Probability F one tail; F critical value one tail. To calculate the F value in which the test statistics has an F-distribution under the null hypothesis. In ANOVA, F-test was used to determine group of trial which differs significantly from an expected value.

Formula used

Sums of squares formula

$$SS_{total} = \sum_{j=1}^{p}\sum_{i=1}^{n_j}(x_{ij} - \overline{x})^2$$

$$SS_{between} = \sum_{j=1}^{p} n_j(\overline{x}_j - \overline{x})^2$$

$$SS_{within} = \sum_{j=1}^{p}\sum_{i=1}^{n_j}(x_{ij} - \overline{x}_j)^2$$

Mean squares Formula

$$MS_{between} \quad \frac{SS_{between}}{df_{between}}$$

$$MS_{within} \quad \frac{SS_{within}}{df_{within}}$$

df is the degree of freedom

F Formula

$$F = \frac{MS_{between}}{MS_{between}}$$

Within instead of between in denominator

Thus, one way ANOVA calculator was used to test the equality of samples by using variance.

F test for normal and diabetic pregnant women after insulin therapy (three hours and seven days) was observed using FTIR spectra (Table1-3)

Interpretation of Table 7

The interpretation of data in normal and diabetic pregnant women with insulin therapy (3hrs and 7 days) was analysed by the intensity test ratio on the sample collected using one way ANOVA method. The result of the insulin therapy as compared to the normal women as per the test hypothesis gives true factor as per the ratio of the two mean squares estimate the same quantity (error variance)using the FTIR spectra. The Effect of insulin using the ANOVA method was verified and mean result shows that the effect of insulin, was within the range of requirement of the Diabetic level control required for the patient through the saliva test within three hours and seven days from the period of insulin therapy. The result of insulin therapy was validated and found to be true in comparison with normal pregnant women.

F test for diabetic pregnant women before administration of insulin and after insulin therapy (three hours and seven days) was observed using FTIR spectra (Table 4-6)

Interpretation of Table 8

Table 1. Squaring of the data -Anova method

x1	$x1^2$	x2	$x2^2$	x3	$x3^2$
0.41	0.1680	0.42	0.1764	0.31	0.0960
0.32	0.1024	0.32	0.1024	0.29	0.0841
0.65	0.4225	0.79	0.6241	0.73	0.5328
1.7	2.8899	1.5	2.25	1.5	2.25
0.18	0.0324	0.21	0.0441	0.18	0.0324

Table 2. Mean, Standard Deviation and Variance of pregnant women (normal and diabetic with insulin therapy).

	x1	x2	x3	Total
Number (n)	5	5	5	15
Σx	3.2600	3.24	3.0100	9.51
Mean	0.652	0.648	0.6020	0.63
Σx^2	3.6153	3.1969	2.9955	9.8079
Variance	0.37	0.27	0.30	
Std.Dev.	0.608	0.520	0.548	
Std.Err.	0.272	0.233	0.245	

Table 3. Anova result

	SS	df	MS	F
Between	0.0079	2	0.0040	0.0127
within	3.7708	12	0.3142	
Total	3.7788	14		

The insulin therapy (after 3hrs and 7 days therapy) used on the diabetic pregnant women was analyzed by the intensity test ratio on the sample collected using one way ANOVA method. The result of the insulin therapy as compared with diabetic pregnant women before administration of insulin, after three hours and seven days on diabetic pregnant women.

Table 4. Squaring of the data -Anova method

x1	$x1^2$	x2	$x2^2$	x3	$x3^2$
0.68	0.4624	0.63	0.3969	0.47	0.2208
0.45	0.2025	0.4	0.1600	0.39	0.1521
0.8	0.6400	0.76	0.5776	0.72	0.5184
0.69	0.4760	0.5	0.25	0.45	0.2025
0.46	0.2116	0.32	0.1024	0.3	0.09

Table 5. Mean, Standard Deviation and Variance of diabetic pregnant women (before and after insulin therapy).

	x1	x2	x3	Total
Number (n)	5	5	5	15
Σx	3.08	2.61	2.33	8.02
Mean	0.616	0.522	0.466	0.53
Σx^2	1.9926	1.4869	1.1839	4.6634
Variance	0.02	0.03	0.02	
Std.Dev.	0.141	0.173	0.141	
Std.Err.	0.063	0.077	0.063	

Table 6. Anova result

	SS	df	MS	F
Between	0.0577	2	0.0289	1.0906
within	0.3179	12	0.0265	
Total	0.3756	14		

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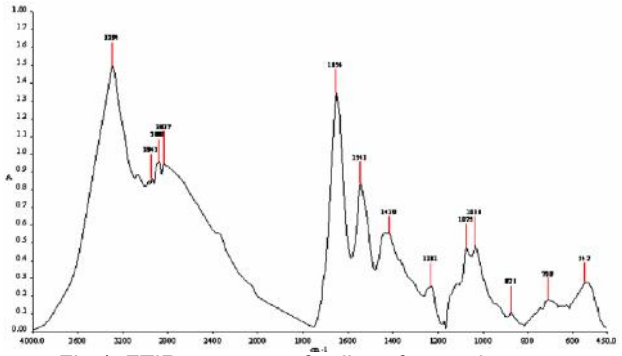

Fig.1. FTIR spectrum of saliva of normal pregnant women

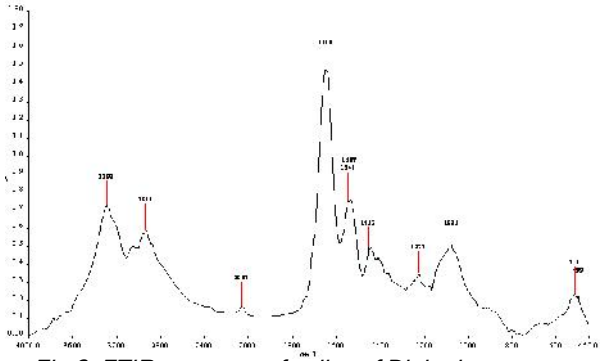

Fig.2. FTIR spectrum of saliva of Diabetic pregnant women

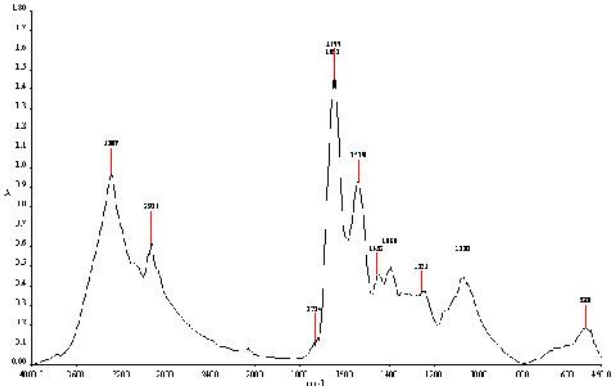

Fig.3. FTIR spectrum of saliva of Diabetic pregnant women (insulin therapy after 3hrs)

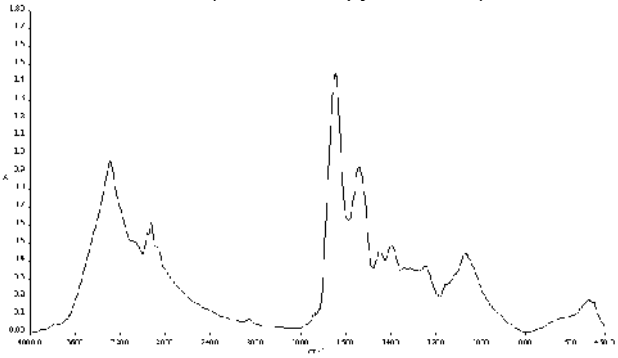

Fig.4. FTIR spectrum of saliva of Diabetic pregnant women (insulin therapy after 7 days)

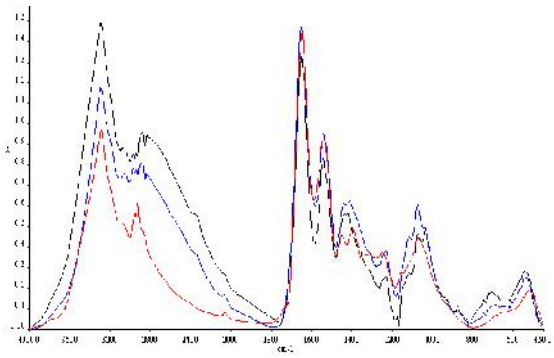

Fig. 5. Comparison of FTIR spectrum of saliva of normal pregnant women and with insulin therapy (after 3hrs and 7 days)

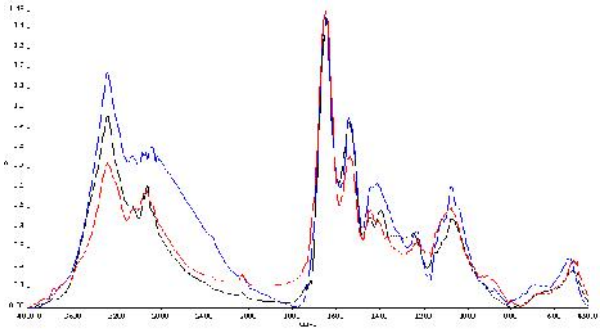

Fig.6. Comparison of FTIR spectrum of saliva of diabetic pregnant women and with insulin therapy (after 3hrs and 7 days).

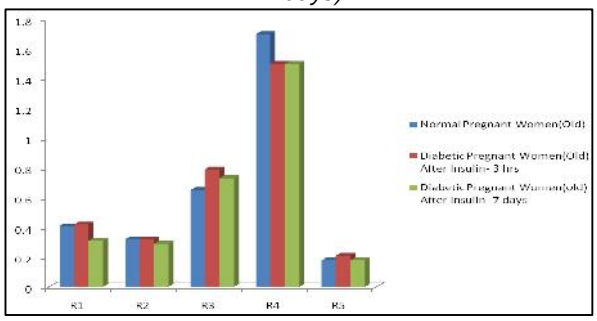

Fig. 7. Comparison of intensity ratio parameters of normal pregnant women and with diabetic insulin therapy (after 3hrs and 7 days).

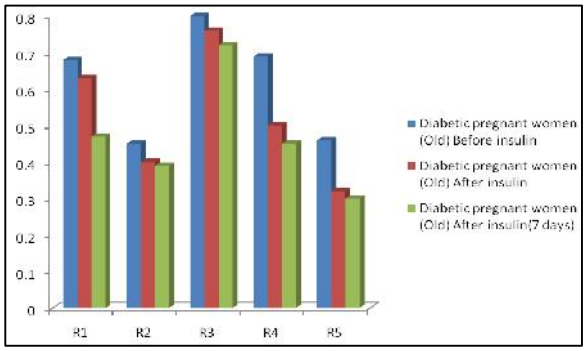

Fig.8. Comparison of intensity ratio parameters of diabetic pregnant women before administration of insulin and with insulin therapy (after 3hrs and 7 days).

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As per the test hypothesis it gave a true factor and as per the ratio of the two mean squares estimate using the FTIR spectra. The Effect of insulin using the ANOVA method was verified and mean result shows that the effect of insulin within the range of requirement of the Diabetic level control required for the patient was effected on a long term on the seventh day as compared to the immediate result of insulin therapy after three hours through the saliva test of insulin therapy on the patient. The Result of insulin response therapy was validated and found to be true in comparison with diabetic pregnant women.

Results and discussion

Oral mucosa has been suggested as an especially suited subject for drug delivery and monitoring of endogenous body metabolites due to histological and physic-chemical properties. FTIR spectroscopy as a bio diagnostic tool to predict changes occur during pregnancy. A representative FTIR absorption spectrum of saliva sample of normal pregnant women is shown in Fig. 1 and their vibrational band assignments with the idea of the group frequencies of the various analytes present in the sample are given in Table 7. Fundamental modes of vibration are identified for normal and diseased pregnant women and their absorbance values are noted. Internal standard at specific modes of vibration is calculated by finding the internal ratio of absorbance of the various bands. The chief organic constituents of saliva are mucin- a glycoprotein. An increase in the secretion of female sex hormones, estrogen by 10-fold and progesterone by 30-fold, is important for the normal progression of pregnancy. The characteristic vibrational peaks are mainly dominated by the protein constituents of the sample (Petibois *et al.,* 2001; Deleris & Petibois, 2003). Hetero aromatic containing an N-H group show their stretching vibrations in the region 3500-3220 cm^{-1}. The position of the absorption band in this region depends upon the degree of hydrogen bonding and hence upon the physical state of the sample or the polarity of the solvent. Primary amines examined in dilute solution display two weak absorption bands one near 3500 cm^{-1} and the other near 3400cm^{-1}. The appearance of the sharp band at 3377 cm^{-1} is assigned as N-H stretching due to the immidazole ring in throphylline. In the present work, the band appearing at 3292 cm^{-1} is due to N-H stretching vibration. The peak at 2933-2923 cm^{-1} is attributed to C-H stretching bands in malignant tissues (Wu *et al.,*2001) and the band appearing at 2931 cm^{-1} is due to asymmetric stretching CH_2.The spectral region from 1630-700 cm^{-1} is due to amide I region and in the present work the peak observed at 1653 cm^{-1} is due to C=O, C=N, N-H of adenine, thymine, guanine, cytosine (Dovbeshko *et al.,* 1997). The spectral region (1500-1300 cm^{-1}) is mainly dominated by the deformations of the methyl, methylene and C-H groups (Gunasekaran *et al.,* 2007) and the band at 1547cm^{-1} is due to aromatic ring stretching and the band at 1400-500 cm^{-1} is due to ring stretching vibration mixed strongly with CH in-plane bending (Schutz *et al.,* 2007) and the observed peak at 1410 cm^{-1} is due to (H-C-H) group.

The lipid contents of these compounds can be evaluated using peak intensity at 2956 cm^{-1} (asymmetric stretching vibration of CH_3 of acyl chains), 2922 cm^{-1} (asymmetric stretching vibration of CH_2 of acyl chains), 2874 cm^{-1} (symmetric stretching vibration of CH_3 of acyl chains), 2852 cm^{-1} (symmetric stretching vibration of CH_2 of acyl chains), and 1600–1800 cm^{-1} (C=O stretching). The specifications of protein contents of biological samples can also be understood from 1717 cm^{-1} (amide I, arising from C=O stretching vibration), 1500-600 cm^{-1} (amide II, N-H bending vibration coupled to C-N stretching), and 1220–1350 cm^{-1} (amide III, C-N stretching and N-H in plane bending, often with significant contributions from CH_2 wagging vibrations). The peaks related to nucleic acids are as follows: 1717 cm^{-1} (C=O stretching vibration of purine base), 1666 cm^{-1} (C=O stretching vibration of pyrimidine base), 1220-1240 cm^{-1} (asymmetric PO_2 stretching), 1117 cm^{-1} (C-O stretching vibration of C-OH group of ribose), 1040–100 cm^{-1} (symmetric stretching of phosphate groups of phosphodiester linkages), and 1050–70 cm^{-1} (C-O-C stretching) (Fabian *et al.,* 1995). Hetero aromatic containing an N-H group shows their stretching vibrations in the 3500-3220 cm^{-1} and in the present work it is observed at 3292 cm^{-1}. The peak at 2933 cm^{-1} is due to C-H stretching vibration in malignant tissues (Wu etal.,2001) and the band appearing at 2931 cm^{-1} is due to asymmetric stretching of CH_2, whereas the progesterone is observed in the region 1690 -1620 cm^{-1}(John *et al.,*2005). The spectral region from 1630-700 cm^{-1} is due to Amide I region and in the present work the peak is observed at 1653cm^{-1} is due to C=O, C=N, N-H of adenine, thymine, guanine, cytosine (Dovbeshko *et al.,*1997). The peak at 1544cm^{-1} is due to Amide II bands (arises from C-N stretching & CHN bending vibrations (Huleihel *et al.,*2002). and the observed peak at 1410cm^{-1} is due to (H-C-H). The prominent absorption peak is at 1224 cm^{-1} is due to symmetric stretching of phosphate groups in phospholipids (Fabian *et al.,* 1995). The glucose or sugar moieties are found to be observed at 950-1180 cm^{-1}, in present work the peak is observed at 1075 cm^{-1} due to glucose and the band at 1075 cm^{-1} is due to C-N stretching absorption of aliphatic amines is weak.

FTIR spectra show significant spectral differences between the normal pregnant women and change in saliva of diabetic pregnant women with insulin therapy i.e., after insulin (3hrs) and after insulin (7days) of pregnant women. In order to quantify the results further five intensity ratio parameters R_1(I_{1653}/I_{1410}), $R_2(I_{2931}/I_{547})$, $R_3(I_{2931}/I_{1410})$, $R_4(I_{2931}/I_{3292})$,$R_5(I_{3292}/I_{547})$ are calculated. The results observed for diabetic pregnant women after insulin therapy (i.e., 3 hrs and 7 days) is more or less similar to that of the normal pregnant women.

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Table 7. Comparison of normal and diabetic pregnant women with insulin therapy

Intensity ratio	Old(3rd trimester)		
	Normal pregnant women (Old)	Diabetic pregnant women(Old) After insulin-3 hours	Diabetic pregnant women(Old) After insulin-7 days
$R_1(I_{1653}/I_{1410})$	0.41	0.42	0.31
$R_2(I_{2931}/I_{547})$	0.32	0.32	0.29
$R_3(I_{2931}/I_{1410})$	0.65	0.79	0.73
$R_4(I_{2931}/I_{3292})$	1.7	1.5	1.5
$R_5(I_{3292}/I_{547})$	0.18	0.21	0.18

Fig.2 presents the spectrum of diabetic pregnant women and infrared spectra of change in saliva of diabetic after insulin therapy (3 hrs and 7 days) are shown in the (Figs. 3-4). In order to quantify the results further five intensity ratio parameters R_1(I_{1544}/I_{1075}), R_2 (I_{1544}/I_{1224}), $R_3(I_{2931}/I_{1075})$, $R_4(I_{3292}/I_{1075})$, $R_5(I_{3292}/I_{1224})$, are calculated and the observed absorbance values increases in diabetic pregnant women and slight decrease in diabetic after insulin therapy 3 hrs and further decreases after 7 days. Fig.5 shows the comparison of normal pregnant women and diabetic with insulin therapy after 3 hrs and 7 days and also the comparison of diabetic pregnant women before and after therapy as shown in Fig.6. Table 8 gives the intensity ratio calculation and the variation of bar diagram shown in (Fig. 7-8).

Analysis with Histogram

The bar diagram shown in Fig.7 and Fig 8 between the intensity ratio parameters and absorbance values were obtained from the FTIR spectra. From the histogram (Fig.7) shows a striking spectral difference between the saliva of normal pregnant women with diabetic after insulin therapy 3 hrs and after 7 days. Also the Fig.8 shows that there is a change in the absorbance value of the diabetic pregnant women with insulin therapy after 3 hrs and after 7 days.

Table 8. Intensity ratio parameter of diabetic pregnant women with insulin therapy of higher age group

Intensity ratio	Diabetic pregnant women (old)		
	Before insulin	After insulin (3 hrs)	After insulin (7 days)
$R_1(I_{1544}/I_{1075})$	0.68	0.63	0.47
$R_2(I_{1544}/I_{1224})$	0.45	0.4	0.39
$R_3(I_{2931}/I_{1075})$	0.8	0.76	0.72
$R_4(I_{3292}/I_{1075})$	0.69	0.5	0.45
$R_5(I_{3292}/I_{1224})$	0.46	0.32	0.3

Conclusion

Role of FTIR spectroscopic techniques in the analysis of saliva of normal and diabetic pregnant women before administration of insulin and after insulin therapy (3 hrs and 7 days) are clearly demonstrated both qualitatively and quantitatively. The absorption of the vibrational peaks of diabetic pregnant women after administrating insulin (3 hrs and 7 days) is similar to that of normal pregnant women. However, the intensity of absorption peaks of the diabetic pregnant women before administration of insulin increases and decreased after intake of insulin (3hrs) and further decreased after insulin therapy (7 days). Thus, striking spectral differences in terms of intensity values were observed between saliva of normal, diseased subjects. This work exhibits how saliva by non-invasive sampling can be used to distinguish between 2 different sample categories (diabetic pregnant women from that of normal pregnant women).

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